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Antiviral Research 28 (1995) 191–212

Antiviral Research

Herpes simplex type 1: *lacZ* recombinant viruses. I. Characterization and application to defining the mechanisms of action of known antiherpes agents

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Received 31 January 1995; accepted 5 June 1995

Abstract

Recombinant viruses with the *lacZ* gene placed under the control of the HSV-1 ICP4, TK and gD regulatory regions were constructed by recombination into the TK locus of HSV-1. Difficulty in isolating ICP4 and gD recombinant viruses with high level, regulated expression of β-galactosidase was overcome by the use of HSV-1 translational initiation sequences of these genes in place of vector-derived sequences. β-Galactosidase expression displayed the kinetics particular to each viral class. The maximal expression of β-galactosidase from the recombinant viruses within a 22-h period (m.o.i. 5) (relative to the ICP4 virus) was gD(3) > gC(2) > ICP4(1) > TK(0.5). The ICP4 virus produces easily quantifiable levels of β-galactosidase activity for multiplicities of infection from 5×10^{-4} through 5 over 48 h postinfection. At multiplicities of infection between 2 and 5, ICP4-driven activity was measurable within 2 h postinfection from a monolayer of 3×10^4 Vero cells in microtiter wells. Mechanisms of inhibition of several antivirals were probed by using the regulated expression of β-galactosidase from the ICP4 virus as a marker for viral growth. An experimental antiviral (E3925, IC_{50} 1 μg/ml) and a neutralizing gD MAb (DUP55306, IC_{50} 0.6 μg/ml) acted prior to immediate early synthesis, consistent with inhibition of viral entry or uncoating. IFN-γ inhibited expression of immediate–early synthesis, while having no effect on viral entry. IC_{50} values for E3925 obtained using either the ICP4 or gD viruses at m.o.i. 0.005, were in good agreement with those obtained by standard plaque assays, but were determined in only 1 day, using a microtiter plate format. Thus, these reporter viruses are useful tools for defining the mechanisms of action of antiherpes agents, while quantitatively reproducing the

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SSDI 0166-3542(95)00048-8

results for IC_{50} determinations from standard plaque assays within 24 h in a microtiter plate format.

Keywords: Herpes simplex virus type 1 (HSV-1); *lac*Z; Recombinant herpes simplex virus type 1

1. Introduction

Site-directed insertion into the genome of HSV has proven to be valuable in studies of the function of herpes genes (Cai and Schaffer, 1989) and their promoters (Post et al., 1981; Post and Roizman, 1981). For example, insertional mutagenesis of the *lac*Z gene has been shown to be a powerful tool for the construction and isolation of null mutants (Goldstein and Weller, 1988; Carmichael and Weller, 1989). It is known that when viral promoters are used to drive heterologous gene expression, the expression of the heterologous gene may be subject to the herpes viral program whose expression is coordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman, 1974). For example, when the *aprt* gene from CHO cells was recombined into the HSV genome within the TK locus, the recombinant virus transcribed an mRNA which was indistinguishable from that synthesized by $Aprt^+$CHO cells (Tackney et al., 1984) and whose appearance was controlled by the TK promoter. Similarly, when the rabbit β-globin gene was placed downstream of the gD promoter and recombined into the HSV genome at the TK locus, the kinetics of β-globin expression followed the native expression of the gD gene (Everett and Dunlop, 1984). More recently, *lac*Z has been used as a reporter gene for studying regulated herpes simplex gene expression when inserted within the TK locus under the control of late herpes promoters (Weir et al. 1990).

In this paper, we report the preparation and characterization of recombinant reporter viruses in which the *lac*Z gene was placed under the control of the HSV-1 ICP4, TK and gD regulatory regions by recombination into the TK locus of HSV-1. We report the use of the ICP4 virus to probe the mechanism of action of several known antiherpes substances: an experimental antiviral, E3925 (a polyoxometalate), a neutralizing monoclonal antibody to the HSV gD protein (DUP55306) and interferon gamma (IFN-γ). In an accompanying paper (Dicker et al., 1995) we report the use of a panel of these recombinant viruses to aid in the identification of points in the herpes life cycle at which antiherpes drug candidates, of unknown mechanism, may be acting.

2. Experimental

2.1. Materials and methods

Baby hamster kidney (BHK21) and African green monkey (Vero) cells were obtained from ATCC (CCL 10 and CCL 81). BSC40 and BSV65 (VP16 stably integrated in BSC40) cells were kind gifts from Dr. John P. Capone, McMaster University, Department of Biochemistry, Ontario, Canada. All cells were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (Gibco), penicillin–strep-

tomycin (Gibco) and 1 mM glutamine (Gibco). IFN-γ was obtained from Hoffman La Roche, Nutley, NJ, HSV-1 (strain KOS) was obtained from Dr. Priscilla Schaffer via Dr. Annie Colberg-Poley and propagated according to standard protocols. β-Galactosidase assays for transient transfections were performed according to the method of Eustice et al. (1991) using chlorophenol red-β-D-galactopyranoside (CPRG) (Boehringer-Mannheim) as substrate. AraT and X-gal were obtained from the Sigma Chemical Co. Plasmid pGal8 and an HSV recombinant in which the expression of β-galactosidase was under the control of the HSV-1 late gene gC regulatory region were kind gifts from Dr. Jerry Weir, Walter Reed Army Institute of Research. Plasmids pSG28B/K and pSG1 were also obtained from Dr. R. Sandri-Goldin via Dr. Annie Colberg-Poley. MAb DUP55306 (a neutralizing MAb against the HSV gD protein) was a kind gift from Dr. Conrad Heilman, DuPont Merck Pharmaceutical Co. Plasmid pMSVP16 was a kind gift from Dr. Kent Vrana, West Virginia University Health Science Center.

