Prostate-Tumor Targeting of Gene Expression by Lentiviral Vectors Containing Elements of the Probasin Promoter

Duan Yu, William W. Jia, Martin E. Gleave, Colleen C. Nelson, and Paul S. Rennie*

The Prostate Center at Vancouver General Hospital and the Department of Surgery, University of British Columbia, Vancouver, British Columbia, Canada

BACKGROUND. Lentiviruses are retroviruses that can infect and stably integrate into the chromatin of non-dividing cells. The purpose of this study was to determine whether lentiviral vectors containing the probasin (PB) promoter displayed prostate-specific, androgen-regulated, and persistent gene expression.

METHODS. Three lentiviral-PB promoter/enhanced green fluorescent protein (EGFP)-reporter vectors together with a control lentiviral-CMV-EGFP, were tested by microscopy and flow cytometry for expression of EGFP after infection of human prostate cancer cells (LNCaP, PC-3, PC-3(hAR), and Du145 cells) and non-prostate cells (COS-1, HeLa, HeLa(hAR), and MCF-7 cells).

RESULTS. All cells infected in vitro with lentiviral-CMV vectors expressed EGFP, whereas with lentiviral-PB vectors (the most potent being Lv-ARR2PB), reporter expression was only observed in LNCaP cells with a small amount seen in androgen-independent PC-3 cells. Stable or transient transfection of androgen receptor only raised EGFP expression in prostate-derived cell lines, but did not change tumor specificity. With Lv-ARR2PB infected LNCaP cells, androgens regulated EGFP both in vitro and in vivo. After intra-tumor injection of this vector, EGFP expression was observed in LNCaP tumors, but not in A-549 lung or CaKi-2 kidney tumors.


KEY WORDS: prostate cancer; androgen regulation; tissue-specific promoter; retrovirus

INTRODUCTION

Prostate cancer is presently the most commonly diagnosed non-skin cancer in men and is the second leading cause of cancer-death in men [1]. While frequently curable in its early stage, many patients present with advanced or metastatic disease with concomitantly fewer curative treatment options and with a median life expectancy of ~3 years [2]. In this regard, gene therapy holds the promise of providing a systemic method for repairing, replacing, or deleting a genetic defect. Hence, the feasibility of using a prostate-specific promoter to express exogenous therapeutic genes in prostate cancers offers an attractive way of potentially targeting locally advanced and metastatic tumors for eradication or control. For this purpose, the promoters that have been most intensively studied for restricting gene expression to the prostate are those
promoters or to constitutively active viral promoters.

While PSA has been the most studied “prostate-specific” promoter, it has been shown to be expressed in a variety of normal and neoplastic tissues including 30–40% of breast tumors and in some salivary duct, colon, and liver cancers [11,13]. The 632 bp proximal promoter of PSA was originally thought sufficient for tissue-specific expression, but it was later found that a strong enhancer at ~4 kb upstream of the start site of transcription was also required for maximal activity [5,14]. Studies using an adenovirus vector with either the 6 kb PB promoter or combining the ~600 bp proximal promoter with a 1,455 bp upstream PSA enhancer sequence have demonstrated preferential in vitro expression of reporter genes in PSA-producing LNCaP cells [6,15]. By comparison, the small fragment (nucleotides −426 to +28), proximal PB promoter (−426/+28 PB) has been shown to be sufficient for prostate-specific expression in transgenic mice [9].

The PB promoter has been extensively characterized with respect to its functional androgen response element, which is referred to as an androgen response region (ARR) and composed of two cooperatively interacting androgen receptor (AR) binding sites (Class I sites) located between nucleotides −236 and −96 [16,17]. An additional set of AR binding sites, called Class II sites, has been found to reside in this region and shown to contribute to the overall androgen regulation of the PB promoter [18]. At the present time, it is not known to what extent any of these AR binding sites govern prostate-specific expression. Recently, non androgen-regulated, cis-acting sites of the PB promoter have been shown to be associated with prostate-specific gene expression [20]. Nevertheless, although −286/+28 PB promoter contains sufficient information to dictate prostate-specific gene expression [21], it has relatively low activity compared to reference CMV promoters or to constitutively active viral promoters.

