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Replication inhibition by nucleoside analogues of a recombinant *Autographa californica* multicapsid nuclear polyhedrosis virus harboring the herpes thymidine kinase gene driven by the IE-1(0) promoter: a new way to select recombinant baculoviruses

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ABSTRACT

The expression of the thymidine-thymidylate kinase (HSV1-TK), (ATP: thymidine 5′-phosphotransferase; EC 2.7.1.21) of herpes simplex virus type 1 endows the host cell with a conditional lethal phenotype which depends on the presence of nucleoside analogues metabolized by this enzyme into toxic inhibitors of DNA replication. To generate a recombinant baculovirus that could be selected against by nucleoside analogs, the HSV1-tk coding sequence was placed under the control of the *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) immediate early promoterm IE-1(0), and this construction was introduced via homologous recombination into the polyhedrin locus of AcMNPV. Two recombinant baculoviruses harboring this gene construct at the polyhedrin locus were isolated and tested for their ability to replicate in the presence of various concentrations of the nucleoside analog 9-(1,3-Dihydroxy-2-propoxymethyl)guanine (Ganciclovir). Neither Sf9 lepidopteran cell viability nor replication of wild type or β-Galactosidase-expressing recombinant AcMNPVs were affected by concentrations of Ganciclovir up to 100 μM. In contrast, replication of the recombinant AcMNPV virus harboring the HSV1-tk gene was inhibited by Ganciclovir in a dose-dependent manner. The inhibition was detectable at 2 μM and complete at 100 μM. This property was exploited in model isolations aimed at purifying new recombinant viruses having lost this counter-selectable gene marker as a result of homologous recombination at the polyhedrin locus after co-transfection of the viral DNA with a replacement vector. After being propagated in the presence of Ganciclovir, the progeny of such co-transfections contained over 85% recombinant viruses, demonstrating that counter-selection of parental HSV1-tk-containing viruses by Ganciclovir constitutes a novel approach for recombinant baculovirus isolation.

INTRODUCTION

Autographa californica multicapsid nuclear polyhedrosis virus is the prototype of a family of insect viruses known as *Baculoviridae*. The viral genome consists of a covalently closed double-stranded DNA molecule of 128 kilobase pairs packaged in a rod-shaped nucleocapsid. After infection with AcMNPV, host cell protein synthesis is gradually shut down so that late in infection, only virally encoded proteins are made. Due to the existence of very late genes expressed at high-levels in the life cycle of baculoviruses that are unnecessary for viral replication in tissue culture (*e.g.* the polyhedrin and p10 genes), the lytic recombinant baculovirus-insect cell expression system, whereby a given cDNA replaces either of these genes by allelic substitution, has recently become the method of choice to express mammalian proteins (1–3). Typically, in this system, the cDNA of interest is placed in a non-fusion configuration under the transcriptional control of the polyhedrin genem and the recombinant virus then infects permissive lepidopteran cells or larvae (4) to produce, in large quantities, recombinant proteins that are authentically folded, proteolytically processed, post-translationally modified, and biologically active. However, recombinant viruses are generated *in vivo* as a result of recombination events taking place between viral DNA sequences and homologous sequences flanking the cDNA in the transfer plasmid vector. The typical frequency of these events is in the range of 0.1–1%, but linearization of viral DNA prior to cotransfection increases the frequency of recombinants among the viral progeny (5). Thus, the widespread use of the baculovirus expression system has been li;ited by the difficulty of identifying recombinant viruses against a high background of parental viruses. Furthermore, the currently frequent use of modified viruses having lost the polyhedrin marker precludes the use of the original plaque assay to discriminate between recombined and unrecombined viral plaques (6). These considerations emphasize the need for the development of new markers to facilitate the isolation of recombinant baculoviruses.

Several nucleoside analogs which are preferential substrates for herpes virus-encoded thymidine-thymidylate kinases (7, 8)

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have been shown to be highly selective against replication of herpes simplex virus, varicella-zoster virus, cytomegalovirus and Epstein-Barr virus (9, 10). The high selectivity of the viral thymidine kinase for these nucleoside analogs (resulting from the low affinity of cellular thymidine kinases for these compounds) makes it possible to selectively kill HSV1-tk expressing cells. Thus, previous gene transfer studies in mammalian cells have demonstrated that the expression of this gene in cultured cells (11, 12), or even in specific tissues of transgenic mice (13, 14), can constitute the basis for a conditional toxic phenotype. To be successful, the use of a similar strategy to inhibit baculovirus replication should allow the toxic intermediate to accumulate in the infected cell before the start of viral DNA synthesis, which takes place 6 hours after infection (15).

To express the HSV1-tk gene as early as possible, we have placed the HSV1-tk coding sequence under the control of an AcMNPV immediate-early gene IE-1 promoter. This gene, which encodes a trans-activator of delayed-early promoters (16, 17), is expressed very early in AcMNPV infection (18). Recently, we and others have identified an upstream IE-1 promoter, IE-1(0), which drives the synthesis of the first viral transcript produced in infected cells (19, 20). This immediate-early promoter was chosen to drive the expression of the HSV1-tk gene, and the AcMNPV IE-1(0) promoter was fused to the coding sequence of HSV1-tk. Using a transfer plasmid containing the gene construct, we created two recombinant viruses harboring this HSV1-tk construct at the polyhedrin locus. The first was a single recombinant (HSV1-tk$^+$), obtained by using wild type viral DNA for homologous recombination, while the other was a double recombinant (SEAP$^+$; HSV1-tk$^+$), obtained by using a viral DNA labelled by the introduction of the human secreted alkaline phosphatase (SEAP) at the p10 locus (21).