2.1.1. Microtiter plate assays

Ninety-six-well microtiter plates (Corning no. 25860) were seeded with Vero cells (50 μl suspension) and incubated overnight (3×10^4 cells/well). Medium was removed and cells were infected at the desired m.o.i. with 25 μl of medium containing virus. After 2 h, the inoculum was removed and replaced with 50 μl of fresh medium or medium containing an anti-HSV agent. At the desired time postinfection, 50 μl of β-galactosidase lysis buffer containing CPRG (final concentration 0.4 mg/ml) was added and the cells were shaken on a plate shaker for 1 min. β-Galactosidase activity versus time was monitored by the change in absorbance at 575 nM on a Molecular Devices Thermomax microtiter plate reader at room temperature.

2.1.2. Preparation of viral DNA

A monolayer of Vero cells was infected with HSV-1 (KOS) at a multiplicity of 5 PFU/cell. After 24 h, $\sim 5 \times 10^6$ cells were harvested and resuspended in 10 ml of LCM buffer (0.5% NP40, 30 mM Tris pH 7.5, 3.6 mM $CaCl_2$, 5 mM $MgCl_2$, 125 mM KCl, 0.5 mM EDTA, 6 mM β-mercaptoethanol). After digestion with 25 μg/ml of DNase I and 25 μg/ml of RNase I for 30 min at room temperature, it was extracted twice with 1 ml Freon® (1,1,2- trichloro-1,2,2-triflouroethane), and the upper (aqueous) phase was layered on top of 45% glycerol and spun in a Beckman SW41 at 26 K rpm for 1 h at 4°C. The nucleocapsid pellet was resuspended in 0.5 ml of TNE (50 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA), treated with 1 mg/ml proteinase K at 50°C for 1–2 h and extracted with phenol–chloroform. The resulting DNA was precipitated overnight with ethanol.

2.1.3. Isolation of stable HSV-1:lacZ recombinants

Approximately 3 μg of each plasmid, containing the desired regulatory region upstream of *lac*Z within pGal8, was cotransfected with 1 μg of HSV DNA onto Vero cells, using calcium phosphate precipitation (Chen and Okayama, 1987). The cells were incubated for 5 h at 37°C before being fed fresh medium. After 5 days, the total progeny virus from the cotransfection was harvested (by pooling the lysed cells), serially diluted and plated onto fresh Vero cells. The cells were overlaid with Dulbecco's modified

minimal essential medium (DMEM) without phenol red (Gibco), containing 100 μg/ml of AraT, 0.5% agarose (FMC) and 2% fetal bovine serum (Gibco) to select for TK^- plaques (Stow et al., 1978). After 2 days, the TK^- plaques were further overlaid with 0.5% agarose in DMEM containing 300 μg/ml of X-gal (no phenol red, no additional AraT). Prospective recombinant virus was identified by the appearance of blue plaques 8–24 h after this overlay. Blue plaques were picked after 24 h and plaque purified a total of 3 times. Titers were typically between 3×10^8 and 1×10^9 PFU/ml.

2.1.4. Transient transfections

BHK21, BSV65, BSC40 or Vero cells were subcultured so that they were 75–80% confluent on the day of the transfection. Approximately 3 μg of the various pHSV*lac*Z constructs (ICP4, ICP0, gD, TK) were transfected individually onto a monolayer of BHK21 cells by the calcium phosphate precipitation method. After 5 h, the medium was removed and the cells shocked for 2 min with 15% glycerol in DMEM containing 10% fetal bovine serum. The cells were washed twice with phosphate-buffered saline and growth medium added. The cells were harvested at different time points and analyzed for β-galactosidase. In some cases, transfections were followed by superinfection with HSV-1 (KOS) for 1 h at different m.o.i.s. The cells were then harvested at various times and β-galactosidase activity determined as before.

2.1.5. Northern analysis

Total RNA was isolated (Promega's total RNA isolation kit) from Vero cells infected at m.o.i. 5 with the HSV-1:ICP4*lac*ZB and the HSV-1:gD*lac*Z viruses at 5.5 and 14 h postinfection with or without cycloheximide and PAA. Total RNA (15 μg) was electrophoresed through 1% formaldehyde-agarose gels according to a standard method (Sambrook et al., 1989). The RNA was blotted to Genescreen™ and prehybridized for 15 h with 50% formamide, 1 M NaCl, 10% dextran sulfate and 1% SDS. ICP4 and gD promoter fragments and *lac*Z DNA were labeled with ^{32}P using the random priming method (5′–3′) according to the manufacturer's instruction. The blots were hybridized separately with the labeled *lac*Z DNA (from pGal8) with 100 μg of sonicated salmon sperm DNA for 15 h and washed twice at room temperature for 5 min with 2 × SSC buffer, once at 60°C for 30 min with 1% SDS/2 × SSC and finally at room temperature for 30 min with 0.1 × SSC. The washed blots were exposed to XAR5 film (Kodak) with a DuPont Croner intensifying screen at −70°C.