To address the problem of low promoter strength, various concatenated forms of the proximal PB promoter have been tested for activity and specificity. For example, androgen induction of a construct composed of three −244 to −96 ARR motifs attached to a thymidine kinase minimal promoter (ARR2tk) was found in the presence of AR to be ~10-fold higher than that seen with the proximal PB promoter [19]. While this construct could not be shown to direct prostate-specific gene expression (R.J. Matusik, personal communication), another concatamer ARR3PB, which is composed of two ARR motifs upstream of the minimal PB promoter, was shown in transgenic animals to target the prostate exclusively and to be strongly induced by androgens [21]. Currently, this is the optimal form of PB promoter for conferring prostate-specific gene expression while possessing substantially higher activity than the proximal promoter.

In addition to a prostate-specific promoter, targeted gene therapy for prostate cancer requires the development of a suitable gene delivery system. To date, various forms of the PB promoter have only been tested with adenoviral vectors [22–25]. While collectively these studies have shown great promise, some of the limitations inherent in using adenovirus as a carrier for therapeutic purposes, such as a very strong host immune response [26], limit their efficacy as a therapeutic and necessitate testing the activity and specificity of the PB promoter when constructed into other viral vectors. In this regard, retroviral vectors are attractive tools for gene therapy since they stably integrate into chromosomes for long-term expression of a therapeutic gene. Also, as they do not transfer viral genes, they do not trigger a strong host immune response; thereby permitting multiple treatment applications to augment the anti tumor effect [27]. HIV-1 based lentiviral vectors have all the advantages of other retroviruses, but additionally are unique in that they are able to infect non-dividing cells [27]; an important clinical requisite for gene therapy of slow growing tumors. Prostate cancers are notoriously slow growing and hence it is particularly important to have a persistent agent like a lentivirus to allow sufficient time for cells to cycle to the point where they are most vulnerable to exposure to a therapeutic gene.

In the present study, we sought to determine whether a viral vector could be created that would combine the best traits of the PB promoter with those inherent to lentivirus. Specifically, we investigated the androgen regulation and tissue specificity of lentiviral vectors expressing enhanced green fluorescent protein (EGFP) under the control of CMV promoter or one of three forms of the PB promoter. The results demonstrated that lentiviral-PB vectors had a persistence after infection, responded to androgen administration or withdrawal, and displayed prostate-specific expression of EGFP under both in vitro and in vivo conditions.

**MATERIALS AND METHODS**

**Cell Culture**

LNCaP, PC-3, and DU145 human prostate cancer cells, MCF-7 human breast cancer cells, COS-1 monkey kidney cells, 293T human embryo kidney cells, A549 lung cancer cells, CaKi-2 kidney carcinoma cells, and HeLa cancer cells were all obtained from American
Type Culture Collection (ATCC; Manasas, VA). A HeLa cell line with a stably transfected flag-tagged human AR (HeLa(hAR)) was a gift from Dr. M. Carey (UCLA). A PC-3(hAR) cell line with a stably transfected human AR was a gift of Dr. T.J. Brown (University of Toronto). A549 cells were cultured in Ham’s F12k medium with 2 mM l-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 10% FBS, whereas Caki-2 cells were cultured in McCoy’s 5a medium with 1.5 mM l-glutamine and 10% FBS. LNCaP and MCF-7 cells were cultured in RPMI 1640 medium with 10% FBS. Human embryo kidney 293T cells, PC-3, Du145, COS-1, HeLa, and HeLa(hAR) were maintained in DMEM medium with 10% FBS. For androgen regulation experiments, cells were also maintained in the RPMI medium with 10% charcoal-stripped FBS. The androgen dihydrotestosterone (DHT) was dissolved in ethanol and diluted into required concentration with the culture medium.

**Plasmid Construction**

The 3.1 kb LacZ component in plasmid pHR-CMV-LacZ (a gift from Dr. I. Verma (Salk Institute)) was replaced with the EGFP reporter (Clontech) and used to create the control lentiviral transfer plasmid pHR-CMV-EGFP as before [28]. The CMV early promoter in the plasmid pHR-CMV-EGFP was replaced by inserting three different PB promoter elements at Cla I and BamH I sites to construct the PB lentiviral transfer plasmids carrying EGFP (Fig. 1). The promoters PB (−426/+28), PB (−286/+28), and ARR2PB were released from pBluescript II-SK by digestion with Cla I and BamH I [19,21]. ARR2PB promoter, which contains two copies of the PB ARR (−244 to −96) upstream of the minimal promoter was a gift from Dr. R. Matusik [21]. Both of the packaging plasmid pCMVΔR8.2, containing viral assembly sequences, and pMD.G, carrying the vesicular stomatitis virus envelop glycoprotein G (VSV-G), were gifts from Dr. I. Verma [29,30].

