REPLICATION OF MUTANT HERPES SIMPLEX VIRUS TYPE 1 IN HUMAN PROSTATE AND NONPROSTATE CANCER CELLS UNDER THE CONTROL OF THE PROBASIN PROMOTER

by

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Department of Surgery

The University of British Columbia
Vancouver, Canada

Date April 25, 2000
ABSTRACT

The use of tissue- or tumor-selective promoters in targeted gene therapy for prostate cancer depends on strong and selective activity. The -426 to +28 bp probasin (PB) promoter has the potential to target selected genes to the prostatic epithelial cells. We showed that in the assay of luciferase reporter gene driven by PB promoter, activation of luciferase expression was 1.5-fold increase in human prostate cancer cell LNCaP relative to non-small cell lung cancer cell H460. Significant increase of luciferase activity was observed in both cell lines when infection with ICP27 (-) HSV-1 was added to PB-luciferase plasmid transfected LNCaP and H460 cells. In addition, an expression vector (PB-ICP27) was generated in which one of the herpes simplex virus type 1 (HSV-1) immediate early (IE) genes required for viral replication--ICP27 was regulated by PB promoter. This vector was transfected into LNCaP, H460 and one other nonprostate cancer cell HepG2 (hepatoma), the cell lines were then infected with ICP27 (-) HSV-1 and resulting viral stock from these cells were used to infect ICP27 expression 2-2 cell. The plaque yields from infected 2-2 cells were counted and reflected the production of ICP27 (-) HSV-1 replication in PB-ICP27 vector transfected cells that can express ICP27 protein. Although we found that the profound viral replication was observed in LNCaP cells compared to that of HepG2 cells, the production of viral replication in H460 cells was surprisingly significant higher than that in LNCaP cells and was contrasted to our PB-driven luciferase assay result. Our study demonstrates that (1). -426 to +28 bp PB promoter shows relative tissue-specific activity; (2). Infection with ICP27 (-) HSV-1 can enhance the expression levels of reporter gene driven by PB promoter and ICP27 (-) HSV-1 can only replicate in the cells expressing ICP27 protein; (3). The vector construct
containing ICP27 gene driven by PB promoter may not be an ideal delivery system for the purpose of prostate tissue-specific gene expression.
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<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>βgal</td>
<td>β-galactosidase</td>
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<tr>
<td>bp</td>
<td>basepair</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyl transferase</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>E. Coli</td>
<td>Escherichia Coli</td>
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<tr>
<td>HSV-1</td>
<td>herpes simplex virus type 1</td>
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<tr>
<td>ICP</td>
<td>infection cell protein</td>
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<tr>
<td>IE</td>
<td>immediate early</td>
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<tr>
<td>Kb</td>
<td>kilobases</td>
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<tr>
<td>KDa</td>
<td>kilodaltons</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>luc</td>
<td>luciferase</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>PB</td>
<td>probasin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PFU</td>
<td>plasque forming unit</td>
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<tr>
<td>PSA</td>
<td>prostate specific antigen</td>
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<tr>
<td>PSBP C3</td>
<td>prostatic steroid binding protein C3</td>
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<tr>
<td>PSMA</td>
<td>prostate specific membrane antigen</td>
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<tr>
<td>rr</td>
<td>ribonucleotide reductase</td>
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<td>--------------</td>
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</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>SV 40</td>
<td>Simian virus 40</td>
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<tr>
<td>tk</td>
<td>thymidine kinase</td>
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<tr>
<td>VSV</td>
<td>varicella zoster</td>
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INTRODUCTION

Developing novel therapeutic approaches for prostate cancer

Adenocarcinoma of the prostate poses a significant health problem in the industrialized western world. This disease is presently the most commonly diagnosed non-skin cancer in men and is the second leading cause of cancer-death in men (Landis et al., 1999). To date, effective therapy for prostate cancer is possible only when the disease has been diagnosed early enough to be still localized within the prostate gland. While frequently curable in its early stage, approximately a third of patients present with advanced disease (Long et al., 1997), the survival for patients with metastatic prostate cancer remains poor. When prostate cancer becomes advanced and acquires increased metastatic potential, endocrine treatment (androgen withdrawal therapy) is commonly used to delay the progression of disease, causing cessation of growth and induction of apoptosis of the androgen-dependent tumor cells. Some 70%-80% of patients experience at least partial remission, but treatment failure and tumor recurrence is almost inevitable several months or years later (Stearns et al., 1992). Several hypotheses for treatment failure involving androgen-dependent mechanisms have been suggested, such as hypersensitivity to residual non-testicular androgens, androgen receptor (AR) amplification, increased androgen biosynthesis from adrenal precursor steroids or mutations of the AR gene resulting in diminished receptor specificity (Labrie et al., 1993; Taplin et al., 1995; Visakorpi et al., 1995). On the other hand, androgen-independent mechanisms have been described, including activation of epidermal, fibroblast or other growth-factor pathways (Thompson, 1990). Some patients who failed hormonal therapy may respond to flutamide withdrawal (Scher et al., 1993). However, most patients do not respond to secondary
endocrine manipulation (Dijkman et al., 1994), and chemotherapy has demonstrated to be of limited value. For these reasons, it is important to develop novel therapeutic approaches.

Targeting gene therapy for prostate cancer by tissue-specific promoter

Recently remarkable advances in the genetics and biology of malignant tumor, and prostate cancer in particular, have contributed greatly to our understanding of tumor development and progression and have given rise to novel therapeutic approaches. Gene therapy has been one area of focus. Prostate cancer has several attributes that allow for the consideration of gene therapy by multiple strategies. The prostate gland is easily accessible transurethrally and transrectally. This anatomic accessibility allows for direct gene therapy approaches. Additionally, prostate cancer is unique because of the presence of tumor-specific markers such as prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), that enhance the potential for strategies of tissue specific delivery of gene therapy. The application of gene therapy to prostate cancer has brought with many challenges unique to this malignancy. Prostate cancer carries great heterogeneity in tumor morphology between both individual tumors and various cell populations within a single tumor. Such heterogeneity makes alterations of cell behavior difficult with a single genetic manipulation. Another major difficulty with gene therapy in prostate cancer is that most gene vectors are nonspecific, and thus tissue targeting except in the case of direct injection, can be difficult. Finally, a therapeutic gene must be chosen that is capable of altering the biological behavior of the cancer cell to slow its
growth, reduce invasive potential, induce apoptosis or even completely eradicate the tumor cell.

Approaches to improve tissue specificity generally involve molecular strategies to improve vector tropism by changing the viral coat, or modulation of the vector regulatory mechanisms that result in restricted transcription (Hart, 1996). Transcriptional targeting of gene transfer agents focuses on restricting transgene expression in target cells through the use of upstream genetic elements such as cell-specific promoters and/or enhancer elements. The basic regulatory apparatus of eukaryotic transcription includes a promoter, enhancers, response elements, and upstream activation sequences and locus control regions. The promoter is a fixed region located not more than 100 bases from the transcription start site and includes a TATA box, a CAAT box or a GC box. Enhancers are elements found at multiple sites within a gene whilst most other regulatory elements are found upstream of the promoter. Both response elements and upstream activation sequences are typically located within 1 kilobase (kb) of the transcription start site and may confer tissue-specific expression and regulate expression of multiple genes. Locus control regions, which are generally far removed from the transcriptional start site of a particular gene, can also play a role in co-ordinating expression between multiple genes. All these elements have been proposed as regulators of transgene expression that could determine tissue-specific expression. Thus far, tissue-specific promoters have been exploited to achieve transcriptional targeting. In establishing a successful prostate cancer gene therapy strategy, it is useful to decide to use a prostate tissue-specific promoter to provide selective prostate-specific gene expression.
Probasin promoter as prostate tissue specific promoter

In the case of prostate, there are several candidates for tissue specific promoters in prostate cancer, and they include the rat prostatic steroid binding protein [PSBP C3(1)] gene (Maroulakou et al., 1994; Shibata et al., 1996), prostate specific antigen (PSA) (Partin et al., 1994; Pang et al., 1997), prostate specific membrane antigen (PSMA) (Troyer et al., 1995; Murphy et al., 1996), and probasin (Rennie et al., 1993). However, expression of C3(1), PSA and PSMA are not restricted exclusively to prostate tissue (Maroulakou et al., 1994; Diamandis et al., 1996; Lintula et al., 1997). For example, PSA has been shown to be present in a variety of normal and neoplastic tissue including 30%–40% of breast tumors and in some salivary duct, colon and liver cancers (Diamandi et al., 1996; James et al., 1996). Use of the 630 basepair (bp) human PSA promoter region to control expression of an activated Ha-ras oncogene in transgenic mice led to the development of salivary gland, but not prostate tumors (Schaffner et al., 1995). Moreover, when a 5-kb region upstream of the rat PSBP c3(1) gene was used to control expression of the simian virus 40 (SV 40) T-antigen, tumors were elicited in the prostate, but gene expression was not highly restricted to the prostate and other tissue abnormalities were also common (Maroulakou et al., 1994).