2.1.6. Plasmids

pICP4*lac*Z: pSG28B/K was cleaved with *Pvu*II/*Acc*I to give a 997-bp segment containing the ICP4 regulatory region. This fragment was cut with *Hin*fI/*Bam*HI, filled in with Klenow and ligated into pGal8 which had previously been linearized with *Sal*I and filled in with Klenow fragment. This clone spans (−613 to +30) of the ICP4 transcriptional start site.

pICP4*lac*Z(361): the *Hin*fI/*Bam*HI ICP4 fragment was cut with *Sma*I; the resulting fragment was filled in with Klenow and cloned into pGal8 (which had been digested with *Sal*I and filled in with Klenow). The final segment spans (−331 to +30) of the transcriptional start.

pICP4*lac*Z(δ123): this clone was isolated as an accidental deletion of 123 base pairs from the ICP4 *Hin*fI/*Bam*HI fragment (−615 to −94) and was cloned into the *Sal*I site of pGal8.

pICP4*lac*ZA: pICP4*lac*Z was cut with *Xba*I/*Bam*HI and ligated to

```
CTAGACAAGAGGCTGCGATGGTTCGTGCAAACAAACGCAACGAGGCTCTACGAATCGGG
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    TGTTCTCCGACGCTACCAAGCACGTTTGTTTGCGTTGCTCCGAGATGCTTAGCCCCTAG.
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pICP4*lac*ZB:pICP4*lac*Z was cut with *Xba*I/*Bam*HI and ligated to

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CTAGACAAGAGGCTGCGATG
    ||||||||||||||||||
    TGTTCTCCGACGCTACCTAG.
```

pICP4*lac*ZC:pICP4*lac*Z was cut with *Xba*I/*Bam*HI and ligated to

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CTAGACAAGCCGCCGCCATG
    ||||||||||||||||||
    TGTTCGGCGGCGGTACCTAG
```

pICP4*lac*ZD:pICP4*lac*Z was cut with *Xba*I/*Bam*HI and ligated to

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CTAGAAAGTACTTACATATGGTTCGTGCAAACAAACGCAACGAGGCTCTACGAATCGGG
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    TTTCATGAATGTATACCAAGCACGTTTGTTTGCGTTGCTCCGAGATGCTTAGCCCCTAG.
```

pICP4*lac*ZE:pICP4*lac*Z was cut with *Xba*I/*Bam*HI and ligated to

```
CTAGACCCGCATCGGCGATG
    ||||||||||||||||||
    TGGGCCTAGCCGCTACCTAG
```