We show in this study that Ganciclovir (GCV) selectively inhibits the replication of the HSV1-tk-containing recombinant virus in a dose-dependent manner, without affecting that of parental viruses. We also show in model isolations of recombinant viruses that this property can be used to purify to more than 85% recombinant baculoviruses in which the HSV1-tk marker has been replaced by a given cDNA following homologous recombination at the polyhedrin locus with a replacement vector by simply propagating them at low multiplicity of infection in the presence of GCV. In addition to being counter selectable, the use of the double recombinant virus (SEAP$^+$; HSV1-tk$^+$) endows the recombinant viruses subsequently obtained with the convenient soluble alkaline phosphatase enzyme label and are, *ipso facto*, p10 deficient, a feature which delays infected cell lysis (22) and should thereby increase recombinant protein production.

MATERIALS AND METHODS

Cell culture and viruses

Suspensions or monolayer cultures of the Sf9 cell line from *Spodoptera frugiperda* were maintained at 27° C in TC 100 medium supplemented with 0.33% Yeastolate (Difco), containing 4% heat inactivated fetal bovine serum, 50 μg/mL penicillin and 50 μg/mL streptomycin. Cell densities were routinely kept between 2.5×10^5 αnd 1.5×10^6 per mL by passaging the cells in fresh medium. These cells were used for DNA transfections, virus propagation, limiting dilutions and gene expression studies. Viral plaques were prepared as described (6) after addition of the chromogenic indicator 5-Bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-Gal) (Boehringer, Mannheim), as described (23). DNA from wild-type and recombinant AcMNPVs was prepared from ECVs or virally infected cells as described (6). The recombinant baculovirus carrying *E.coli* β-Galactosidase (AcMNPV-LacZ) was kindly supplied by Dr. G. Devauchelle, and AcMNPVRP23-LacZ (5) by Dr. Possee. The double recombinant virus AcMNPVRP23-LacZ-p10SEAP, harboring human soluble alkaline phosphatase cDNA at the p10 locus and the the Lac-Z gene coding sequence at the polyhedrin locus, will be described (21).

Transfection and isolation of recombinant baculoviruses

To generate recombinant viruses, DNA from recombinant plasmids (20 μg) was co-transfected with AcMNPV DNA (10 μg) by electroporation using a standard cuvette with a 0.4 cm gap, containing a 1 mL cell suspension ($0.3-1.0\times10^7$ cells/mL) in BNP (25 mM BES pH 6.95, 140 mM NaCl, 1.5 mM NaH_2PO_4 and 1 mM Glucose), exposed to the exponential discharge of a 1030 μF capacitance charged under 220 Volts in the presence of 500 μg/mL herring sperm DNA (24, 25). Four to seven days after transfection, the medium containing the mixed viral progeny was harvested, centrifuged and titrated. The mixed viral progeny was then submitted to a purification procedure based on limiting dilution of the viral suspension in 96 well microtiter plates (26, 27). Depending on the nature of the viral DNA used in the transfection, detection of recombinant viruses was achieved either by hybridization or by screening for the loss of the β-galactosidase marker using SEAP as a virus marker as described (21), or by a combination of both. Hybridization was performed after having dissolved the cellular layer in 100 μL of 0.4 M NaOH and transferred it onto a nylon filter (Gene Screen Plus Dupont) via a dot blot manifold (28). After neutralization, the filter was hybridized to a ^{32}P-labelled HSV1-tk probe (29), as described (30). For the isolation of the double recombinant baculovirus containing the HSV1-tk gene at the polyhedrin locus, DNA from a double recombinant virus for SEAP at the p10 locus and for *E.coli* β-Galactosidase at the polyhedrin locus was used (21). A first enrichment cycle was necessary in which recombinant viruses were detected by hybridization to a HSV1-tk probe. Supernatant of positive wells was diluted at limiting dilutions and the resulting positive wells were screened for the loss of β-Galactosidase activity.

Amplification of 606 bp DNA fragment from AcMNPV IE-1 gene

The region chosen for amplification was a 606 bp fragment whose 3′ extremity terminates 11-bp upstream from the translational start site of the open reading frame encoded by exon 0 of the immediate-early gene IE-1 in the *Eco*RI B fragment of the AcMNPV genome (18). This DNA fragment contains the IE-1(0) promoter driving the synthesis of the 2.1 kb IE-1 immediate early transcriptional unit active in lepidopteran cells without requiring any additional viral function (19, 20). A 39-mer oligonucleotide (forward primer) corresponding to its 5′ end was synthesized with the sequence: (5′gcgaattcTACGTAGATCGCGCTCTTGTTT-GAAATCCAG3′), creating an additional *Eco*RI site (underlined) (lower case represents non-AcMNPV DNA). A second 39 mer oligonucleotide (backward primer) was synthesized with the sequence: (5′cgggatccCGTTATCAATTACTTATACTATCCG-GCGCGC3′), adding an extra *Bam*HI site. After purification, these two primers were used in a polymerase chain reaction with AcMNPV DNA as template using a GeneAmp DNA amplification kit (Perkin-Elmer Cetus). The resulting 622 bp

fragment with 3'*Bam*HI and 5' *Eco*RI extremities was purified and subcloned as described below.