Probasin (PB) is an androgen- and zinc-regulated protein first characterized in the dorsolateral prostate of the rat. Immunohistochemistry with polyclonal and monoclonal antibodies has shown dual cellular localization of PB within the ducts and nucleus of epithelial cells of the prostate (Spence et al., 1989). PB gene expression is developmentally regulated in the prostate, the minimal -426 to +28 bp PB promoter
fragment carries sufficient information to direct developmentally and hormonally regulated expression of a heterologous gene specifically to the prostate in transgenic mice. Furthermore, this expression was both male specific and restricted to the epithelial cells of the lateral, dorsal, and ventral prostatic lobes. Constructs containing the -426 to +28 bp promoter region of the PB gene linked to the chloramphenicol acetyl transferase (CAT) reporter gene have resulted in hormonally regulated CAT gene expression in human prostate cancer cell lines (Rennie et al., 1993; Kasper et al., 1994). Furthermore, -426 to +28 bp of the PB 5'-upstream region had the ability to target CAT gene expression to the prostate in a tissue-specific manner while maintaining developmental and androgen-specific regulation (Greenberg et al., 1994). The ras oncogene, rasT24, linked to the -426 to +28 bp PB promoter facilitated the development of prostate epithelial cell hyperplasia (Barrios et al., 1996), while SV40 T-antigen linked to the -426 to +28 bp PB promoter resulted in the development of both benign prostatic hyperplasia and prostate cancer (Greenberg et al., 1995). Thus, the minimal -426 to +28 bp PB promoter region has the potential to target selected genes to the prostatic epithelial cells.

The selection of HSV-1 as viral-based cancer gene therapy

Several gene therapy strategies have been examined for their therapeutic potential in the treatment of cancer. These approaches have been based on 1) Introduction of genes that produce multiple copies of RNA decoys; 2) Expression of transdominant proteins that can functionally replace mutant or deleted cellular proteins; 3) Modification of adoptively transferred T cells; 4) Expression of cell surface antigens to enhance the antitumor response; 5) Expression of ribozymes that cleave specific DNA sequences; 6)
Intracellular production of antibodies to bind to specific proteins; 7) Expression of foreign enzymes that render cells susceptible to otherwise nontoxic prodrugs; 8). Production of intracellular toxins that lead to cell death; 9). Modification of hematopoietic stem cells to decrease toxicity from chemotherapy; or 10). Infections with oncolytic replicating viruses that can themselves destroy tumor cells (Clark, 1996).

The idea of using replicating viruses to treat tumors was suggested as early as 1904 by George Dock (Dock, 1904), who reported the case of a woman who experienced a dramatic remission of her leukemia following a presumed attack of influenza. In fact, a number of clinical trials were conducted in the 1950s, 1960s and early 1970s with some success (Smith et al., 1956; Asada, 1974), although interest in the use of viral-based cancer therapy waned thereafter. Advances in virology and molecular biology now allow the "engineering" of viruses with specific properties, suggesting that the idea of viral-base cancer therapy should be revised.

There are two major approaches to use viruses for cancer gene therapy. The first is to use the viruses as vectors, such as retroviruses or adenoviruses, to shuttle antitumor genes into the tumor cells. The oncolytic effect is not produced by the virus but by administrating a relatively nontoxic pro-drug, which is converted into a toxic metabolite, poisoning the infected cell and, to some extent, adjacent cells by a "bystander effect". Death of the infected cell limits and terminates the therapeutic process. In addition to the inherent inability of replication incompetent viruses to spread beyond the initial cells infected, some of the disadvantages of these systems are the variability in transduction
rates, the need for implantation of producer cells, and the possibility of insertional mutagenesis causing secondary tumors (e.g., retroviruses). The second major approach is using a genetically engineered virus, not as a vector to transfer genes, but as a direct tumor cell killer. Safety is an important consideration in the development of viral-based gene therapy as an approach to treat cancer. The ideal solution would be a virus that replicates selectively in the tumor cells, but can neither infect nor damage normal host tissues.

Currently, Herpes simplex viruses type 1 (HSV-1) has attracted considerable interest for its potential as a gene transfer vector and viral based gene therapy. Much has been known about HSV-1 at the molecular biological level and it is able to infect a wide range of replicating and nonreplicating cells. HSV-1 is one of the human herpes viruses, the other members of this DNA family include HSV-2, varicella zoster (VSV), cytomegalovirus (CMV), Epstein-Barr virus (EBV). At first, HSV-1 was explored as a vehicle for gene transfer into the central nervous system, but studies using HSV-1 have demonstrated cytotoxicity from cellular lysis (Johnson et al., 1992). Entry of wild-type HSV-1 into cells leads to a sequential cascade of viral gene expression that ultimately results in the production of multiple progeny virions and cell death (Roizman et al., 1985). A permissive infection with HSV-1 results in profound alterations in the host cell at a number of different levels. Most wild-type strains of HSV-1 induce cell rounding early in infection. This event is associated with a breakdown of components of the cytoskeleton, including the action containing microfilaments (Heeg et al., 1986), and a concomitant loss of cell surface matrix proteins such as fibronectin, leading to cell detachment.
Infection with HSV-1 also leads to rapid inhibition of host cell DNA synthesis (Roizman et al., 1964) and to the induction of chromosomal damage (Hampar et al., 1961).

The use of genetically engineered HSV-1 has been designed to kill tumor cells while sparing normal tissue. Malignant tumor cells are by nature a dividing cell population, whereas mature neurons and many somatic cells are postmitotic. HSV-1 mutants can be genetically engineered to require a dividing host-cell population for efficient replication. In some instances, investigators have attempted to achieve this by creating HSV mutants deficient in one of the genes coding for enzymes needed for nucleic acid metabolism and replication in non-dividing cells, i.e., thymidine kinase (tk') or ribonucleotide reductase (rr'). Without these genes, the virus is unable to replicate unless the cell that it infects supplies the deficient enzymes, which are present normally only in dividing cells. Thus, viral replication is able to proceed in dividing cells and causes cell death. Previous studies demonstrated that attenuated, single-gene mutants of the HSV-1 was effective in killing malignant nervous system tumors (Markert et al., 1992) and other system tumors (Carroll et al., 1996; Kucharczuk et al., 1997; Yoon et al., 1998). However, each of these viruses has limitations that could preclude use in humans. Therefore, to provide adequate safeguards for possible clinical trials. Further studies would be necessary to engineer HSV-1 to have increased tumor cell specificity with decreased cytopathogenicity toward normal cells. By deleting the genes for tk, rr simply or in combination, the above strategies of vector construction selectively target features that are common to many
cancers. However, if viral replication can be confined to specific types of cancer cells, it should be possible to use less attenuated and presumable more efficacious vectors.