pICP4*lac*ZF:pICP4*lac*Z was cut with *Xba*I/*Bam*HI and ligated to

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CTAGACCCGCATCGGCGATGGTTCGTGCAAACAAACGCAACGAGGCTCTACGAATCGGG
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    TGGGCGTAGCCGCTACCAAGCACGTTTGTTTGCGTTGCTCCGAGATGCTTAGCCCCTAG
```

pICP0*lac*Z: the *Sac*I/*Bbv*IA fragment from the ICP0 regulatory region (Perry et al., 1986) was obtained by PCR from HSV-1 (KOS) genomic DNA. The region was successfully amplified by PCR using *Taq*® polymerase, but only in the presence of

10% glycerol. The fragment was filled in with Klenow and cloned into pGal8 within the *Xba*I/*Sal*I sites (−807 to +51) of the transcriptional start.

pICP0*lac*ZA:pICP0*lac*Z was cut with *Xba*I/*Bam*HI and ligated to

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CTAGACATACGACCCCCATG
 |||||||||||||||||
    TGTATGCTGGGGGTACCTAG
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pTK*lac*Z was prepared by cleaving pGal8 with *Hin*dIII, filling in overhanging ends with Klenow and religating with T4 DNA polymerase. This represents from −727 to +495 relative to the TK transcriptional start site.

pgD*lac*Z: a 409-bp *Sac*I/*Hin*dIII fragment was obtained from pRWF6, containing the 6.4-kb J fragment of HSV-1 (Patton) (Watson et al., 1983). This segment was treated with Klenow (*Sac*I blunted, *Hin*dIII filled in) and ligated into the *Eco*RV site of Bluescript SK$^+$. The gD sequence was then excised with *Sma*I/*Sal*I and ligated into pGal8 (which had been cut with *Hin*dIII, filled in with Klenow then cut with *Sal*I). The resulting clone contains the gD regulatory region (−423 to −20) relative to the transcriptional start.

pICP0*lac*ZA: pICP0*lac*Z: was cut with *Xba*I/*Bam*HI and ligated to

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CTAGAGTGCGTTCCGGTATG
 |||||||||||||||||
    TCACGCAAGGCCATACCTAG
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3. Results

3.1. Construction of the panel of HSV-1:lacZ reporter viruses

3.1.1. ICP4 reporter viruses

The vector pGal8 (Weir et al., 1990) was used to prepare *lac*Z positive, TK-deficient HSV recombinants. This vector contains the *lac*Z gene inserted within the HSV-1 TK gene and contains a polylinker upstream of the *lac*Z gene. Viral promoters (Fig. 1), obtained either by subcloning or PCR from genomic or plasmid HSV DNA, were

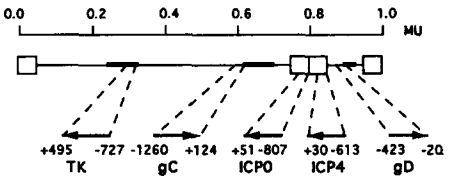

Fig. 1. HSV genome, showing regulatory regions (relative to transcriptional start sites) used in this study.

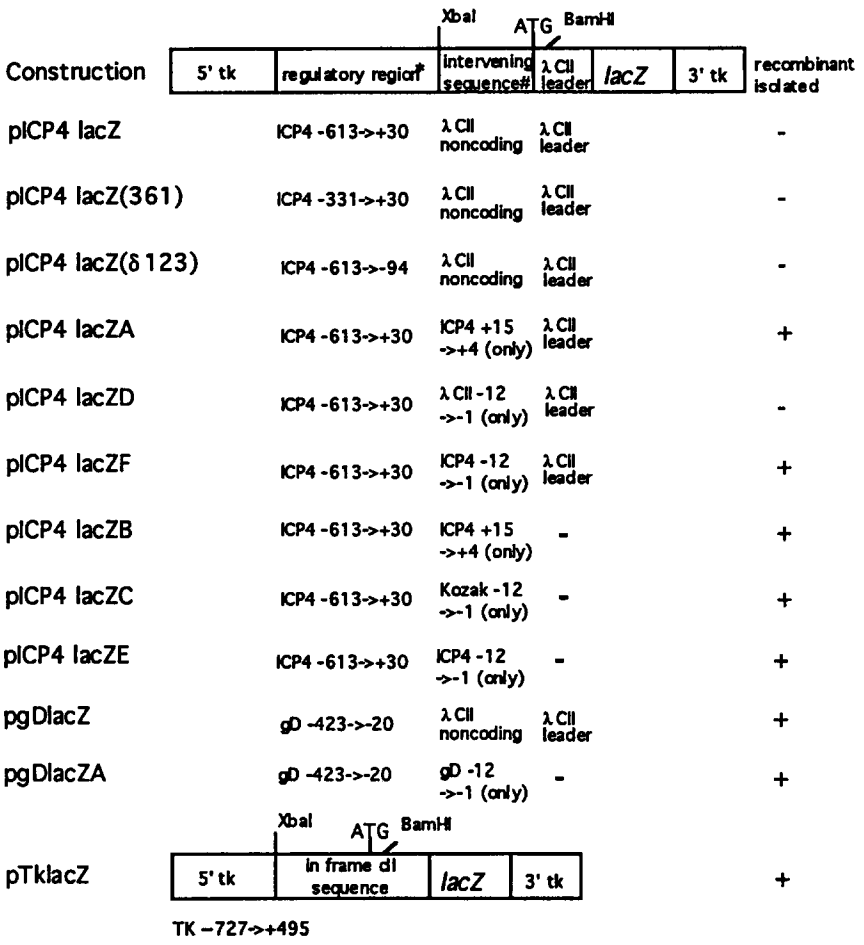

Fig. 2. Organization of constructions made for this study showing partial or total replacement of CII non-coding (intervening) and CII leader sequences. *, relative to transcriptional start, #, relative to lacZ translational start. Plasmid pTK*lac*Z: the CII leader is in frame with the *lac*Z gene and encodes a fusion protein. + or −, a recombinant virus could (+) or could not (−) be isolated.

inserted into one of several unique restriction sites. Thus, pICP4*lac*Z (Fig. 2) was constructed and evaluated by transient transfection into BHK21, BSC40 (VP16-negative) and BSV65 (VP16-positive) cells (Werstuck et al., 1990). BSV65 is a derivative of BSC40 which constitutively expresses VP16, the potent transactivator of HSV immediate–early gene expression (Batterson and Roizman, 1983; Campbell et al., 1984). In the absence of additional VP16 (supplied by pMSVP16, Liu et al., 1990), β-galactosidase activity in BHK21, BSC40 and BSV65 cells was increased 1.3-, 1.2- and 2.9-fold, respectively (versus pGal8 as control). In the presence of additional VP16 supplied by cotransfection with pMSVP16, β-galactosidase activity was increased 9.8-, 6.0- and 16-fold, (versus pGal8 as control). Apparently, BSV65 cells supply the necessary