Construction of replacement vectors

All plasmids were constructed and purified according to standard recombinant DNA techniques (31), except that transformation of *E.coli* hosts was performed by electroporation (32). Conditions for restriction endonuclease digestions were those suggested by the manufacturer. DNA fragments were isolated from agarose gels by electroelution. The construct used in this study is shown schematically in Figure 1. The replacement vector used was the non-fusion pVL941 (33), kindly provided by Drs. M. Summers and the pVL1392 (Stratagene). To clone the SEAP cDNA into the pVL1392 transfer vector, the SEAP coding sequence was first excised from pRSV-SEAP (34) by double digestion with *Hind*III and *Xho*I. The 1.9 kbp fragment was isolated and subcloned into the Bluescript vector (Stratagene) digested with the same enzymes. A recombinant plasmid was then digested with *Xho*I, filled-in with *E.coli* DNA polymerase large fragment, and finally cut with *Not*I. The resulting fragment was isolated and ligated to the transfer vector pVL1392 digested with *Not*I and *Sma*I. DNA from the recombinant plasmid pVL1392-SEAP was isolated and transfected with viral DNA by electroporation. To create a replacement vector for polyhedrin targeting of the *E.coli* β-Galactosidase gene, *E.coli* LacZ gene was excised from pMS1 plasmid (35) by *Xba*I and *Bgl*II digestion and ligated to the pVL1393 vector digested with the same restriction endonucleases, thus yielding the pVL1393-LacZ replacement vector.

To construct pVL941IE-1-tk, in which the HSV1-tk coding sequence is placed under the control of the AcMNPV IE-1(0) promoter, the complete HSV1-tk gene was excised from pAG0 (36) βy *Pvu*II digestion and this fragment was subcloned into the *Sma*I and *Eco*RV-digested Bluscript vector (Stratagene). A plasmid containing the HSV1-tk gene in the adequate orientation was chosen and subsequently digested with *Eco*RI and *Bgl*II restriction endonucleases to excise the 131 bp fragment containing the HSV1-tk gene promoter elements (37, 38). To this plasmid was then ligated the *Eco*RI and *Bam*HI-digested 622-bp PCR fragment containing the IE-1(0) promoter from the IE-1 AcMNPV gene (20). A *Bam*HI-*Kpn*I fragment from the resulting plasmid was then isolated and ligated into *Bam*HI and *Kpn*I-digested pVL941 to yield the transfer plasmid pVL941IE-1-tk, subsequently used to generate the AcMNPVIE-1-tk recombinant virus.

Drugs

9-(2-Hydroxyethoxymethyl)guanine (Acyclovir) (10) was obtained from Burroughs Wellcome (Research Triangle Park, NC) and 9-(1,3-Dihydroxy-2-propoxymethyl)guanine (Ganciclovir) (9, 39) from Synthex (USA) Inc., Palo Alto, CA. 1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU) (40), 1-(2'-Deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) (41) and 1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU) (40) were gifts from Bristol-Myers (Syracuse, NY). (E)-5-(2-Bromovinyl)-2'-deoxyuridine (42) was a gift from Dr. E. De Clercq (Rega Institute, Leuven, Belgium).

Enzyme assays

Human soluble alkaline phosphatase activity was determined essentially as described (34). Briefly, 20 μL of culture medium were incubated in a buffer containing 200 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$, 1.0 mM *p*-nitrophenylphosphate, 10 mM L-Homoarginine in a total volume of 200 μL in microtiter wells. The absorbance at 405nm was measured at the end of the incubation period in an ELISA plate reader. *E.coli* β-Galactosidase was measured using a similar protocol using the buffer described by Miller (43), with 1.0 mM Chlorophenolred-β-Galactoside (Boehringer Mannheim) as substrate. The absorbance at 550 nm was determined at the end of the incubation period in an ELISA plate reader or in a spectrophotometer. Cell viability was estimated by the (3-(4,5-dimethyl thiazol-2yl)-2,5 diphenyl tetrazolium bromide) (MTT) dye method essentially as described (44).

RESULTS

Isolation of two recombinant baculoviruses harboring the HSV1-tk gene under the control of the IE1(0) promoter

To insure that HSV1-tk would be expressed as early as possible during the replicative cycle of AcMNPV, the HSV1-tk coding sequence was placed under the transcriptional control of the AcMNPV immediate early promoter IE-1(0) by fusing it to a PCR fragment comprising this promoter activity (19, 20). To target this gene construct to the polyhedrin locus of AcMNPV, this DNA fragment was cloned in a replacement vector. The resulting plasmid, pVL941IE-1-tk (figure 1), was cotransfected with two different viral DNAs. The first viral DNA was that of the wild type AcMNPV. A first recombinant baculovirus (AcMNPV-IE-1-tk) was isolated using the procedure combining limiting dilution and hybridization to a HSV1-tk specific probe. To isolate a virus labelled with SEAP at the p10 locus, a second HSV1-tk-harboring recombinant baculovirus was isolated by co-transfecting pVL941IE-1-tk with the viral DNA from the previously described double recombinant virus (AcMNPVRP-

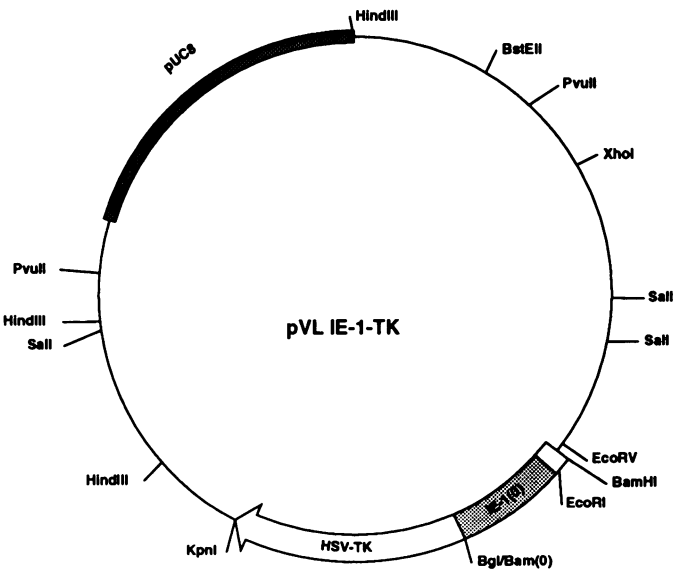

Figure 1. Map of the replacement vector comprising the IE-1(0)-HSV1-tk construct. The pVL941-IE-1-tk gene in which the HSV1-tk coding sequence was placed under the control of the AcMNPV IE-1(0) promoter, was used to target this construct to the polyhedrin locus. The open arrow represents the HSV1-tk coding sequence and the light stippled box represents the 606-bp fragment of AcMNPV IE-1 gene containing the IE-1(0) promoter activity.