**Targeting HSV-1 replication to prostate cancer by placing immediate-early gene essential for viral replication under the control of PB promoter**

The lytic infection of a susceptible cell by HSV-1 consists of a temporally regulated program of viral gene expression, viral DNA replication, and virion assembly. The genome of HSV-1 has been completely sequenced, it encodes approximately 80 viral proteins that are expressed sequentially in three major kinetic classes: immediate early (IE or \( \alpha \)), early (\( \beta \)), and late (\( \gamma \)). (McGeoch et al., 1986; McGeoch et al., 1988). Soon after the virus penetrates the cell, the viral genome enters the nucleus, where the viral genes are transcribed by the host RNA polymerase II (Constanzo et al., 1977). The first genes to be transcribed are called IE genes, the IE genes encode \( \alpha \) proteins that regulate the infectious process. One function of the \( \alpha \) proteins is to induce the transcription of the \( \beta \) genes, transcription of \( \beta \) genes is dependent upon the activities of the \( \alpha \) proteins (Honess et al., 1975). The protein products of the \( \beta \) genes are largely enzymes involved in viral DNA synthesis. Synthesis of \( \beta \) proteins signals the onset of viral DNA synthesis, which in turn is required for maximum expression of late genes. Soon after the expression of \( \beta \) proteins, viral DNA replication begins. The process of viral DNA replication is critical for the expression of the late set of viral genes, the \( \gamma \) genes (Holland et al., 1980). \( \gamma \) genes specify virion structural proteins and have been divided into two groups, \( \gamma_1 \) and \( \gamma_2 \). Transcription of \( \gamma_1 \) genes from input genomes occurs at low levels in the absence of viral DNA synthesis but is maximal when progeny viral DNA is
synthesized. By contrast, the accumulation of $\gamma_2$ transcripts is stringently dependent upon viral DNA synthesis (Holland et al., 1980; Ohare et al., 1985). In addition to the requirement for viral DNA synthesis, maximum expression of both $\gamma_1$ and $\gamma_2$ genes requires functional $\alpha$ proteins (Honess et al., 1975). The $\gamma$ genes for the most part encode proteins that are components of the virus particle or are involved in its assembly. Following $\gamma$ gene expression, virions are assembled in the cell nucleus and then mature and exit the cell via a complex interaction with the host secretory apparatus. In summary, IE genes encode the major HSV regulatory proteins, $\beta$ genes code for proteins involved in viral DNA synthesis, and $\gamma$ genes specify virion structural proteins. Control of gene expression during HSV-1 infection appears to occur primarily at the level of transcription (Smith et al., 1988).

IE genes are expressed in the absence of prior viral protein synthesis (Clements et al., 1977). HSV-1 encodes four $\alpha$ proteins, which have been shown to affect the expression of follow HSV-1 genes (Honess et al., 1974). These proteins are termed infected cell protein (ICP) 4, ICP0, ICP27, and ICP22. The fifth $\alpha$ protein, ICP47, does not appear to affect HSV-1 gene expression (Mavromara-Nazos et al., 1986). All IE proteins except ICP47 are nuclear phosphoproteins, and act to regulate their own synthesis as well as the synthesis of proteins of later proteins (Mavromara-Nazos et al., 1986; Quinlan et al., 1985). Genetic studies have demonstrated that two of the five $\alpha$ proteins have regulatory roles which are absolutely essential for viral growth (Deluca et al., 1985; McCarthy et al., 1989). One of these, infected cell protein (ICP4), which is encoded by IE3 gene, is the major transcription-activating protein of HSV-1; it is required throughout infection for
the transcription of the β and γ genes (DeLuca et al., 1988). Since ICP4 is critically required for expression of viral β and γ genes, an IE3 mutant expresses very few viral genes and is completely replication defective (DeLuca et al., 1985). The second essential α protein is the 63-kDa ICP27 polypeptide. The analysis of viral mutants containing temperature-sensitive mutations in the IE2 gene (encodes the expression of ICP27 protein) first demonstrated that ICP27 has an essential regulatory role during infection and this role is required after the onset of early gene expression and viral DNA synthesis (Sacks et al., 1985). Thus, ICP27 appears to mediate the transition between the β and γ phases of viral infection. Recently, a replication-defective virus bearing a deletion in the ICP27 gene (ICP27 (-) HSV-1) was isolated and characterized. Mutants lacking ICP27 fail to synthesize early or late viral polypeptides but continue to express the other IE genes (e.g., ICP4, ICP0). This mutant expresses no detectable ICP27 polypeptide and can only be replicated in ICP27 expression cells. Under nonpermissive conditions, this mutant replicated a reduced amount of viral DNA, was defective in inducing many γ-1 proteins, and failed to induce γ-2 proteins (McCarthy et al., 1989).

From these findings it is presumed that the replication and cytotoxicity of HSV-1 results from the expression of HSV-1 IE gene products and therefore it follows that elimination of these gene products may result in the production of non-cytotoxic HSV viruses. In gene therapy for prostate cancer using HSV-1, it would be desirable to create vectors that allow exogenous control of expression of HSV-1 regulatory protein required for an essential lytic function.
It is anticipated that deletion of ICP27 gene will essentially shut down viral gene expression upon infection of non-complementing cell lines, rendering the virus safe, non-cytotoxic and capable of establishing latency in most non-dividing cells. A vector containing the ICP27 gene controlled by prostate tissue-selective promoter can be constructed, and transferred into prostate cancer cell lines. The transfected cells will then be infected with replication defective virus bearing a deletion in the ICP27 gene (ICP27 (-) virus). The vector construct will express ICP27 protein as a complement to the ICP27 (-) HSV-1 to allow the virus to propagate and replicate only in the prostate cancer cells since the PB promoter should only be active in prostate cells. In this way, HSV-1 viral replication, and subsequent intrinsic prostate cancer cell death will only occur when the ICP27 genes are transcribed initiated by the prostate tumor-selective promoter. The concept of using a tissue-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. Since prostate tissue specific promoters and viral transcription factors are readily amenable to manipulation, it may now be possible by genetic engineering to generate selective viral gene expression in target cell through the use of upstream genetic elements such as prostate cell-specific promoters.

Objective of the present study
As mentioned above, among the promoters and cell types tested, the best level of expression in prostate cells combined with limited expression in other cell types is seen with the -426 to +28 bp promoter region of the PB gene, and HSV-1 ICP27 protein is essential for virus replication. The hypothesis to be tested in the present study is that a
unique combination of -426 to +28 bp PB promoter and the HSV-1 ICP27 gene controlled by the promoter will express ICP27 protein only in prostate cancer cells and complement ICP27 (-) HSV-1 resulting in prostate specific oncolytic viral replication. To test this hypothesis, the present study aimed to construct a plasmid containing the ICP27 gene controlled by -426 to +28 bp PB promoter. The constructed plasmid PB-ICP27 will be transfected into LNCaP Cells (androgen-dependent prostate cancer cell line) and other non-prostate cancer cell lines. After transfection, cells will be superinfected with ICP27 (-) HSV-1 virus (viral deletion mutants which do not express ICP27, this mutant is defective for growth in normal cells but can be efficiently propagated and replicated in ICP27-expressing cell lines). Plaque assays will be performed on 2-2 cells (ICP27-expressing cells which carry a stable integrated copy of the ICP27 gene) by virus stocks from the transfected and superinfected prostate and nonprostate cancer cells to determined whether ICP27 (-) HSV-1 could be complemented by the expression of ICP27 protein in PB-ICP27 transfected cell lines and allow the virus to replicate. If PB-ICP27 transfected cells can express ICP27 protein, then ICP27 (-) HSV-1 could be complemented and replicate in these cells, the viral stock from these cells can then infect 2-2 cells and result in 2-2 cells death and produce the plaques; otherwise there will be no plaque yield. The tissue-specific expression of the luciferase reporter gene controlled by PB promoter will be studied first by luciferase reporter assay in which the vector contains the -426 to +28 bp PB promoter controlling luciferase report gene will be transfected into the prostate cancer cell line and nonprostate cancer cell lines. Following transfection, the cells are assayed for the activity of the firefly luciferase protein.
MATERIALS AND METHODS

General enzymes and chemicals

DNA modifying enzymes including restriction endonucleases were from Gibco BRL (Burlington, ON, Canada), New England (Mississauga, ON, Canada), and Pharmacia (Baie d'Urfe, QE, Canada). Fine chemicals were purchased from Sigma (Mississauga, ON, Canada). Cell culture medium and transfection reagents were also purchased from Gibco BRL.