Table 1
Comparison of the fold induction of β-galactosidase expression by ICP4 reporter plasmids in BHK21 cells

Plasmid	Fold induction [a]
pICP4*lac*Z	1.4±0.2
pICP4*lac*Z(361)	1.6±0.3
pICP4*lac*Zδ123	1.0±0.2
pICP4*lac*ZA	3.0±0.7
pICP4*lac*ZB	9.5±2.8
pICP4*lac*ZC	3.9±1.0

[a] Approximately 3×10^5 BHK21 cells were cotransfected with 3 μg of pICP4*lac*Z±3 μg pMSVP16. After 24 h, cells were harvested and β-galactosidase activity was assayed, as described in Materials and methods. Numbers are averages of duplicate experiments and represent the ratio of fold induction over the vector control in the absence of VP16 (basal expression) divided by fold induction over the vector control in the presence of VP16, with S.D.

functions for transactivation of pICP4*lac*Z, as expected, but this activity is further increased by additional VP16 supplied by cotransfection with pMSVP16.

Despite confirmation of the responsiveness of pICP4*lac*Z to VP16 by transient analysis, a recombinant containing the ICP4 regulatory region could not be isolated. Since pGal8 contains lambda CII sequences upstream of the *lac*Z translational start, we questioned whether this may have interfered with *lac*Z expression in the viral context. Therefore, the CII non-coding and CII leader sequences were completely or partially replaced by HSV-1 sequence to give derivative plasmids pICP4*lac*ZA-F (Fig. 2).

Plasmids pICP4*lac*ZA, B and C were studied by evaluating their ability to respond to transactivation by VP16, in comparison to pICP4*lac*Z and pICP4(361) (a shorter 5′-regulatory region than pICP4*lac*Z) and pICP4δ123. The numbers in Table 1 represent the ratio of fold induction over the vector control in the absence of VP16 (basal expression) divided by fold induction over the vector control in the presence of VP16. Simple replacement of the CII non-coding sequence, while retaining the CII leader sequence upstream of the ATG start by an alternative translational initiation (TI) sequence, conferred VP16 responsiveness (pICP4*lac*ZA, 3-fold induction). Greater VP16 responsiveness was observed when the entire intervening sequences (CII non-coding as well as the CII leader) were replaced with a control sequence (pICP4*lac*ZB, 9.2-fold) or with a Kozak TI sequence (Kozak, 1987; Cavener and Ray, 1991) (pICP4*lac*ZC, 3.9-fold). By comparison, plasmids pICP4*lac*Z and pICP4*lac*Z(361) were only modestly transactivated by VP16 (1.4- and 1.6-fold, respectively) while pICP4*lac*Z δ123 was unresponsive, as expected.

Similar constructions were then made with replacement of the CII non-coding sequence by the native −12 to −1 region (pICP4*lac*ZF) of the ICP4 gene or of the entire intervening sequence (CII non-coding and CII leader) with the native sequence (pICP4*lac*ZE). Lastly, pICP4lacZ was altered only slightly by removing part of the intervening sequence, leaving a 12-bp TI region from lambda CII (pICP4*lac*ZD).

Recombinants were readily isolated from pICP4*lac*ZA, B, C and E, but not from pICP4*lac*ZD, clearly indicating the importance of a suitable TI sequence for proper expression of β-galactosidase within the viral context. The unsuitability of the CII

Table 2
Translational initiation regions preceding the ATG start codon within the ICP4 plasmids [a]

pICP4 *lac*ZA&B:	CAA CAG GCT GCG	(+15 → +4 of ICP4)
pICP4 *lac*ZE&F:	CCC GCA TCG GCG	(−12 → −1 of ICP4)
pICP4 *lac*Z&D:	AAG TAC TTA CAT	(lambda CII within pGAL8)
pICP4 *lac*ZC:	CAA GCC GCC GCC	(Kozak Consensus)