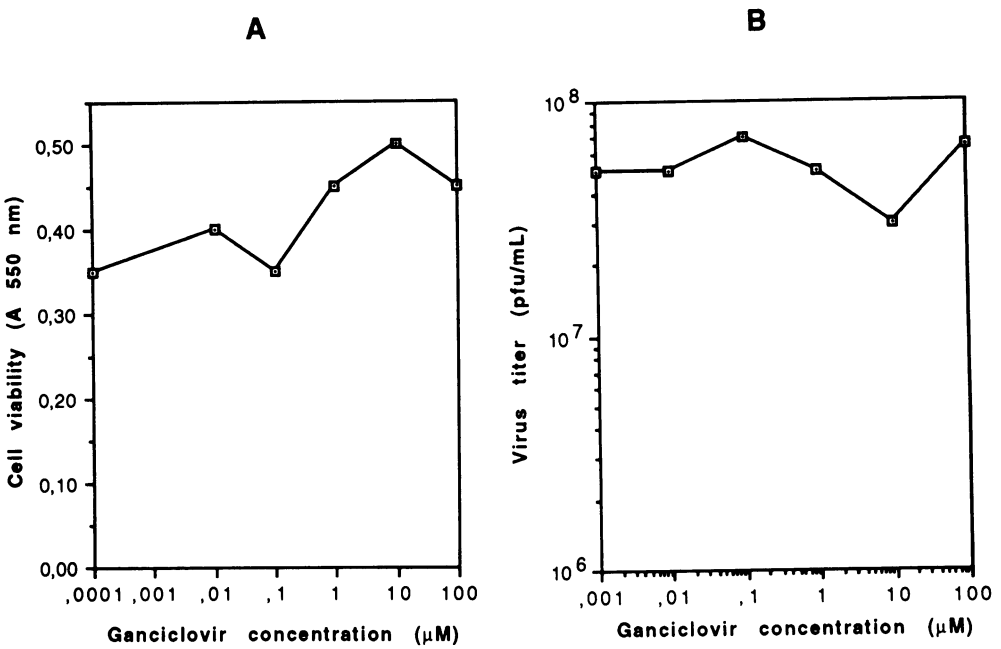

Figure 2. Effect of Ganciclovir on Sf9 cells survival and on replication of wild type AcMNPV. Sf9 cells were seeded in 96 well microtiter plates in triplicate in the absence or presence of various concentrations of GCV. Seventy two hours later, cell survival was assessed by addition of MTT, as described (44) (Panel A). Cells were infected by wild type AcMNPV and cultivated in the absence or presence of various GCV concentrations. At the end of infection, individual viral progenies were collected and titrated (Panel B).

23-p10SEAP), producing the SEAP and *E. coli* β-Galactosidase activities *in lieu* of the p10 or polyhedrin proteins, respectively (21). Using the procedure based on the loss of β-Galactosidase activity (21), a recombinant baculovirus containing the HSV1-tk coding sequence at the polyhedrin locus and carrying the resident SEAP marker from the p10 locus (AcMNPV-IE-1-tk-p10SEAP) was isolated.

Ganciclovir specifically inhibits replication of HSV1-tk-harboring recombinant baculoviruses

In experiments aimed at using GCV as a selective agent on the replication of this virus, we first tested the possible toxicity of this nucleoside analogue on Sf9 cells. As shown in Figure 2A, concentrations of GCV as high as 100 μM had no effect on the viability of these cells, suggesting that lepidopteran cells behave like mammalian cells with respect to the inability of their thymidine kinase to metabolize this nucleoside analogue into a toxic compound. We then sought to determine whether GCV could affect AcMNPV replication in cells treated with increasing concentrations of the drug, using the wild type AcMNPV virus. As indicated in Figure 2B, the virus titer obtained after infection of Sf9 cells with AcMNPV was unaffected by concentrations of GCV up to 100 μM, when the drug and the virus were added simultaneously to the culture. This result suggests that none of the virally-encoded enzymes expressed during infection are able to metabolize GCV into a compound deleterious for viral replication.