Construction of recombinant PB-ICP27 plasmids

1. Preparing the vector for cloning

The PBSBH vector contains the -426 to +28 bp PB promoter was supplied by Dr. Paul Rennie (Prostate Research Center, Vancouver General Hospital). It was constructed briefly as described below: a 454-bp Hind III-BamH I fragment containing -426 to +28 bp PB promoter sequences (Figure 1) from PBH500 plasmid was subcloned into the Hind III-BamH I sites of Stratagene pBluescript SK (+/-) vector (Figure 2). The total size of this vector was 3.382-kb, and carried a marker gene for ampicillin resistance. The fragment of 7-bp was taken out from PBSBH vector using the restriction enzymes Not I and Xba I as described below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBSBH DNA</td>
<td>5 ul</td>
</tr>
<tr>
<td>Not I</td>
<td>3 ul</td>
</tr>
<tr>
<td>One-phor-all buffer, 10x</td>
<td>3 ul</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>19 ul</td>
</tr>
<tr>
<td>Final volume</td>
<td>30 ul</td>
</tr>
</tbody>
</table>
Figure 1. Sequence of PB 5'-upstream DNA. The start of transcription is shown as V with the number starting immediately after as +1. All negative numbering is relative to the start site of transcription. The CAAT box and TATAA box are underlined.
Figure 2. The circle map of PBSBH vector. The -426 to +28 bp PB promoter (454 bp) was inserted into the Hind III/ BamH I site of pBluescript SK (+/-) plasmid. This construct (3.382-kb) containing the -426 to +28 bp PB promoter was named PBSBH.
Prepared in autoclaved microcentrifuge tube and incubated at 37°C for 1.5 hours. Then added

<table>
<thead>
<tr>
<th>Xba I</th>
<th>3 ul</th>
</tr>
</thead>
<tbody>
<tr>
<td>One-phor-all buffer, 10x</td>
<td>3 ul</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>24 ul</td>
</tr>
</tbody>
</table>

Final volume 60 ul

Incubated at 37°C for 1 hour. After the digestion, added 10ul of blue 6xloading dye to the reaction and proceeded to gel analysis as described below:

(1) To prepare the 0.9% agarose gel, added 0.27 g of agarose to 30 ml of TBE 1x buffer;
(2) Heated the mixture in a microwave oven for the minimum time required to allow all the agarose to dissolve;
(3) Cooled the solution to 50°C-60°C, added the ethidium bromide into the gel at a concentration of 0.5 ug/ml, then poured the gel to the minigel apparatus, and allowed the gel to form completely (30 min at room temperature);
(4) Loaded the above digestion samples into the wells. Connected the gel apparatus to an electrical power supply and applied the 90 voltages to the gel;
(5) After electrophoresis was complete, removed and placed the gel on an UV lightbox and excised the desired DNA (3.36-kb) band using a clean, sterile scalpel.

2. Preparation of the insert ICP27 DNA fragment

The PH27 plasmid was obtained from Dr. William Jia (The Prostate Center, Vancouver General Hospital). This plasmid contained the ICP 27 gene, the expression cassette
containing the 2.7 kb ICP 27 gene was excised from this plasmid by Not I and Drd I digestion as follow:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid PH27 DNA</td>
<td>10 ul</td>
</tr>
<tr>
<td>Not I</td>
<td>4 ul</td>
</tr>
<tr>
<td>Drd I</td>
<td>4 ul</td>
</tr>
<tr>
<td>One-phor-all buffer, 10x</td>
<td>8 ul</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>5 ul</td>
</tr>
<tr>
<td>Final volume</td>
<td>60 ul</td>
</tr>
</tbody>
</table>

Incubated at 37°C for 1.5 hours. After digestion, added 10 ul of blue 6xloading dye to the reaction and proceeded to gel analysis as described above. Finally excised the desired DNA (2.7-kb) band using a clean scalpel.

3. Ligation of plasmid vector and insert DNA

The excised PBSBH vector and insert ICP 27 DNA were added together and purified by QIA quick Gel Extraction Kit (QIAGEN, Mississauga, ON, Canada). The procedures as follow:

1. Weighted the gel slice in a colorless tube. Added 3 volumes of Buffer QG to 1 volume of gel;

2. Incubated at 50°C for 10 min until the gel slice has completely dissolved. To help dissolve gel, mixed by vortexing the tube every 2-3 min during the incubation;

3. After the gel slice dissolved completely, checked up that the color of the mixture was yellow (similar to Buffer QG without dissolved agarose), and added 1 gel volume of isopropanol to the sample and mix;

4. Placed a QIAquick spin column in a provided 2 ml collection tube;
(5). To bind DNA, applied the sample to the QIAquick column, and centrifuged for 1 min;

(6). Discarded flow-through and placed QIAquick column back in the same collection tube;

(7). Added 0.5 ml of Buffer QG to QIAquick column and centrifuged for 1 min;

(8). To wash, added 0.75 ml of Buffer PE to QIAquick column and centrifuged for 1 min;

(9). Discarded the flow-through and centrifuged the QIAquick column for an additional 1 min at 13,000 rpm;

(10). Placed QIAquick column into a clean 1.5 ml microcentrifuge tube;

(11). To elute DNA, added 30 µl elution nuclease-free water to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuged for 1 min.

After the PBSBH vector and insert ICP27 DNA were eluted, added 1ul T4 DNA ligase, 8 ul ligase 5xBuffer and nuclease-free water to final volume of 40ul. Incubated at room temperature for 1 hour. Immediately cooled the tube on ice, then added 1ul T4 DNA polymerase and 2ul dNTP, incubated at 16°C for 30 min. After that, added another 1ul T4 DNA ligase and incubated at 16°C overnight.

4. Transformation of competent cells

Thawed a 200ul aliquot of competent cells (Escherichia Coli Strain) on ice, added 2ul of aboved ligated DNA to the competent cells. Then mixed by gently swirling the pipet tip, incubated on ice for 30 min, heated the tube at 37°C for 45 seconds, placed on ice again to cool for 2 min. Added 2ml of LB medium (bacterial culture media, including 10g
tryptone, 5g yeast extract, and 10g NaCl in per liter deionized water), and incubated for 45 min at 37°C with shaking at 150 rpm, then plated 100 ul of the transformation mixture onto the selection LB plate containing 100 ug/ml ampicillin, incubated overnight at 37°C. Each of 12 clones was picked up and transferred to the 4ml LB medium containing 100ug/ml ampicillin, this was allowed to grow in 4ml LB medium overnight at 37°C with vigorous shaking to replicate plasmid DNA.