**Lentiviral Vector Preparation and Infection**

The method for lentiviral preparation that we used in this study was as described previously [28,29]. Briefly, 1.5 × 10^6 293T cells were seeded onto 10 cm plates and co-transfected the following day by calcium phosphate precipitation method (Promega Profection

---

Fig. 1. The four lentiviral transfer vectors used in these studies. The control vector Lv-CMV-EGFP, which uses the cytomegalovirus (CMV) early promoter to drive expression of the enhanced green fluorescent protein (EGFP) reporter gene, was constructed as described in the lentiviral vectors Lv-(−426/+28)PB-EGFP, Lv-(−286/+28)PB-EGFP and Lv-(−286/+28)ARR2PB-EGFP were made by replacing the CMV promoter with different compositions of PB promoters at the Cla I/BamH I site.
Mammalian Transfection Systems) with 10 µg of transfer plasmids, 10 µg of pCMV-AR8.2, and 5 µg of pMD.G for 12–16 hr before the medium was changed. The vector-conditioned medium was collected after 48 hr and passed through a 0.45 µm filter to remove debris. The virus-containing medium can be collected for 3–4 days and stored at −70°C for future use. For assessment of lentiviral concentration, the p24 viral protein was assayed by ELISA and the virus titer was estimated as before [28]. Viral vectors were also measured by counting the number of EGFP-positive cells 72 hr post infection of 2 × 10⁴ LNCaP cells per well in 24-well plates. To concentrate the viral vectors, the conditioned medium stocks were ultracentrifuged at 50,000 × g for 90 min using a SW-28 rotor. The virus pellets were suspended with serum-free medium and aliquoted in small volumes for later use.

For lentiviral vector infections, cells were seeded in 24-well plates at a density of 1 × 10⁴ cells/well in 0.5 ml or in 6-well plates at a density of 1 × 10⁵ in 2 ml. Twenty-four hours later virus stocks were added to the cell cultures at a multiplicity of infection (MOI) of 10–60. The medium was changed 16 hr after infection and the EGFP-positive cells were counted at 48 hr under UV microscopy. The cells were then trypsinized and washed with 1 × PBS and suspended in 1 ml of 1 × PBS for flow cytometer assay (FACS).

**AR Transfections and Western Blotting**

To increase intracellular AR, the plasmid pRc-CMV-AR6 which has the full length rat AR cDNA cloned into vector pRc/CMV (Invitrogen, San Diego, CA) was used for transfection of cell lines in this study [16,19]. Briefly, about 1 × 10⁷ cells of each cell line were grown in 6-well plates until ~70% confluent and then 3 µg of pRc-CMV-AR6, 18 µl of lipofectAMINE™ Reagent (GIBCO BRL), and 200 µl of serum-free DMEM were well mixed and left for 30 min at room temperature before addition to the cells and adjustment to a final volume of 2 ml. The cells were washed with 1 × PBS and changed to fresh medium with 10% FBS 12–16 hr post-transfection. At 48 hr after transfection, the cells were used for lentiviral infection, nuclear protein extraction, and Western blot assay.

Western blotting was performed as previously described [19]. Briefly, the cells were lysed on ice for 30 min using RIPA buffer. Cell debris was removed by centrifugation at 12,000 × g for 10 min and the supernatant was assayed for protein. Equal amounts of protein lysate (20 µg) together with sample buffer were run on a 8% sodium dodecyl sulfate (SDS) polyacrylamide gel. After transfer to nylon membranes, the membranes were probed with rabbit polyclonal anti-AR (Affinity BioReagents) and anti-actin antibodies (Sigma), followed by polyclonal goat anti-rabbit antibody (Bio-Rad). The corresponding AR and β-actin bands were detected by ECL (Amersham Biosciences). The density ratio of AR/actin was assayed by the software Quantity One (Bio-Rad).