We then tested AcMNPVIE-1-tk-p10SEAP carrying the HSV1-tk gene under the control of the IE-1(0) promoter for its ability to replicate in the presence of various concentrations of GCV. As shown on Figure 3 (upper panel), viral replication, as measured by the amount of SEAP produced, was inhibited in a dose-dependent manner by GCV. The inhibition could be detected at GCV concentrations as low as 2 μM and the half maximal inhibitory concentration was around 10 μM, the effect being maximal for a 100 μM concentration. We used the doubly labelled parental virus, AcMNPVRP23-p10SEAP, as a control. As expected, its replication was unaffected by GCV when assessed by either SEAP production as shown on Figure 3 (upper panel), or β-Galactosidase production (results not shown). We also tested the effect of other nucleoside analogs generating toxic compounds when metabolized by HSV1-TK, such as FIAU (40), BVdU (42), FIAC (41) and FMAU (40). Whereas all of these compounds selectively inhibited the replication of AcMNPVIE-1-tk-p10SEAP, they also exhibited, to a lesser extent, a toxic effect on the replication of control viruses (results not shown). In contrast, 9-(2-Hydroxyethoxymethyl)guanine (Acyclovir), whose inhibitory activity on herpes type 1 viral replication has been shown to require the simultaneous activity of HSV1-TK and HSV1 DNA polymerase (45), had no effect on AcMNPVIE-1-tk-p10SEAP replication (figure 3; upper panel), as expected in the absence of herpes virus DNA polymerase. Taken together, these results demonstrate that the introduction of the HSV1-tk gene under the control of the immediate-early promoter IE-1(0) into the AcMNPV genome can lead to a conditional lethal phenotype, which depends on the presence of GCV (or other related drugs).

To demonstrate directly the inhibition of AcMNPVIE-1-tk-p10SEAP virus replication by GCV, viral replication was quantitated with a hybridization assay using the 606-bp amplified AcMNPV *Eco*RI B fragment as a probe for the presence of virus. As shown in Figure 3 (lower panel), viral replication was strongly inhibited by GCV concentrations of 10 μM and 100 μM, thus confirming the observation made using SEAP activity measurements as an indirect index of viral replication. Overall, these results indicate that replication of the virus carrying the herpes simplex virus thymidine kinase gene under the control of the IE-1(0) promoter is selectively inhibited by concentrations

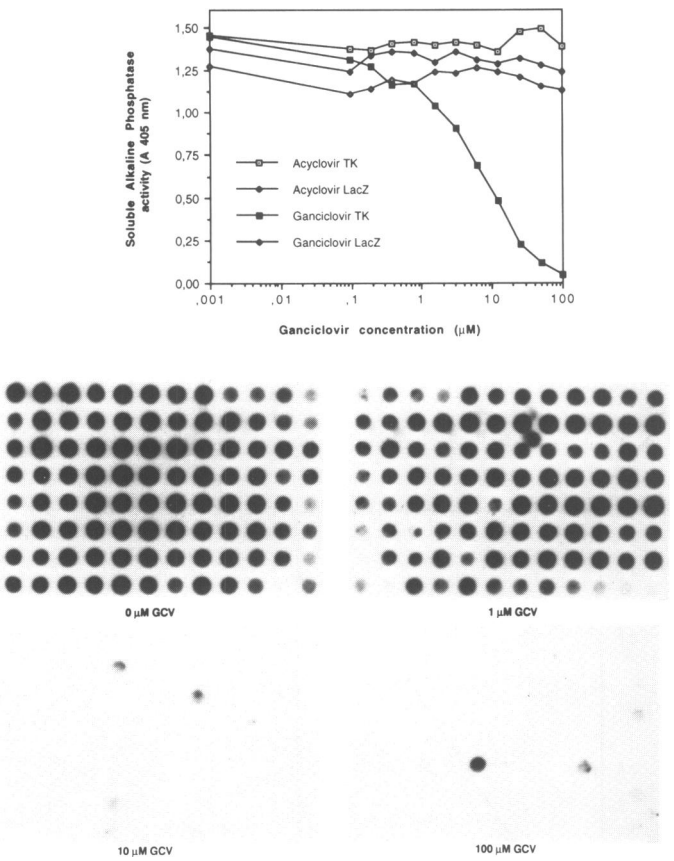

Figure 3. Replication of a recombinant virus harboring the HSV1-tk gene under the control of the viral immediate-early promoter IE-1(0) is inhibited by Ganciclovir. Recombinant virus AcMNPVIE-1-tk-p10SEAP and the parental virus AcMNPVRP23LacZ-p10SEAP were used to infect Sf9 cells at a moi < 1 in the presence of various concentrations of either Ganciclovir or Acyclovir, as indicated. Cultures were incubated for 5 days, after which time SEAP activity was determined in the cell-free supernatant (panel A). A stock from recombinant virus AcMNPVIE-1-tk-p10SEAP was diluted 10^5 times, and used to infect cell Sf9 cell cultures (approximately 100 pfus per well), which were subsequently seeded in microtiter plates in the presence of various concentrations of GCV. At the end of the infection period, DNA from infected cells was transfered to nylon filters which were hybridized using the 606-bp amplified viral DNA fragment from the AcMNPV *Eco*R1 B fragment as a probe. After stringent washing, the filters were autoradiographed (panel B).

of GCV in the range of $10-100$ μM. It is worth noting that the replication of a similar virus harboring HSV1-tk driven by its authentic herpes virus promoter, similarly introduced at the polyhedrin locus, could not be inhibited by GCV (results not shown).

Incubating mixed infected cells with Ganciclovir leads to an enrichment in HSV1-tk$^-$ baculoviruses

Since GCV inhibits specifically the replication of HSV1-tk$^+$ viruses, treatment of cell cultures infected with a mixture of HSV1-tk$^+$ and HSV1-tk$^-$ viruses should result in an enrichment of the progeny in the GCV-insensitive HSV1-tk$^-$ species. We first mixed AcMNPVIE-1-tk and AcMNPV-LacZ viruses in a proportion of 99 to 1. This viral mixture was used to infect Sf9 cells at a moi < 1 which were further incubated in the presence of increasing concentrations of GCV. After completion of the infectious cycle, the composition of the resulting viral progeny was analyzed in two different ways. First, β-Galactosidase activity

Table 1. One step isolation of recombinant baculoviruses at the polyhedrin locus by Ganciclovir counter-selection of the HSV1-tk-harboring parental viruses

Viral DNA used in co-transfection	Plasmid DNA used in co-transfection	Number of viral clones examined	Number of recombinant clones	Fraction of recombinant clones (%)
AcMNPVIE-1-tk-p10-SEAP	pVL1393LacZ	21 #	21 #	100.0%
AcMNPVIE1-tk	pVL1392SEAP	8*	7*	87.5%

Replacement vectors were transfected with the indicated viral DNA harboring the HSV1-tk gene in Sf9 cells. The resulting viral progeny was propagated in the presence of Ganciclovir and submitted to limited dilution for clonal analysis.