5. **Analysis of transformant (Identify the clone of interest)**

Inoculated 4ml of LB medium (starter culture) containing 100ug/ml ampicillin with a single bacterial colony picked up from a fresh culture in ampicillin selective plate as described above. Used a sterile 15 ml tube for adequate aeration and incubated at 37°C with vigorous shaking overnight. Then placed 1.5 ml of the overnight culture into a microcentrifuge tube and centrifuge at 13,000 rpm for 1 min. Removed the LB medium by aspiration, leaving the bacterial pellet as dry as possible. After that, using the QIAprep Spin Miniprep Kit (QIAGEN, Mississauga, ON, Canada; designed for purification of up to 20ug of high-copy plasmid DNA from 1-5 ml overnight cultures of E. Coli in LB medium) to isolate plasmid DNA. The procedure was described below:

(1) Resuspended pelleted bacterial cells in 250ul of Buffer P1 (Resuspension buffer) and transfered to a microfuge tube;

(2) Added 250ul of Buffer P2 (Lysis buffer) and gently inverted the tube 4-6 times to mix;

(3) Added 350ul of Buffer P3 (Neutralization buffer) and inverted the tube immediately
by gently 4-6 times;

(4). Centrifuged at 13,000 rpm for 10 min. During the centrifugation placed a QIAprep spin column in a 2ml collection tube;

(5). Applied the supernatants from above to the QIAprep column by decanting, then centrifuged at 13,000 rpm for 1 min, discarded the flow-through;

(6). Washed QIAprep spin column by adding 0.5ml of Buffer PB and centrifuging 13,000 rpm for 60 seconds. Discarded the flow-through;

(7). Washed QIAprep spin column by adding 0.75ml of Buffer PE and centrifuged at 13,000 rpm for 60 seconds;

(8). Discarded the flow-through, and centrifuged at 13,000 rpm for an additional 1 min to remove residual wash Buffer;

(9). Placed QIAprep column in a clean 1.5ml-microfuge tube. To elute DNA, added 30ul of nuclease-free water to the center of QIAprep column, let stand for 1 min, and centrifuged at 13,000rpm for 1 min.

Recombinant plasmid obtained from cloning was screened by restriction digestion from above miniprep DNA with restriction enzyme Not I or Sal I and gel analysis. The digestion procedures were set up in the reaction microcentrifuge.

| PB-ICP27 DNA from above miniprep | 1 ul |
| Not I | 1 ul |
| Reaction buffer 3 | 1 ul |
| Nuclease-free water | 7 ul |
| Final volume | 10 ul |

PB-ICP27 DNA from above miniprep | 1 ul |
Sal I | 1 ul |
Reaction buffer 2 1 ul
Nuclease-free water 7 ul
Final volume 10 ul

6. Preparation of PB-ICP27 plasmid DNA used for transfection

If the above miniprep DNA screened by Not I and Sal I digestion and gel analysis was confirmed to be the desired clone, taken 1ml from above starter culture into 500ml LB medium containing 100ug/ml ampicillin. Grown at 37°C for overnight with vigorous shaking (250 rpm).

Harvested the bacterial cells by centrifugation at 7,500rpm for 15 min at 4°C. Then purified plasmid DNA using QIAGEN Plasmid Maxi kit (QIAGEN, Mississauga, ON, Canada). The procedure was described below:

(1). Resuspended the bacterial pellet in 10ml of Buffer P1 (Resuspension buffer containing 50mM Tris.Cl, pH8.0; 10mM EDTA; and 100ug/ml RNase A);
(2). Added 10ml of Buffer P2 (Lysis buffer containing 200mM NaOH, and 1% SDS), mixed gently but thoroughly by inverting 4-6 times, and incubated at room temperature (RT) for 5 min;
(3). Added 10ml of chilled Buffer P3 (Neutralization buffer containing 3.0M potassium acetate, pH5.5), mixed immediately but gently by inverting 4-6 times, and incubated on ice for 20 min;
(4). Centrifuged at 15,000 rpm for 30 min at 4°C, removed supernatant containing plasmid DNA promptly, applied the supernatant to a prewetted, folded filter;
(5). Equilibrated a QIAGEN-tip 500 by applying 10ml Buffer QBT (Equilibration buffer
containing 750mM NaCl; 50mM MOPS, pH7.0; 15% isopropanol; and 0.15% Triton X-100), and allowed the column to empty by gravity flow;

(6) Applied the supernatant from above filter to the QIAGEN-tip and allowed it to enter the resin by gravity flow;

(7) Washed the QIAGEN-tip with 2x30ml Buffer QC (wash buffer containing 1.0M NaCl; 50mM MOPS, PH7.0; and 15% isopropanol); Eluted DNA with 15ml Buffer QF (Elution buffer containing 1.25M NaCl; 50mM Tris.Cl, pH8.5; 15% isopropanol);

(8) Precipitated DNA by adding 10.5ml RT isopropanol to the eluted DNA. Mixed and centrifuged immediately at 15,000 rpm for 30 min at 4°C. Carefully decanted the supernatant;

(9) Washed DNA pellets with 5ml RT 70% ethanol, and centrifuged at 15,000 rpm for 10 min. Carefully decanted the supernatant without disturbing the pellet;

(10) Air-dried the pellet for 10 min, and redissolved the DNA in 200ul 100mM Tris.Cl. pH 8.5 buffers.

To measure the yield, PB-ICP27 plasmid DNA concentration and purity were determined by spectrophotometers to measure the A260 value and A260/A280 ratios.

Prostate and Nonprostate Cancer Cell Lines and Cell Culture

Androgen-dependent prostate cancer cell line (LNCaP) was obtained from Dr. Paul Rennie (Prostate Research Center, Vancouver General Hospital) and cultured in RPMI 1640 medium, containing 10% fetal bovine serum, 2mM L-glutamine, and 100 units/ml
of penicillin/streptomycin. Human hepatoma cell line (HepG2) and human large lung cancer cell line (H460) were obtained from Dr. William Jia and cultured in Dulbecco's Minimal Essential Medium (DMEM), containing 10% fetal bovine serum, 2mM L-glutamine and 100 units/ml of penicillin/streptomycin.

**Evaluation of Expression of Luciferase Reporter Constructs in Prostate and Nonprostate Cell Lines**

To characterize the PB promoter activity and specificity, the recombinant plasmid containing -426 to +28 bp PB promoter fragment fused to PGL-2 luciferase reporter vector (PB-Luc) was obtained from Dr. Luke Bu (Prostate Research Center, Vancouver General Hospital) and constructed briefly as described below: the -426 to +28 bp PB promoter was cloned upstream of the Luciferase gene in Promega's pGL-2 Luciferase Reporter Vector (Figure 3).

The effect of -426 to +28 bp PB promoter on the tissue specific expression of luciferase reporter gene activity was tested by transfection of the PB-Luc plasmid into the LNCaP cells and H460 cells. The cells were cultured in 12-well tissue culture plate and grown in the appropriate complete growth medium. When cells were grown about 60% confluent, the 1ug purified PB-Luc plasmid DNA was transfected using Lipofect AMINE plus Reagent (Gibco BRL, Burlington, ON, Canada) as described below:
Figure 3. The circle map of PB-luc reporter vector. The -426 to +28 bp PB promoter was inserted upstream of the luciferase gene in promega's pGL 2-basic vector.
(1). Pre-complexed the PB-luc plasmid DNA with the PLUS Reagent: diluted 1ug DNA into 50ul serum-free dilution medium (using the medium normally used to culture the target cells), each well culture cells used 1ug PB-luc DNA mixed 5ul PLUS Reagent and incubated at RT for 15 minutes;

(2). Diluted 2ul Lipofect AMINE Reagent into 50ul serum-free dilution medium (for one well culture use) in a second tube, and mixed;

(3). Combined pre-complexed DNA (from step 1) and diluted Lipofect AMINE Reagent (from step 2); mixed and incubated for 15 minutes at RT;

(4). While complexes were forming, replaced complete medium on the culture cells with 0.4 ml serum-free transfection medium;

(5). Added 0.1ml DNA-PLUS-Lipofect AMINE Reagent complexes to the each culture well containing 0.4ml fresh serum-free medium, mixed gently and incubated at 37°C at 5% CO₂ for 3 hours;

(6). After 3 hours incubation, added 0.5 ml fresh, appreciate complete medium to each well, and incubated at 37°C at 5% CO₂ for 48 hours.