### Measurement of EGFP Fluorescence of Cells

Two to five days after lentiviral infection, cells expressing the EGFP transgene were photographed under a fluorescence microscope and then analyzed by FACS. Briefly, infected and non-infected control cells were trypsinized, washed with 1 × PBS, suspended in 0.5 ml of 1 × PBS and silvered with a 70 µm cell strainer (Falcon). After setting of the background level of the fluorescence using non-infected control cells, EGFP expression was analyzed using the FACS-Calibur system (Beckton-Dickenson).

### In Vivo Lentiviral Vector Studies

Three groups of four nude mice (Charles River Labs) were inoculated subcutaneously on both flanks with 2 × 10⁶ cells with either LNCaP prostate cancer cells, A-549 lung cancer cells or CaKi-2 kidney carcinoma cells. Tumors usually grew to a diameter of ~0.5 cm (estimated as before using calipers [31]) at 30–40 days post-inoculation. Two types of concentrated lentiviral vectors, Lv-ARR₂PB-EGFP and control Lv-CMV-EGFP, were injected intratumorally in 2 × 10⁰ µl at a concentration of 10⁹ infectious units (IU) per ml. Mice were sacrificed and the tumors were removed 5 days post-injection. Each tumor was minced into pieces of ~1 mm in diameter and EGFP expression was detected by fluorescence microscopy.

Two groups of eight nude mice were inoculated subcutaneously on both flanks with 2 × 10⁶ of LNCaP-EGFP cells, previously infected in vitro with Lv-CMV-EGFP or Lv-ARR₂PB-EGFP, along with a group of LNCaP tumor controls. When tumor diameters reached ~0.5 cm, half the mice in each group were castrated. Ten days after castration, EGFP-fluorescent tumors from both castrated and non-castrated mice were excised and examined for EGFP fluorescence UV microscopy. Five hundred grams of tissue from each tumor were homogenized and lysed on ice for 30 min with 0.5 1 ml RIPA buffer. After centrifugation for 10 min at 14,000 rpm, supernatant was collected and protein concentration was measured. Fifty micrograms of protein from each tumor extract were used to measure EGFP fluorescence levels in a fluorescence plate reader (Fluoroskan Ascent FL, MTX Labs, Inc.) using wavelengths of 485 (excitation) and 527 nm (emission).
RESULTS
Promoter Activity of Three Different PB-Lentiviral Constructs

Since the initial demonstration that the proximal PB promoter \((-426/+28\) PB) could direct prostate-specific expression in a transgenic mouse [9], there have been several reports comparing the relative activity and tissue specificity of various PB promoter constructs in vitro [10,19,21,32]. While the \(-286/+28\) PB form was shown to be more active than \(-426/+28\) PB, at least one report suggested that the enhanced activity was at the price of lower tissue specificity [32]. Similarly, concatamers of the ARR motif (nucleotides \(-244\) to \(-96\)) linked to a thymidine kinase (tk) minimal promoter were reported to be several fold more active than the basic proximal PB promoter, but lost stringent prostate-specific expression [19,33]. The most potent form of PB promoter that has been shown to retain prostate-specific and hormone-regulated expression is a structure referred to as ARR2PB, which is composed of an ARR motif attached to \(-286/+28\) PB [21]. Since the presence of viral enhancers and other viral promoter elements have been shown in some cases to interfere with normal tissue and hormonal regulation of exogenous transgenes [25,34,35], we performed experiments to compare the relative level of EGFP reporter expression in prostate and non-prostate cancer cells infected with the Lv-CMV-EGFP and Lv-PB-EGFP lentiviral constructs prepared from the transfer plasmids indicated in Figure 1.

The Lv-PB-EGFP and Lv-CMV-EGFP vector particles were generated by co-transfection of 293T cells with lentiviral packaging plasmids. Approximately \(3 \times 10^6\) virus particles per ml were obtained from the vector-conditioned media, which was then concentrated by centrifugation to between \(3 \times 10^8\)–\(10^9\) particles/ml viral stock. Human prostate cancer cell lines (LNCaP, PC-3, and DU145) and non-prostate lines (MCF-7, HeLa, COS-1) were grown to \(\sim 70\%\) confluence and then infected at an MOI of 30 with the lentiviral vectors shown in Figure 1. When present, EGFP expression was detected by fluorescence microscopy between 24–48 hr after infection and reached the highest level by day-5 post-infection. EGFP expression was seen in about 90–95% of cells in all lines infected with the CMV lentivector. However, with the three types of PB-lentiviral vectors, EGFP expression was only seen in two of the human prostate cancer cell lines used (Fig. 2); LNCaP cells, and to a lesser extent PC-3 cells. By comparison, EGFP fluorescence was not detected in MCF-7 human breast cancer cells (Fig. 2) nor in DU145 human prostate cancer cells, HeLa cells and COS-1 cells (data not shown). The results indicated that the lentiviral components of these constructs did not interfere with the expected relative potency nor the preferential expression in prostate cells seen previously with these PB promoters [32].