These clones originated from a propagation step of the original progeny performed on bulk cultures in T25 tissue culture flasks in the presence of 50 μM Ganciclovir prior to clonal analysis. After limiting dilution of the resulting progeny, clonal analysis was carried out by measuring in each individual well secreted alkaline phosphatase activity and β-Galactosidase activity in replicate microtiter plates. Recombinant viruses were positive for both enzyme activities.

*These clones originated from a propagation step of the original progeny inoculated at about 100 virus per well in 96 well microtiter wells in the presence of 20 μM Ganciclovir prior to clonal analysis. After 5 days, the supernatants of each culture were individually screened for the presence of SEAP activity. Several positive wells containing a candidate SEAP recombinant baculovirus were identified and the purity of one of them (F7), was further investigated.by limiting dilutions used to infect fresh cultures seeded in 96 well microtiter plates. Five days later, the culture supernatants were serially assayed for the presence of SEAP activity. Residual cells were used to transfer viral DNA on Nylon filters which were hybridized to the ^{32}P-labelled 606-bp amplified viral probe from AcMNPV *Eco*R1 B fragment and autoradiographed Recombinant viruses were those which were positive in the hybridation assay and which produced SEAP activity simultaneously.

was determined in each culture, this enzymatic activity being taken as a proliferation index of the GCV-insensitive virus. As shown in Figure 4 (panel A), β-Galactosidase activity increased gradually in these cultures with GCV concentration. At the highest GCV concentration (100 μM), β-Galactosidase activity produced in infected cells was 150 times higher than in cultures infected in the absence of the drug. This increment is close to the value of 100 that one would have expected from the initial (99:1) composition of the viral mixture. These results suggest that the replication of HSV1-tk$^+$ viruses was efficiently repressed by GCV, and that, consequently, HSV1-tk$^-$, GCV-insensitive viruses expressing β-Galactosidase could develop at their expense, thus giving rise to a higher enzyme level as replication inhibition of the counter-selectable HSV1-tk$^+$ species by GCV became more efficient. The progenies of these infections were also analyzed directly using a plaque assay measuring β-Galactosidase-producing viruses by counting blue plaques made in the presence of the chromogenic substrate X-Gal. As shown in Figure 4 (panel B), while the total virus titer did not change, the fraction of β-Galactosidase positive viruses increased with increasing GCV concentrations, reaching 100% at 100 μM. These results demonstrate that GCV treatment of insect cells infected at low moi with a viral mixture composed of a minority of HSV1-tk$^-$ viruses and a vast majority of HSV1-tk$^+$ viruses yields a progeny considerably enriched in this minor (HSV1-tk) viral species.

One step isolation of a recombinant baculovirus at the polyhedrin locus by Ganciclovir counter-selection

We then tested, in two model systems, the feasibility of using GCV counter-selection for isolation of a recombinant baculovirus generated by homologous recombination between a replacement vector containing a given cDNA and the DNA of either of the