The luciferase activity in each sample was normalized for variations in transfection efficiency by measuring the level of β-galactosidase expression with the co-transfected of 1ug pCMV-βgal plasmid. This plasmid is designed as a positive control vector for monitoring transfection efficiencies of mammalian cells. The CMV promoter and enhancer drive transcription of the bacterial lacZ gene which, in turn, is translated into the β-galactosidase enzyme. β-galactosidase is an excellent reporter enzyme which can be assayed quickly and directly in cell extracts using spectrophotometric assay. In order
to determine whether the infection with ICP27 (-) HSV-1 will influence the expression of the luciferase reporter gene, the superinfection with 20 ul ICP27 (-) HSV-1 viral stock generated previously in the lab was added to these two cell lines after 2 days PB-Luc and pCMV-βgal plasmid co-transfection. One day after the superinfection with ICP27 (-) HSV-1, the cells were harvested for luciferase activity and β-galactosidase enzyme assays as described below:

1. Removed the growth medium from the cells, washed the cells twice with phosphate-buffered saline (PBS), being careful not to dislodge any of the cells. Removed as much of the final wash as possible with a pipet tip;

2. Added 250ul of 1xReporter lysis Buffer (Promega, Madison, WI, USA) to cover the cells. Rocked the culture plate slowly several times to ensure complete coverage of the cells;

3. Incubated the culture plate at room temperature for 15 min, slowly rocked the plate several times through the incubate period;

4. Scraped all areas of the plate surface, taken care to scrape down all visible cell debris, using a pipet, transferred the cell lysate to a microcentrifuge tube and placed the samples on ice;

5. Vortexed the tube for 10-15 seconds, then centrifuged at top speed in a microcentrifuge for 2 min at 4°C, then transferred the supernatant to a fresh tube;

6. Ten microliters of cell extract supernatant was mixed with 100ul luciferase assay reagent (Promega, Madison, WI, USA) and the luciferase activity was measured with the Lumat Luminometer (Berthold, Bad Wildbad, Germany);
(7) Pipeted another 67ul of each cell extract supernatant into labeled 96-well with 33ul 1xReporter lysis buffer, added 100ul of β-Galactosidase Assay 2xBuffer (Promega, Madison, WI, USA) to each of the tubes, mixed by vortexing briefly, then incubated the reactions at 37°C for 30 minutes, read the absorbance at 420nm with a spectrophotometer (U-2000, Hitachi, Japan)

The cells with PB-Luc plasmid transfection only, and that without PB-Luc plasmid transfection and ICP27 (-) HSV-1 superinfection were also measured. Results were controlled for transfection efficiency in the cells by dividing the luciferase expression from the PB-Luc construct to the β-galactosidase expression from the cotransfected pCMV-βgal plasmid. All values were determined from triplicate determination and the results are the mean of these three measurements.

Transfection of PB-ICP27 plasmid DNA into Prostate and Nonprostate Cancer Cells

The LNCaP, HepG2 and H460 cell lines were grown in six-well tissue culture plate (each cell line cultured in 2-well) with 2ml appropriate complete growth medium. The cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO2 incubator until the cells were 60% confluent. One-well of each culture cells was transfected with 3ug purified PB-ICP27 plasmid DNA (the other culture well without transfection) using Lipofect AMINE plus Reagent (Gibco BRL) as described below:

(1). Pre-complexed the PB-ICP27 plasmid DNA with the PLUS Reagent: diluted 3ug DNA into 100ul serum-free dilution medium (using the medium normally used to
culture the target cells), each well used 3ug PB-ICP27 DNA mixed 6ul PLUS Reagent and incubated at RT for 15 minutes;

(2). Diluted 4ul Lipofect AMINE Reagent into 100ul serum-free dilution medium (for one well using ) in a second tube, and mixed;

(3). Combined pre-complexed DNA (from step 1) and diluted Lipofect AMINE Reagent (from step 2); mixed and incubated for 15 minutes at RT;

(4). While complexes were forming, replaced complete medium on the culture cells with 0.8 ml serum-free transfection medium;

(5). Added the DNA-PLUS-Lipofect AMINE Reagent complexes to the one-well culture of each cell line containing 0.8ml fresh serum-free medium, mixed gently and incubated at 37°C at 5% CO₂ for 3 hours;

(6). After 3 hours incubation, added 1ml fresh, appreciate complete medium to each well, and incubated at 37°C at 5% CO₂ for 48 hours.

Superinfection with ICP27 (-) HSV-1 after Transfection of PB-ICP27 Plasmid DNA

The complementation of ICP27 (-) HSV-1 mutants by ICP27 proteins expressed transiently from cloned PB-ICP27 gene was carried out as described below. After 48 hours of transfection, replaced the medium with 400ul appreciate complete medium and added 100ul ICP27 (-) HSV-1 generated previously in the lab to each well (including plasmid PB-ICP27 transfection and no transfection wells) for infection. One hours after superinfection, replaced the infection medium containing ICP27 (-) HSV-1, washed the cells with 2.0 ml PBS gently for 3 times without rinsing the cells (in order to delete the residual ICP27 (-) HSV-1 in the medium), then 1.5ml appropriate complete medium was
added to the infected cells. After 48 hours, cells and supernatant were harvested, exposed to three freeze-thaw cycles, and centrifuged at 2000 rpm for 10 minutes, after centrifugation to remove denatured debris, the supernatants were recovered and used as viral stocks at various titers to infect confluent monolayer 2-2 cells cultured in 12-well plate. After 48 hours infection, removed the medium by aspiration, 2-2 cells were fixed and stained with Mythelene Blue, and plaques (plaques are areas cleared of cells surrounded by 2-2 cells) were counted by light microscopy, and the average number of plaques was determined from 2 wells.

Statistical Analysis

Statistical Analysis of all the data in the present study was performed using student t-test. A probability value of p<0.05 was our criteria for the significant difference.
RESULTS

Production of Recombinant Plasmid Containing the ICP27 Gene under the Control of the -426 to +28 PB Promoter

The Xba I and Not I cut the PBSBH vector and taken out 7 bases, releasing the 3.375-Kb fragment containing both -426 to +28 PB promoter and marker gene for ampicillin resistance (Figure 4). The 2.7-Kb fragment containing ICP27 gene was released from the PH27 plasmid by Not I/Drd I double digestion (Figure 5). The total size of the recombinant PB-ICP27 plasmid is 6.075-kb. This PB-ICP27 plasmid DNA was identified by digestion with two different enzymes, Not I and Sal I. There is only one unique recognition site for Not I digestion in this plasmid, the Not I cut the PB-ICP27 plasmid, releasing the 6.075-kb full-length band (figure 6). There are two sites found for the Sal I restriction endonuclease in the PB-ICP27 vector, one in 674 site of the original pBluescript SK (+/-) plasmid, the other one in the 3230 site of the ICP27 DNA sequence. After the Sal I digestion, the PB-ICP27 plasmid will release two fragments, one is 1.246-kb fragment, the other is 4.829-kb fragment (figure 6).

Tissue Specificity of the PB promoter by Luciferase Activity Measurement

The expression of the firefly luciferase gene controlled by the -426 to +28 bp PB promoter in the plasmid PB-luc was measured after transfection into LNCaP and H460 cell lines. As noted in Figure 7, the PB-luc transfected LNCaP cell lines had 1.5-fold higher luciferase activity than H460 cell lines. Although it was not statistically
Figure 4. Restriction digestion of PBSBH DNA with Xba I/Not I
Lane 1: 1-kb plus DNA ladder
Lane 2: PBSBH DNA digestion with Xba I and Not I, releasing 3.375-kb fragment
**Figure 5.** Restriction digestion of PH27 DNA with Drd I/Not I
Lane 1: 1-kb plus DNA ladder
Lane 2: PH27 DNA digestion with Drd I and Not I, and showing 2.7-kb fragment of ICP27 gene
Figure 6. Restriction digestion of purified PB-ICP27 DNA with Not I and Sal I
Lane 1: 1-kb plus DNA ladder
Lane 2: 6.075-kb full fragments of the PB-ICP27 after Not I digestion
Lane 3: 4.829-kb and 1.246-Kb fragment of PB-ICP27 DNA after Sal I digestion
Figure 7. Comparison of luciferase expression in prostate (LNCaP) and nonprostate cell (H460) lines following transfection with PB-Luc construct with or without the superinfection of ICP27 (-) HSV-1. Cells were cotransfected with pCMV-βgal construct. For each cell line, the ratio of luciferase activity: β-galactosidase activity was calculated, and the results were expressed as the percentage ratio of PB-Luc construct activity: CMV control. The cells without transfection and superinfection were also measured as baseline controls. All values were determined from triplicate measurement.
significant (p=0.471), this result suggested the relative prostate tissue specific expression of reporter genes driven by -426 to +28 bp PB promoter and was consistent with previously published report (Brookes et al., 1998). To determine whether ICP27 (-) HSV-1 exhibits the altered patterns of transcription, the superinfection of ICP27 (-) HSV-1 was added to the PB-luc transfection LNCaP and H460 cell lines. The summary of results was shown in Figure 7. In PB-luc transfected LNCaP cells, superinfection with ICP27 (-) HSV-1 significantly stimulated 4.8-fold increases in luc activity (P=0.017), whereas superinfection with ICP27 (-) HSV-1 also stimulated 3.2-fold increase of luc (P=0.008) in PB-luc transfected H460 cells. These data suggest that superinfection with ICP27 (-) HSV-1 can induce high levels of PB promoter controlled luc activity.