Fig. 2. Expression of EGFP after infection with lentiviral vectors. Prostate cancer cell lines LNCaP, PC-3 and the breast cancer cell line MCF-7 were seeded at \(10^4\) cells per well and infected on the following day with control Lv-CMV-EGFP vector and the three Lv-PB-EGFP vectors (MOI = 30). EGFP expression was observed and photographed under fluorescence microscopy on the 5th day post-infection.
Furthermore, the low but detectable expression of EGFP in PC-3 cells indicates that the viral constructs have some activity even in androgen-independent prostate cancer cells. However, LNCaP cells, the more highly differentiated and androgen-regulated variety of prostate cancer cells used in this comparison, displayed the highest levels of PB-mediated EGFP fluorescence.

**Relative Potency of PB-Lentiviral Constructs**

For a more quantitative numerical comparison of the relative potency of the three forms of PB vectors, lentiviral infected LNCaP cells were assayed by flowcytometry and analyzed using the FACS-Calibur program for number of EGFP positive cells (EGFP percentage) and the mean fluorescent intensity of expression per cell (EGFP mean) at 5 days post-infection (MOI = 30). As can be seen in Figure 3a, the number of EGFP positive cells was highest in experiments with Lv-CMV-EGFP \( (P < 0.05, t\text{-test}) \), followed next by Lv-ARR2PB-EGFP and Lv\((-286/+28)\)PB, which were essentially equivalent in this regard \( (P > 0.05) \). With respect to mean EGFP intensities, the order \( (P < 0.05) \) was Lv-CMV-EGFP, Lv-ARR2PB-EGFP, Lv\((-286/+28)\)PB, and then Lv\((-426/+28)\)PB-EGFP with the lowest activity. The number of EGFP positive cells increased with higher viral titers such that with Lv-ARR2PB-EGFP at an MOI of 100, almost 100% of LNCaP cells were EGFP positive and the mean intensity of EGFP fluorescence was approximately equivalent to that seen in experiments with Lv-CMV-EGFP (Fig. 3b). Thus, the very high levels of EGFP expression seen using a very strong non-discriminating promoter like CMV can be approximated in LNCaP cells by increasing the dosage of the ARR2PB promoter.

**Effects of AR Expression on Lv-ARR2PB-EGFP Activity**

To investigate whether the relative activity of Lv-ARR2PB-EGFP in different cell lines was due to the presence of AR, prostate (LNCaP, PC-3, DU145) and non-prostate (MCF-7, COS-1, HeLa) cell lines were transiently transfected with an AR expression plasmid (pRc-CMV-AR6) [16] and then grown for 48 hr in charcoal-stripped sera supplemented with 10 nM of the potent androgen, DHT. Levels of AR expression in transfected cells and in non-transfected controls were compared by Western blotting with endogenous levels of \( \beta\)-actin (Fig. 4). With the exception of LNCaP cells (Fig. 4, lane 1), which have a functional yet mutated form of endogenous AR [36], none of the non-transfected cell lines had detectable AR protein. For comparison, HeLa(hAR) and PC-3(hAR) cells with a stably transfected AR [37,38] were also examined. Each of the transfected lines expressed AR, although the levels were somewhat higher in LNCaP, MCF-7 and COS-1 cells (Fig. 4, lanes 2, 8, and 10). After densitometry, the AR: \( \beta\)-actin ratios were compared and also indicated that significant AR expression could be seen in LNCaP, MCF-7, COS-1, as well as Hela(hAR) and PC-3(hAR).