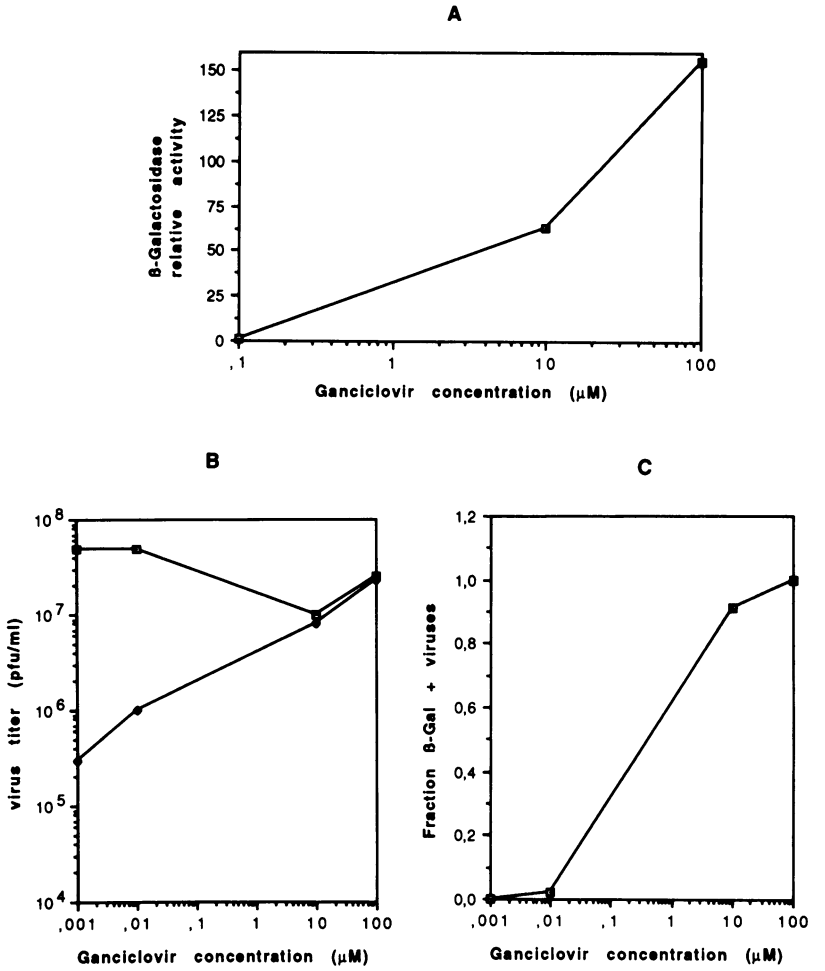

Figure 4.: Ganciclovir-enrichment of HSV1-tk$^-$ viruses in mixed infections. A mixture consisting of AcMNPVIE-1-tk-p10SEAP virus and AcMNPVRP23LacZ-p10SEAP virus in a proportion of 99:1 was used to infect Sf9 cell cultures at a moi < 1, which were subsequently incubated in the presence of variable concentrations of GCV. At the end of the infection period, virus-containing supernatants were saved and residual cells were used to determine β-Galactosidase activity. The activity measured in a given culture was normalized to that of the untreated, control culture (panel A). The virus-containing supernatants were diluted and used to count β-Galactosidase-expressing viruses in a plaque assay made in the presence of the chromogenic indicator X-Gal. Open symbols: total virus titer; closed symbols: β-Galactosidase-expressing viruses (panel B). The fraction of β-Galactosidase positive viruses has also been represented (panel C).

two previously isolated viruses harboring the HSV1-tk construct after co-transfection. In a first co-transfection, the replacement vector pVL1393-LacZ, encoding *E.coli* β-Galactosidase, was used together with AcMNPVIE1-tk-p10SEAP viral DNA as recombination partners. The progeny of this cotransfection was propagated in the continuous presence of 50 μM GCV after infecting Sf9 cells at low moi. To assess the frequency of recombinants in this viral population, the viral stock obtained was further diluted and used to clonally infect fresh Sf9 cells in 96 well microtiter plates. After completion of the infectious cycle, each well was individually tested for SEAP activity (indicating the presence of virus) and for β-Galactosidase to detect recombinant viruses at the polyhedrin locus. As shown in table one, 21 wells scored positive for SEAP activity indicating the presence of 21 viral clones. When assayed for β-Galactosidase activity, all of these 21 clones contained high levels of enzyme activity, indicating that 100% of the viral clones were recombinant for the LacZ gene at the polyhedrin locus.

We also performed a second model isolation of recombinant baculovirus using a slightly different protocol for GCV counter-selection. In this co-transfection, aimed at introducing the SEAP cDNA at the polyhedrin locus, the SEAP replacement vector, pVL1392-SEAP, was used with AcMNPVIE-1-tk (a virus devoid of SEAP) viral DNA as recombination partner, expecting that recombinant virus identification would be simplified by the ease with which this gene product can be detected (21, 34). The resulting viral progeny was diluted 10^4 times (a dilution factor that corresponds to inoculating each well with approximately 100 pfu), and this dilution was used to infect Sf9 cell microcultures in 96 well microtiter plates, which were subsequently incubated in the presence of a 50 μM concentration of GCV. The screening of these individual cultures for SEAP activity identified several positive wells, indicating the presence of candidate recombinant baculoviruses. The purity of one of these viral clones (F7) was investigated by further diluting the content of the F7 well at limiting dilutions to infect microcultures, which were seeded in a new set of microtiter plates in the absence of GCV. At the end of the infection period, culture supernatants were individually assayed for alkaline phosphatase activity and the viral DNA from infected cells was transferred on a filter which was hybridized to the 606-bp fragment from the *Eco*R1 B fragment of AcMNPV. As shown in table 1, 7 of the 8 (87.5%) viral clones detected