Replication of ICP27 (-) HSV-1 in Plasmid PB-ICP27 Transfection and Nontransfection Human Prostate Cancer and Nonprostate Cancer Cell lines.

As an alternative means of determining tissue specific expression of the PB-ICP27 construct, complementation test and plaque assay was carried out in 2-2 cells, to determine the tissue-specific effect of the PB promoter on the induction of the ICP27 gene and whether the expression of ICP27 protein in prostate or nonprostate cancer cells could complement the ICP27 (-) HSV-1 and allow the virus replication.

One human prostate cancer cell line, LNCaP, and two nonprostate cancer cell lines, HepG2 and H460, were used as a target cells for the PB-ICP27 transfection combined with the superinfection of ICP27 (-) HSV-1. The cells and supernatant were harvested
and used as viral stock with 1 ul each to infect 2-2 cells. The summary of results was shown in Figure 8 and 9. If the cancer cell lines (including prostate cancer and nonprostate cancer cells) received ICP27 (-) HSV-1 infection only, the virus could not propagate and replicate in the infected cell lines. No more than $1 \times 10^3$ PFU/ml was detected in 2-2 cells, this probably represented the occasional residue of the ICP27 (-) HSV-1 during infection of tumor cells and preparation of virus stock. As shown in Figure 9, the plaque yields by infection with viral stock from PB-ICP27 plasmid transfected LNCaP cells is significantly higher than that from HepG2 cells ($16 \times 10^3$ vs $2.5 \times 10^3$ PFU/ml, $p=0.017$). However, the highest plaque yield ($10^{4} \times 10^3$ PFU/ml) was observed from H460 cells, not from LNCaP cells, which reflected more viral replications in H460 cells than that in LNCaP cells. This data suggests that PB promoter may lose its tissue specificity in some nonprostate cells such as H460, and/or there may contain factors able to increase the viral production in H460 cells.
Figure 8. The plaque yields from 2-2 cells infected by viral stock from PB-ICP27 transfected H460 cells with the superinfection of ICP27 (-) HSV-1. Plaques were areas cleared of cells surrounded by 2-2 cells. Microscopic photographs taken at 4x magnification.
Figure 9. Replication of ICP27 (-) HSV-1 in PB-ICP27 transfected and nontransfected LNCaP, HepG2 and H460 by plaque assay in 2-2 cells. The PB-ICP27 transfected and nontransfected LNCaP, HepG2, and H460 were superinfected with ICP27 (-) HSV-1. The cells were scraped into the medium and disrupted by freeze-thawing, and 1ul of each virus stock from the resulting supernatants was used to infect the confluent layers of 2-2 cells. Value represented the yield of plaque from duplicate infected cultures and were representative of repeated experiments.
DISCUSSION

General discussion

The ultimate goal of cancer therapy is a maximum of tissue-specific cytotoxicity with a minimum of toxic side effects in nonmalignant cells. An additional requirement for a successful cancer therapy is the elimination of metastatic cancer cells in addition to treatment of the local tumor. Gene therapy using tissue-specific promoters provides a way of selectively targeting therapeutic genes to malignant cells (Hart et al., 1996). This serves as a method of maximizing cytotoxicity to the target tissue where the gene of interest is expressed and minimizing exposure to cells that do not express it. Under the classification of Deonarain et al., prostate may represent a normal tissue that is nonessential. Men can survive without a prostate gland and, if gene-mediated ablation, rather than surgical ablation of this tissue were possible, it would be unlikely to cause detrimental side effects (Deonarain et al., 1995). We therefore set out to optimize tissue-specific control of expression using the prostate-specific promoter as a model.

It has been established that a 426-bp region upstream promoter of the PB gene can confer prostate-specific expression on a reporter gene, and it is the shortest fragment that has demonstrated ability to target expression of the heterologous genes specifically to the prostate in transgenic mice and maintain the spatial pattern of transgene expression independent of the random nature of transgene integration (Greenberg et al., 1994). The PB promoters are more active than the core PSA630 promoter in LNCaP cells and also show good activity in PC-3 cells expressing androgen. The 426-bp PB promoter shows the highest level of specificity in that its expression is extremely low in nonprostate cell
types examined (Brookes et al., 1998). Therefore, it is feasible to use the -426 to +28 bp PB promoter to direct expression of reporter gene, such as HSV-1 immediate-early gene IE 2, which encodes regulatory protein ICP27 that is essential for viral replication in prostate cancer cells.

As a result of evolutionary forces, viruses have necessarily evolved efficient mechanisms to deliver their genetic material into cells, avoid cellular defenses, and induce host cells to transcribe and translate viral genes. Accordingly, several viruses have been adapted for delivery of therapeutic genes to both normal cells and cancer cells. Most gene therapy research has been devoted to the development of strategies that allow viruses to deliver their genetic payload without subsequent viral replication. The overwhelming majority of cancer gene therapy strategies reported to date use replication-incompetent viruses (Roth, 1997). These viruses have served principally as vehicles for delivery of therapeutic genes. However, viruses engineered to remain replication-competent may be exploited for cancer therapy because viral replication within cancer cells results in oncolysis and produces progeny virion that can infect adjacent cancer cells.

HSV-1 is a double-stranded DNA virus that has been adapted for cancer therapy. HSV1-based vectors were initially examined as potential vehicles for gene transfer into the central nervous system. Unwanted cytopathic effects resulting from viral infection plagued these early experiment (Johnson et al., 1992). However, these cytopathic effects have subsequently been exploited to treat cancer. Entry of wild-type HSV1 into cancer cells leads to a sequential cascade of viral gene expression that ultimately results in
production of multiple progeny virions and cell death. Progeny virion can then infect adjacent cancer cells to enhance the anti-tumor effects (Roizman et al., 1996).

HSV-1 whose replication is limited to specific cell types or tumor cells should be especially useful in tumor therapy. Development of cell type-specific replication and oncolysis with minimal side effects is the ultimate goal of HSV-1 based anticancer therapy. In this study, we would like to determine whether the PB promoter driven ICP27 gene construct was able to express ICP27 protein to complement ICP27 (-) HSV-1 and finally make viral replication, and the viral replication and cell lysis was solely restricted to the cells of prostate origin.