Five days after infection (MOI = 30) of AR-transfected and non-transfected cells with Lv-ARR2PB-EGFP and growth in 10 nM DHT, the cells were analyzed by fluorescence microscopy and by FACS (Fig. 5). The DU145 cells and all of the non-prostate cell lines tested in this manner showed essentially background levels of EGFP-positive cells and this did not change appreciably as a consequence of either transient or stable transfection of AR. Again, LNCaP cells had the highest proportion of EGFP positive cells, which changed only slightly (76–83%) after AR transfections (Fig. 5). The biggest incremental increase as a consequence of AR transfection was seen in PC-3 prostate cancer cells (26–37%). PC-3(hAR) cells, which stably
express AR, had a slightly lower level of EGFP expression than the wild type PC-3 line, but higher than non-prostate cell lines. These results suggest that while additional AR may enhance expression of EGFP in some Lv-ARR2PB-EGFP infected prostate cancer cell lines, the presence of AR is not in itself sufficient to impart cell-specific expression of this lentiviral construct.

Androgen Regulation of EGFP Expression in Lv-ARR2PB-EGFP Infected LNCaP Cells and Tumors

Although the lentiviral component of the Lv-ARR2PB-EGFP vector apparently did not interfere with EGFP expression in some prostate-derived cell lines, it was uncertain whether the ARR2PB promoter could still be androgen regulated. To test this, LNCaP cells infected with Lv-ARR2PB-EGFP were grown in charcoal-stripped media and then treated with 10 nM DHT. At various times following initiation of androgen treatment, the amount and intensity of EGFP fluorescence were monitored by microscopy and by FACS. In the absence of androgen, the number of fluorescent cells detected and the intensity of fluorescence was negligible (Fig. 6a). However, addition of DHT to the media resulted in a doubling of relative fluorescent intensity within 4 hr and a further linear increase until approximately 16 hr, after which it reached a plateau (Figs. 6c and 7A) and remained relatively constant as long as DHT was present in the media (Fig. 6b,f). In contrast to the rapid induction of EGFP by androgens, loss of EGFP fluorescence following androgen withdrawal was a relatively protracted process requiring several days (Fig. 7B). After transfer of LNCaP cells to charcoal-stripped media following growth in DHT, EGFP fluorescence declined in an almost linear fashion over the next few days, returning to near basal levels of detectability by day 6 (Figs. 6d and 7B). By comparison, the levels of fluorescence in LNCaP cells infected with Lv-CMV-EGFP were unaffected by changes in the androgen milieu (Fig. 6f). These results indicate that Lv-ARR2PB-EGFP can be tightly regulated in vitro by androgen addition or withdrawal.

The androgen dependence of EGFP expression was also determined in experiments where LNCaP cells pre-infected with Lv-ARR2PB-EGFP were grown as tumors in mice. LNCaP cells were infected in vitro with Lv-ARR2PB-EGFP or Lv-CMV-EGFP and then cultured for 5 days. Approximately $2 \times 10^9$ EGFP-expressing cells were injected subcutaneously into two groups of male nude mice. When the tumors attained a diameter of $\sim 0.5$ cm [31], one group of animals was castrated and another sham operated. After a further 5-day period, the tumors were excised and assayed for fluorescence. The results shown in Figure 8 indicate that after castration the levels of fluorescence declined by approximately 75% (relative to non-castrated controls) in LNCaP tumors infected with Lv-ARR2PB-EGFP, whereas only about a 20% reduction was seen in castrate animals bearing Lv-CMV-EGFP infected tumors. Thus the ARR2PB promoter in the lentiviral vector was androgen regulated in vivo such that there was maintenance of EGFP expression in non-castrated hosts, but a substantial reduction following androgen withdrawal.

In Vivo Expression of EGFP in Tumors Infected With Lv-ARR2PB-EGFP

To determine whether Lv-ARR2PB-EGFP could infect and express EGFP in a prostate-specific manner in vivo, groups of nude mice were inoculated subcutaneously with $2 \times 10^9$ LNCaP prostate cancer cells or an equivalent number of either A-549 lung carcinoma cells or CaKi-2 kidney carcinoma cells. When the tumors attained a diameter of approximately 0.5 cm, they were injected intratumorally with $2 \times 10^9$ IU of either Lv-CMV-EGFP or Lv-ARR2PB-EGFP. After a further 5 days, the tumors were excised, minced into pieces of about 1 mm in diameter and examined by
fluorescence microscopy for EGFP expression (Fig. 9). In experiments with Lv-CMV-EGFP, EGFP expression was detected in all LNCaP tumors, three out of four A-549 lung tumors, and four out of four CaKi-2 kidney tumors (Fig. 9a–c), indicating that the lentiviral vector could readily infect and express EGFP in all the tumors tested. However, in comparable experiments using Lv-ARR2PB-EGFP, only LNCaP tumors expressed EGFP (Fig. 9d) with no EGFP fluorescence evident in either type of non-prostate tumors (Fig. 9e,f). Thus the Lv-ARR2PB-EGFP vector retained a prostate expression preference in tumors growing in vivo.