[a] Regions of identity with the native HSV-1 −12 to −1 translational initiation regions of ICP4 are boxed.

sequence is probably due to its poor fit with the consensus sequence (Table 2), particularly a C residue at the −3 and a T residue at the −1 position (Kozak, 1987).

In contrast to the results with the ICP4 promoter, recombinants from the ICP0 promoter were readily obtained without any need to replace the region from pGal8. Moreover, when a replacement of the region was made, the ICP0*lac*ZA virus was indistinguishable from the parent ICP0*lac*Z in terms of β-galactosidase expression (data not shown).

3.1.2. Temporal expression of β-galactosidase from the ICP4lacZB recombinant virus

Confluent monolayers of Vero cells in 96-well microtiter plates (average cell number 3×10^4 per well) were infected with ICP4*lac*ZB (m.o.i. 5×10^{-4} through 5) to explore the kinetics of β-galactosidase expression (Fig. 3). At the lowest m.o.i. (5×10^{-4}), β-galactosidase activity was not detectable until 46.5 h postinfection (h p.i.), reflecting

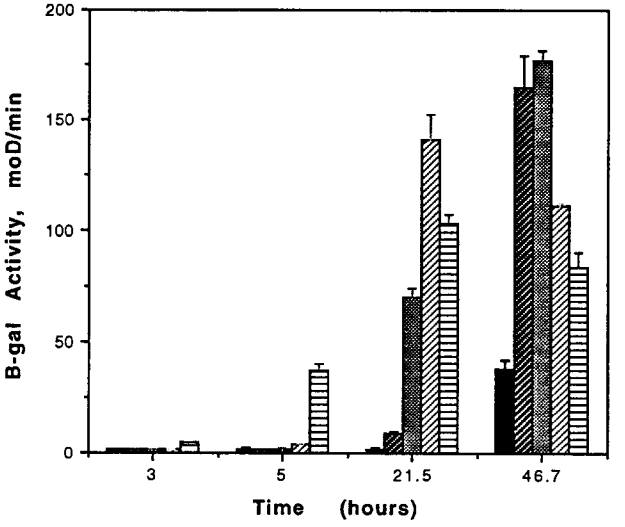

Fig. 3. Kinetics of β-galactosidase expression from infection of Vero cells by ICP4*lac*ZB in 96-well microtiter plates between m.o.i. 5×10^{-4} through 5, as described in Materials and methods. m.o.i.: 0.0005 (■), 0.005 (■), 0.05 (■), 0.5 (▨), 5 (▤), bars indicate standard deviation of quadruplicates.

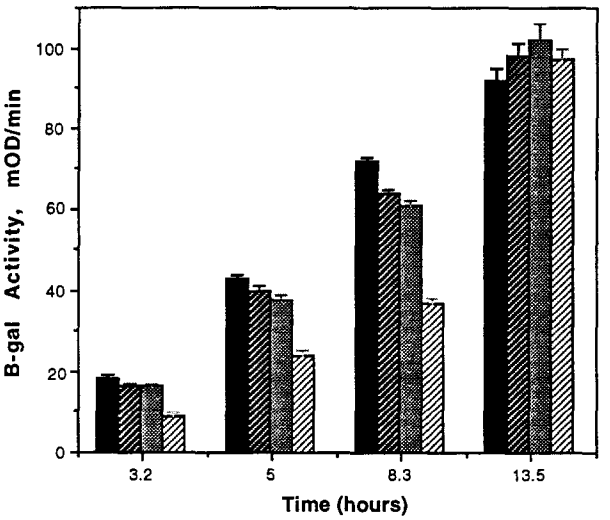

Fig. 4. Sensitivity of signal (β-galactosidase activity) to changes in viral input. ICP4*lac*ZB m.o.i. were varied from 2 to 5 and assayed between 3.2 and 13.5 h, as described in Materials and methods. m.o.i.: 2 (■), 4 (▨), 4.5 (■), 5 (■), bars indicate standard deviation of duplicates.

expression from reinfection after subsequent rounds of replication. At intermediate m.o.i., activity appeared earlier. At the highest m.o.i. (5), β-galactosidase activity was detectable within 3 h, increasing steadily and reaching a maximum after 1 day. This followed the pattern of expression expected for this immediate–early promoter (Clements et al., 1977). Maximal activity (180-fold over background) was observed at m.o.i. 0.05 assayed at 47 h p.i.

3.1.3. Infection at relatively high m.o.i.

The response at relatively high m.o.i. was investigated for the ICP4*lac*ZB virus. Fig. 4 profiles the dynamic range for detection of β-galactosidase activity. Differences in activity at m.o.i.s between 2 and 5 were resolvable at early times postinfection, with the greatest differentiation observed at 8 h p.i. At 13.5 h p.i., there was little difference in signal. Thus, this recombinant virus can be used as a sensitive and rapid marker for assaying small differences in viral input at high multiplicities of infection within short times postinfection.

3.1.4. Transient vs viral expression of β-galactosidase

Expression of β-galactosidase activity from recombinants ICP4*lac*ZA,B and C was compared to the activity observed by transient analysis of the corresponding plasmids (Fig. 5). Expression levels followed the rank order of expression observed transiently, thus validating the use of transient expression to compare semi-quantitatively the reporter plasmids and to serve as a guide for the construction of reporter viruses.

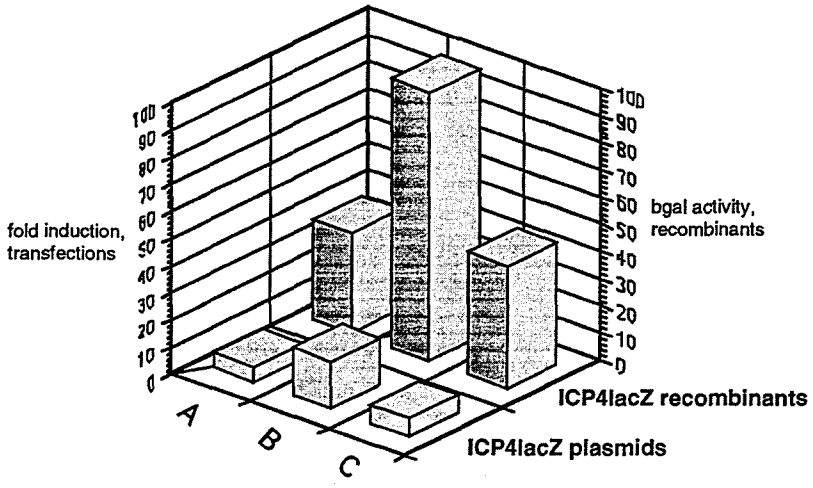

Fig. 5. Comparison of expression of β-galactosidase activity from ICP4 recombinant viruses A, B and C (3×10^4 Vero cells, m.o.i. 5, 22 h) to the expression observed by transient expression (see Materials and methods) from the corresponding plasmids in BHK21 cells.

3.1.5. TK and gD reporter viruses