To characterize this purpose, the -426 to +28 bp fragment of the PB promoter inserted upstream to a luciferase reporter gene was constructed to test the tissue-specificity of the PB promoter. This construct was then transfected into LNCaP and H460 cells to confirm whether the PB-regulated expression was prostate cell type-specific. As expected, the PB-luc transfected LNCaP cells in our study exhibited a higher level of luciferase activity compared with PB-luc transfected H460 cells, suggesting that PB promoter has relative regulated prostate tissue-specificity though the result is not statistically significant. The finding of markedly increased luciferase activity in both LNCaP and H460 cells after superinfection with ICP27 (-) HSV-1 suggests that a high level of transcription would be induced in the cells. It raises an interesting question as to whether heterologous promoters used to express transgenes may be affected and enhanced by HSV-1 infection.
HSV-1 encodes five IE proteins, designated ICP0, 4, 22, 27 and 47 (Honess et al., 1995). ICP4 and ICP27 are absolutely essential for virus replication, while ICP0 is required during viral infection for the expression of all classes of HSV genes (Sacks et al., 1987). The IE proteins of HSV-1 have multiple and varied effects on the expression of viral genes, mostly, but not entirely, acting to increase gene expression. For example, many studies have shown that ICP0 will induce the expression of most test genes in transient assays (Everett et al., 1988; O'hare et al., 1985). ICP0 has been shown to interact with components of the transcription (Lees-Miller et al., 1996) translation (Kawaguchi et al., 1997), and cell cycle (Kawaguchi et al., 1997) of the cell. Therefore, it has the potential to affect many aspects of the host cell metabolism. Samaniego has reported that ICP0 could significantly increase the transcription rates of genes in the absence of ICP4 and ICP27 (Samaniego et al., 1997). The genes of HSV-1 are standard polymerase II transcription units (Costanzo et al., 1977). Therefore, heterologous polymerase II promoters used to express transgenes may be affected by HSV-1 IE proteins in the same way as HSV-1 viral gene promoters are affected. Our current study also demonstrates that PB promoter used to express luciferase reporter gene can be stimulated by ICP27 (-) HSV-1, and this increase in luciferase gene expression may be largely due to the elevation of transcription induced by IE proteins such as ICP0.

The alternative strategy that described in our study is to construct a vector in which a ICP27 gene is regulated by PB promoter which has the potential to target selected gene to the prostatic epithelial cells. This constructed PB-ICP27 vector is transfected to prostate cancer cell and nonprostate cancer cell. To test whether this vector construct is active and
prostate-specific, the same panel of prostate and nonprostate cancer cell lines transfected with PB-ICP27 are infected with ICP27 (-) HSV-1. If transfected cells can express ICP27 protein and complement the ICP27 (-) HSV-1. The virus will propagate and replicate in the cells, and the plaques will yield after using the viral stock from these cells to infect ICP27 expression 2-2 cells. The plaque yield will reflect the production of ICP27 (-) HSV-1 replication in the PB-ICP27 plasmid transfected cells which express ICP27.

As shown in our results, profound viral replication was observed in LNCaP cells after PB-ICP plasmid transfection with the superinfection of ICP27 (-) HSV-1. In contrast, nearly no viral replication was observed in HepG2 cells, suggesting that the capability of ICP27 (-) HSV-1 replication is restricted in the prostate not in hepatic cells and due to the promoter specificity that controls the expression of ICP27 gene in the PB-ICP27 vector. However, the production of replication ICP27 (-) HSV-1 in PB-ICP27 plasmid transfected H460 cells are surprisingly significantly higher than that in LNCaP cells, because H460 cells were derived from a nonprostate tissue, it suggests that the cell specificity of PB promoter may be lost, giving high levels of ICP27 gene expression when the ICP27 (-) HSV-1 and the PB-ICP27 vector are co-located in the H460 cells. As we know, promoter is composed of multiple genetic elements, or modules, allowing its transcription to be regulated in response to diverse intracellular signals (Dynan, 1989). Oncogenic mutations often lead (directly or indirectly) to changes in the activity of abundance of various transcription factors (Lewin, 1991). In addition, malignant cells may be less stringently controlled by trans-acting regulatory signals because they grow in an autonomous, uncontrolled manner. Here we consider that the oncogenic mutations in
H460 cell line may account for the changes in the control of transcription to increase the expression of ICP27 gene. By virtue of increased expression or activity, the oncogenic mutation could turn up transcription of genes whose products can be tolerated only in a small amount. However, this result contrast with our PB controlled luciferase reporter gene assay, which showed higher level of luciferase activity in PB-luc transfected LNCaP cells than H460 cells. We suspected that the difference is likely to be the inefficient replication of ICP27 (-) HSV-1 in slow proliferating LNCaP cells rather than PB promoter partially lost its cell specificity in H460 cell line. The production of replication ICP27 (-) HSV-1 may not reflect the actual expression of the ICP27 gene in different cell lines in vitro, where cell proliferation rate is different. For example in our cell culture, H460 cells had shown about double growth rate than that of LNCaP and HepG2 cells. There will be more viral replication and production in fast proliferating H460 cells if only a small amount of “leakage” of ICP27 protein occurs. On the other hand, we also suspect the role of IE protein from the ICP27 (-) HSV-1 in the transactivation of PB promoter in H460 cells. To verify this hypothesis, it is necessary to identify the increase of ICP27 protein expression in PB-ICP27 plasmid transfected H460 cells with the superinfection of ICP27 (-) HSV-1.

Summary and conclusions

Tissue -or cell-specific targeting of vectors is critical to the success of gene therapy. We described a novel approach to HSV-1 mediated gene therapy by constructing a vector PB-ICP27 in which a viral IE gene required for replication (ICP27) is regulated by -426 to +28 bp PB promoter which has the potential to target selected gene to the prostatic
cells. To identify the tissue-specificity of this construct and the ability of expression ICP27 protein to complement the replication-competent ICP27 (-) HSV-1, Lipofection mediated gene transfection was performed with this vector in the human prostate cancer cell line (LNCaP) and two other nonprostate cell lines, HepG2 (human hepatoma), and H460 (human lung cancer). After transfection, cells were superinfected with ICP27 (-) HSV-1 and the plaque assays were performed on 2-2 cells with the viral stocks from these cells. A PB-driven luciferase reporter gene, PB-luc, was also used to estimate the tissue-specific expression of the PB-driven transcripts in LNCaP and H460 cell lines.

The luciferase reporter analysis demonstrated that PB-luc transfected LNCap cells had higher luciferase activity compared with that of H460 cells, and superinfection with ICP27 (-) HSV-1 can significantly enhance the luciferase expression controlled by PB promoter. As expected, the PB-ICP27 plasmid transfected LNCap cells with the superinfection of ICP27 (-) HSV-1 in our study exhibited significant viral replication compared to that of HepG2 cells. However, PB-ICP27 transfected H460 cells displayed a surprisingly significantly more viral replication than LNCap cells, and contrast with our PB-driven luciferase assay, which showed higher level of luciferase activity in PB-luc transfected LNCap cells than that in H460 cells.

In conclusion, this study demonstrated that (1) -426 to +28 bp PB promoter was active and showed relative tissue-specific production of functional protein in LNCap cells; (2) ICP27 (-) HSV-1 was able to replicate only in the target cells that express ICP27 protein; (3) -426 to +28 bp PB promoter may lose its tissue-specific expression in H460 cells; (4) ICP27 (-) HSV-1 can enhance the activity of -426 to +28 bp PB promoter. Our
finding also suggest that the construct in which the -426 to +28 bp PB promoter used to drive the expression of HSV-1 ICP27 gene is not suitable to use as the prostate tissue targeting gene delivery vector.

Future investigations

Based on the preliminary results, the future studies should be focused on the following aims:

(1). To verify the missing region of important upstream regulatory sequences of PB promoter.

Although -426 to +28 bp PB promoter was sufficient to direct prostate-specific expression, the low levels of transgene expression in our study suggested that important upstream regulatory sequences may be missing. To enhance transgene expression, the large fragment of PB promoter which impact high levels of prostate-specific expression needed to be identified, and will be inserted back to the promoter-reporter gene construct to test its activity and specificity and will be compared with that of -426 to +28 bp promoter.

(2). To identify the factors in the HSV-1 which contribute to enhance levels of transgene expression in the absence of ICP27.

The preliminary results showed that ICP27 (-) HSV-1 can increase PB promoter driven gene expression and this may be due to IE gene (e.g. ICP0 or ICP4). It is therefore necessary to examine and compare the level of PB promoter driven gene expression as a
consequence of infection with different IE mutant HSV-1 and to determine which IE or combination are responsible for this action.
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