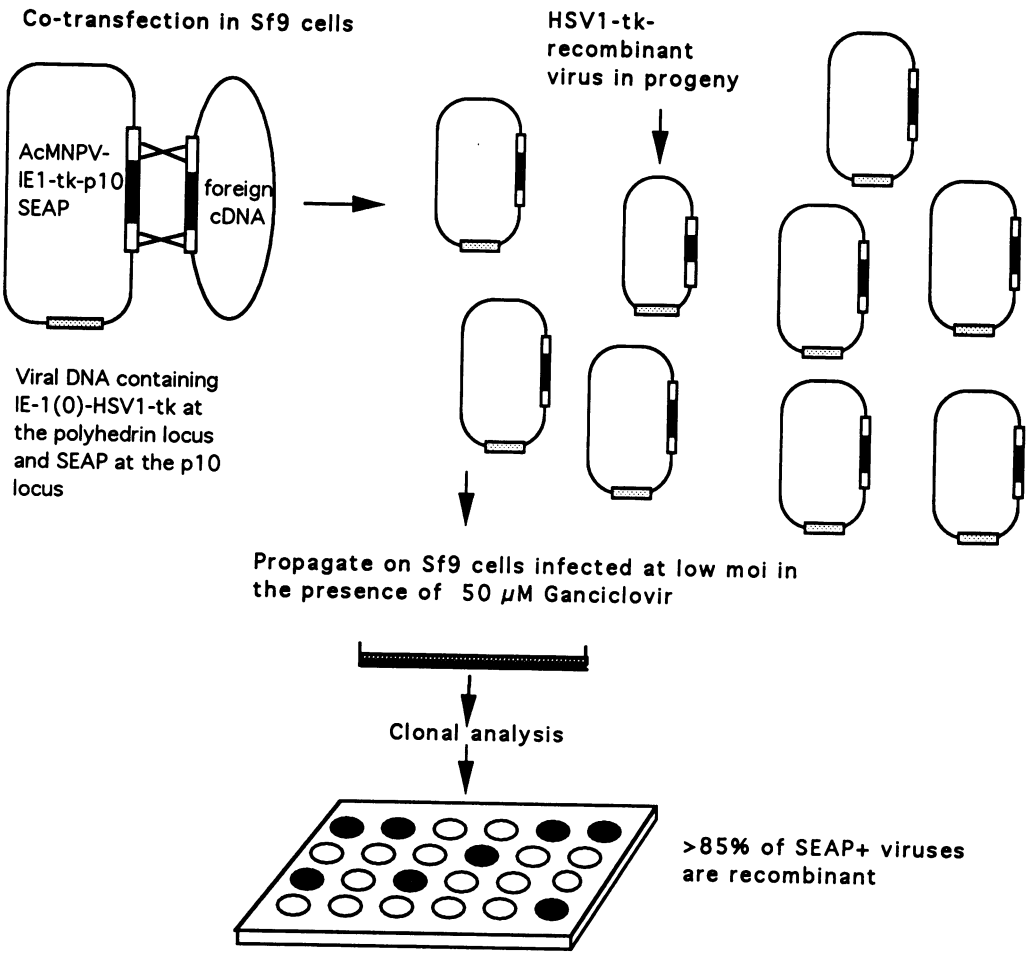

Figure 5. Scheme of Ganciclovir counter-selection of HSV1-tk-harboring parental viruses. AcMNPVIE-1-tk-p10-SEAP viral DNA is used with a polyhedrin-targetted replacement vector containing a cDNA of interest to cotransfect Sf9 lepidopteran cells. The mixed progeny is then propagated at moi < 1 on Sf9 cells in the presence of 50 μM Ganciclovir. When appropriately diluted to clonally infect fresh Sf9 cells in 96 well microtiter plates, over 85% of the viral clones are recombinant for the cDNA of interest and are, in addition, endowed with the readily assayable secreted alkaline phosphatase gene marker.

by hybridization to the viral probe were also recombinant for SEAP cDNA at the polyhedrin locus.

In conclusion, GCV treatment of cells infected with a mixture of baculoviruses whose genome harbors the presently described HSV1-tk gene construct can lead to the negative selection of HSV1-tk$^+$ viruses and to a reciprocal enrichment in HSV1-tk$^-$ viruses, and specifically of recombinant viruses expressing other genes at the polyhedrin locus as a result of homologous recombination with the parental virus. These characteristics permit the design of a selection method using the presently described viral DNA as a recombination partner with replacement vectors to target new cDNAs to the polyhedrin locus of AcMNPV in order to isolate new recombinant viruses via a simple one-step procedure schematically represented in Figure 5.

DISCUSSION

The present work describes the development of a conditional lethal viral vector resulting from the introduction into the viral genome of the HSV1 thymidine kinase gene, whose product can convert a benign nucleoside analog into a toxic intermediate leading to the inhibition of viral replication. Cellular systems using the same strategy have already been developed for cultured cells (11, 12), and for specific tissues of transgenic mice (13, 14). Our goal was to transpose this approach to a replicative viral system where such toxic intermediates could interfere with viral replication, leading to the counter selection of viruses expressing this activity. Given the organization of the viral replicative cycle, it was crucial that the toxic inhibitor of DNA replication be present at the onset of viral DNA synthesis, which starts 6 hours after infection (15). Consequently, HSV1-TK had to be produced in sufficient amounts during the first few hours following infection. This requirement eliminates at once the use of very late promoters such as the the polyhedrin promoter, which are active only after viral replication is completed. The success of our strategy has thus critically depended on the use of an AcMNPV immediate early promoter, IE-1(0), to drive the expression of the HSV1-tk gene. This promoter is active in Sf9 lepidopteran cells and does not require the presence of other viral functions (19, 20). In contrast, recombinant viruses harboring a construct in which the HSV1-tk gene is driven by the authentic HSV1-tk herpes virus promoter are not sensitive to treatment by GCV (results not shown), probably because this promoter is poorly recognized in lepidopteran cells (20). A selectable viral system using the bacterial gene *eco* gpt as a dominant marker for the selection of recombinant Vaccinia viruses has recently

been described (46). The dominant marker was also placed under the control of the 7.5K polypeptide promoter, a promoter also expressed early in infection.

The ability of GCV to inhibit replication of the recombinant baculovirus harboring HSV1-tk by GCV, permitted us to select viruses having lost this counter selectable marker as a result of homologous recombination with a replacement vector containing a candidate cDNA for gene substitution. The selection method described here, allowed us to obtain a recombinant fraction of more than 85%, which is significantly higher than the previously described method where viral DNA is linearized prior to cotransfection in order to increase the recombinant fraction in the range of 15–25% (5). This is the highest fraction of recombinant viruses reported thus far in the litterature. We have been able to use this method for the one-step isolation of several recombinant viruses which exhibited the expected activity in one step. The method described here should thus make recombinant virus isolation an easier and faster procedure.

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ABBREVIATIONS

AcMNPV: *Autographa californica* multicapsid nuclear polyhedrosis virus; SEAP: secreted human placental alkaline phosphatase; bp: base pair; HSV1-TK: herpes simplex virus type 1 thymidine kinase; GCV: 9-(1,3-Dihydroxy-2-propoxymethyl) guanine (Ganciclovir); PCR: polymerase chain reaction; moi: multiplicity of infection; X-Gal: 5-Bromo-4-chloro-3-indoyl β-D-galactopyranoside.

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