Plasmid constructions were validated by cotransfection and/or superinfection studies. Plasmid pTK*lac*Z was validated by cotransfection into BHK21 cells with pSG28B/K or pSG1 which express the ICP4 and ICP0 proteins, respectively (Sekulovich et al., 1988). In the presence of these transactivators (DeLuca and Schaffer, 1985; Gelman and Silverstein, 1985), β-galactosidase activity was increased 2.9- and 2.1-fold, respectively, versus pTK*lac*Z-only control, as assayed 20 h p.i. Superinfection studies (Table 3) also demonstrated upregulated expression of pTK*lac*Z. By this technique (Everett and Dunlop, 1984), a foreign promoter, introduced by transfection, is transactivated by viral gene products introduced by infection. In this case, BHK21 cells were transfected with pTK*lac*Z followed 24 h later by infection with HSV-1 (strain KOS). β-Galactosidase activity was elevated from 2 to 6.5 h p.i., declining to background by 15 h p.i.

Table 3
Time dependence of fold induction of β-galactosidase expression transfection/superinfection [a]

Plasmid	Hours postinfection			
	2	4	6.5	15
pICP4*lac*Z	1.7±0.3	1.7±0.3	2.7±0.6	2.7±0.5
pICP4*lac*Z(δ123)	1.2±0.3	1.2±0.2	1.2±0.2	1.1±0.2
pTK*lac*Z	1.1±0.3	2.5±0.5	2.3±0.4	0.9±0.2
pgD*lac*Z	1.1±0.2	1.1±0.2	1.3±0.3	4.2±0.9

[a] BHK21 cells were transfected with pgD*lac*Z, pTK*lac*Z, pICP4*lac*Z and pICP4*lac*Zδ123. At 24 h post-transfection, cells were infected with HSV-1 (strain KOS) at m.o.i. 5 and subsequently assayed at times shown for β-galactosidase activity, as described in Materials and methods. Data are from duplicates, with standard deviations, expressed as fold induction compared to vector control plus virus.

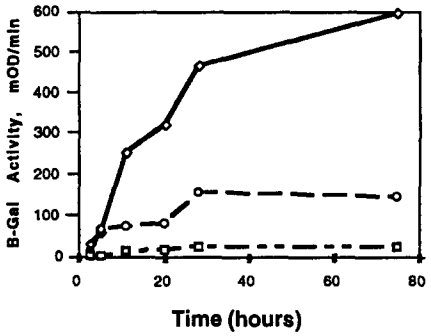

Fig. 6. Comparison of β-galactosidase expression from gD*lac*Z (□), gD*lac*ZA (◇) and ICP4*lac*ZB (○) at m.o.i. 10 over a 75-h time course. Monolayers of 3×10^4 Vero cells were infected with viruses and assayed at the indicated times, as described in Materials and Methods.

The pgD*lac*Z construction was validated by transfection/superinfection studies similar to those done for the pTK*lac*Z (Table 3). β-Galactosidase activity was apparent at 15 h p.i., as expected for this delayed early promoter (Everett, 1983; O'Hare and Hayward, 1984). The recombinant virus derived from this construction (gD*lac*Z) expressed a level of β-galactosidase activity far lower than that observed from both ICP4*lac*ZB or ICP0*lac*Z, though expression of β-galactosidase activity followed the delayed early time course (Ikura et al., 1983; Everett, 1986). Therefore, the CII non-coding and leader sequences within the gD construct were replaced by the −1 to −12 region from the gD gene of HSV-1. Vero cells infected with this derivative virus, gD*lac*ZA, produced approximately 20-fold higher levels of β-galactosidase than cells infected with gD*lac*Z and 3–4 times the level of expression (depending on when assayed) of ICP4B*lac*ZB over a 75-h time course at m.o.i. 1, 5 and 10. Data for m.o.i. 10 are shown in Fig. 6.

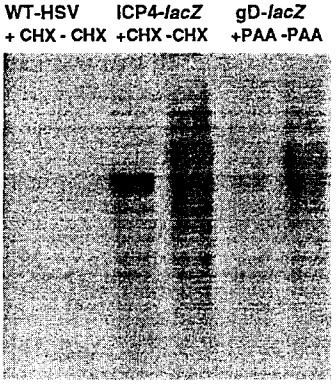

Fig. 7. Comparison of *lac*Z messages produced by the ICP4*lac*ZB and gD*lac*Z viruses as a function of treatment with either cycloheximide (C) or phosphonoacetic acid (PAA). Total RNA was isolated from Vero cells infected at m.o.i. 5 with or without cycloheximide (CHX) or phosponoacetic acid (PAA) with HSV (KOS), HSV-1:ICP4*lac*ZB and the HSV-1:gD*lac*Z viruses at 5.5, 5.5 and 14 h p.i., respectively.

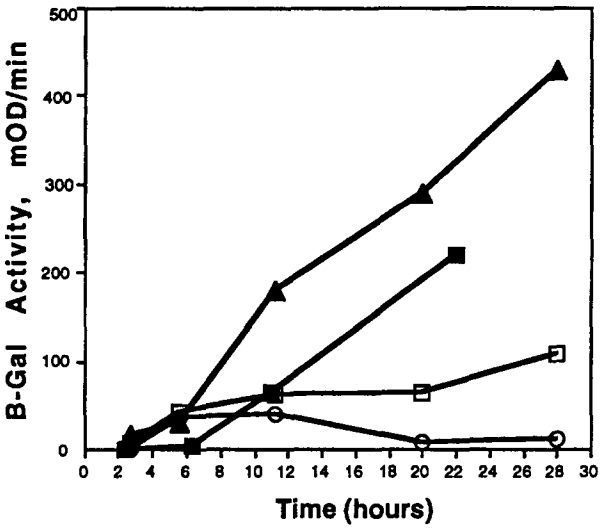