**DISCUSSION**

The concept of using a tissue-specific promoter to express therapeutic genes in prostate cancers offers a very attractive way of potentially targeting locally advanced and metastatic tumors for eradication or control. In addition to developing a prostate-specific
promoter with sufficient activity, targeted gene therapy for prostate cancer requires the successful integration of transgenes into a suitable gene delivery system. The appropriate selection of an exogenous promoter and viral vector combination that may eventually serve as the backbone for a gene therapeutic is not a trivial issue, as either component may have properties that interfere with transgene expression or tissue delivery. For example, it has been shown that proteins encoded in the HSV-1 viral genome, can activate the tetO/minimal CMV promoter and interfere with tetracycline-regulated transgene expression [34]. We have made similar observations with respect to interference of tissue-specific gene expression by the transactivating immediate early gene products of HSV-1 [35]. The purpose of the present study was to determine whether vector constructs composed of the HIV-1 derived lentivirus containing different forms of the PB promoter could drive prostate-specific and androgen-regulated expression of a reporter gene, EGFP.

Various forms of the PB promoter have been well characterized with respect to their hormonal regulation and prostate-specific expression [19,20,32,33] and where direct comparisons have been made, have been shown to possess a higher degree of prostate-specific expression than PSA promoter forms [32]. While the PB promoter possesses relatively low activities in its wild type forms (i.e., $-426/+28$ PB and $-286/+28$ PB), engineered concatamers containing two or more $-244$ to $-96$ ARR motifs linked to the minimal tk or PB

**Fig. 6.** Androgen regulation of EGFP expression in Lv-ARR$_2$PB-EGFP infected LNCaP cells. LNCaP cells, infected with Lv-ARR$_2$ PB-EGFP (MOI = 30), were cultured for 5 days in (a) RPMI medium with charcoal-stripped 10% FBS or (b) in the same medium with 10 nM DHT present. LNCaP cells cultured in charcoal-stripped medium for 5 days (as in a) and then DHT was added for 24 hr. (c) LNCaP cells were initially grown in the presence of DHT (as in b), transferred to charcoal-stripped media, and then cultured for an additional 6 days in the absence (d) or presence (e) of DHT. LNCaP cells infected with control Lv-CMV-EGFP (MOI = 30) and cultured for 6 days in RPMI medium with charcoal-stripped 10% FBS (f).
promoter have been shown in vitro to act as strong androgen-inducible promoters [19,32]. The ARR2PB promoter combined with an adenoviral vector has been used to direct expression of apoptosis inducers, Bax or caspase 9, and subsequently kill prostate tumor cells [22–24]. While clearly demonstrating proof of principle for targeting with the PB promoter, the low level of persistent expression associated with adenovirus and the very strong immunological response elicited following multiple exposure to adenoviral vectors generally make them unsuitable as useful delivery systems for gene therapy of slow growing human prostate cancers [26,27]. By comparison, lentivirus are better suited for this purpose since by virtue of not transferring viral genes, they do not trigger a strong immune response and can therefore be used for multiple treatments of slow growing tumors [27]. Furthermore, unlike most retroviruses, lentiviruses do not require dissolution of the nuclear membrane for gene transduction; rather, they can access the nucleus of non-dividing cells through the nuclear pore and then integrate into the host genome [27]. This provides for long term expression of the transgene in a manner comparable to that achieved with stable transfections and hence allows for a persistent therapeutic effect in slow growing prostate tumors.

Inclusion of the $-426/+28$ PB, $-286/+28$ PB, and the ARR2PB promoters into the lentiviral vector did not interfere with preferential prostate-specific expression of the EGFP reporter in vitro, with virtually no expression detected visually or by FACS analysis in all three non-prostate cell lines tested (Fig. 2). In poorly differentiated PC-3 prostate cancer cells, the levels of PB promoter activity were low or in the case of DU145 cells, absent. In the more well-differentiated LNCaP prostate cancer cells much stronger EGFP expression was evident, with the relative rank of promoter activities being $-426/+28$ PB $< -286/+28$ PB $<$ ARR2PB PB, respectively (Fig. 3). Furthermore, based on comparable levels of EGFP expression, approximately a 3-times greater MOI was required for Lv-ARR2PB-EGFP to give fluorescence levels similar to those seen in LNCaP cells after infection with Lv-CMV-EGFP control. Thus, by adjusting the dose of infectious particles, a very high level of prostate-specific gene expression can be achieved with a lentivirus containing the ARR2PB promoter.

The preferential high levels of EGFP expression seen in LNCaP cells treated with Lv-ARR2PB-EGFP relative to the non-prostate and other prostate cancer cell lines were not due to the presence of an endogenous, functional AR. Transient transfection of AR expression...
Retention of androgen responsiveness of the ARR2PB promoter linked to a lentiviral backbone was clearly demonstrated with LNCaP cells treated with Lv-ARR2PB-EGFP (Figs. 6 and 7). Treatment with androgens resulted in a rapid linear increase in promoter activity which reached a plateau between 16 and 24 hr. Conversely, removal of androgens from the media caused a gradual decline in EGFP fluorescence with about 50% of the original amount remaining after about 4 days. This long half life probably reflects the turnover of the EGFP protein rather than incremental decreases in promoter activity. By comparison, in the continued presence of androgens, EGFP expression was maintained at near constant levels for the 6-day duration of the experiment. Essentially equivalent results were obtained in vivo; where prior infection with Lv-ARR2PB-EGFP resulted in tumors that continuously expressed EGFP in the presence of androgens (non-castrated hosts). Similarly, castration resulted in a 75% reduction in EGFP expression (Fig. 8). In essence, infection with Lv-ARR2PB-EGFP is the functional equivalent of creating a stable, androgen-regulated EGFP expression cell line. In the context of a gene therapy, the stable integration and persistence of the Lv-ARR2PB promoter activity would ensure a long duration of therapeutic efficacy for treating slow growing prostate cancers.

The preferential induction of the ARR2PB promoter in LNCaP cells in vitro relative to other tumor lines was also observed under in vivo conditions where lentivirus was injected directly into tumors (Fig. 9). All tumors tested after in vivo administration of Lv-CMV-EGFP showed similar levels of fluorescence, whereas only LNCaP tumors were fluorescent after infection with Lv-ARR2PB-EGFP. Thus, while all the tumors were equally infectable by lentiviral vectors, gene expression was restricted to LNCaP prostate tumors by the ARR2PB promoter.

The MOIs of 30–100 used in our in vitro experiments to obtain high levels (>80%) of infectivity and intensity of expression are similar to MOIs used by others in in vitro experiments with lentis and retroviruses [40–42], but may not be readily achievable under in vivo conditions. Although we cannot directly calculate an MOI for our in vivo experiments, we can obtain a rough estimate using tumor weight and injected dose. If in the case of LNCaP tumors we assume approximately 2.5–10^8 cells per gram of tumor [43], then each ~0.5 g tumor contains approximately 1–5 × 10^8 cells, depending on the blood and stromal cell components. Using these estimates, an intra tumor dose of 2 × 10^9 IU yields an MOI of ~4–20. This is approximately 35–87% lower than the lowest MOI of 30 used in our in vitro experiments. However, based on microscopy fields of green fluorescence seen throughout the LNCaP
tumors following injections at three sites, this was still sufficient virus to infect 40–50% of the cells with Lv-ARR2PB-EGFP or 60–70% of cells following injections with Lv-CMV-EGFP (Fig. 9). However, in the context of a gene therapy designed to kill all prostate tumor cells, one must effectively achieve 100% infectivity and gene expression through an enhanced delivery system or possibly development of a conditional, replication-competent viral vector.

CONCLUSIONS

Together our findings suggest that the Lv-ARR2PB vector may serve as an excellent targeting vehicle for in vivo delivery and for persistent, high levels of prostate-specific expression of an anti prostate cancer therapeutic gene.

ACKNOWLEDGMENTS

We are thankful for the technical assistance of Ms. Mary Bowden and Mr. Luk Bu.

REFERENCES

24. Lowe SL, Rubinchik S, Honda T, McDonnell TJ, Dong JY, Norris JS. Prostate-specific expression of Bax delivered by an...