Fig. 8. Comparison of the β-galactosidase activity generated by the recombinant *lac*Z viruses from one member of each class of viral gene expression: □, ICP4*lac*ZB, immediate–early (IE); ○, TK*lac*Z, early (E); ▲, gD*lac*Z, delayed early (DE); and ■, gC*lac*Z, late (L). Monolayers of 3×10^4 Vero cells were infected with viruses at m.o.i. 5 and assayed, as described in Materials and methods.

The level of resistance of the recombinant viruses to AraT and acyclovir were determined using ICP4*lac*ZB at m.o.i. 0.04 by assaying for β-galactosidase activity at 27 h p.i.: AraT IC_{50} > 100 μg/ml (27% inhibition at 100 μg/ml), acyclovir IC_{50} 25 μg/ml, IC_{90} 120 μg/ml. By plaque assay, ICP4*lac*ZB was 24-fold more resistant than HSV-1 (KOS) in the presence of Acyclovir (IC_{50} 7.1 vs 0.3 μg/ml). Transcriptional regulation of the *lac*Z message was studied from the ICP4*lac*ZB and gD*lac*Z viruses (Fig. 7). As shown in the Northern blot, the *lac*Z message from the ICP4*lac*ZB virus was amplified in the presence of cycloheximide while *lac*Z transcripts from the gD*lac*Z virus were completely eliminated by treatment with PAA, thereby confirming the immediate–early and delayed early characteristics of these messages, respectively (a longer *lac*Z transcript from the ICP4*lac*ZB virus eliminated by cycloheximide might indicate readthrough from the upstream TK promoter). Thus, the resistance to DNA synthesis inhibition, together with the Northern analysis, confirmed integration of the heterologous genes within the TK locus.

3.1.6. Comparison of β-galactosidase activity between classes

The β-galactosidase activity generated by the recombinant viruses was studied in Vero cells at m.o.i. 5 for one member from each class of viral gene expression: immediate–early (IE), early, delayed early and late (Fig. 8).

At an m.o.i. of 5, the ICP4 virus produced detectable β-galactosidase activity within 2.7 h p.i. (3.8-fold over background), the level rising to 72-fold over background at 28 h p.i. In contrast, activity from the TK*lac*Z virus rose rapidly to 50% maximal at 6 h p.i. and reached a maximum at 11 h p.i. Subsequently, activity fell to 20% maximal by 28 h

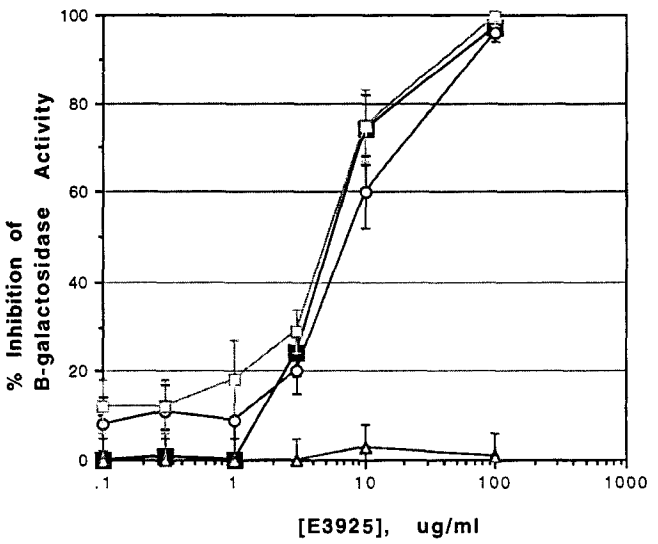

Fig. 9. Comparison of the effects of E3925 on β-galactosidase expression from ICP4*lac*ZB as a function of the order of E3925 addition. Monolayers of 3×10^4 Vero cells were treated as follows: a (■), E3925 and cells preincubated 75 min before adding HSV (m.o.i. 5), assayed 5 h p.i.; b (○), E3925/HSV preincubated 1 h before adding to cells (m.o.i. 5), assayed 5 h p.i.; c (△), E3925 added 2 h p.i. (m.o.i. 5), assayed 5 h p.i.; d (□) E3925 added 2 h p.i. (m.o.i. 0.005), assayed 21.5 h p.i.

p.i., indicating further expression of β-galactosidase did not take place and that previously synthesized β-galactosidase was being degraded. β-Galactosidase activity from the gD*lac*ZA virus appeared at early times postinfection (2.7 h) but at that time was very low compared to levels at later times. Activity rose dramatically in mid-cycle, between 5.5 and 20 h p.i. The gC*lac*Z virus behaved similarly to the gD*lac*ZA virus, except that there was virtually no β-galactosidase activity from the gC*lac*Z virus, on an absolute basis, until 6 h p.i., whereas the gD*lac*ZA virus produced a considerable level of activity at 6 h.

The absolute levels of β-galactosidase activity expressed by these viruses are significantly different from one another. In particular, the gD*lac*ZA virus produced 1.5–3 times the activity of the gC*lac*Z virus (depending on when it was measured in the cycle) while the gC*lac*Z virus produced roughly twice the activity of ICP4B (at later times of infection). The lowest activity was produced by the TK*lac*Z virus; it was approximately 10-fold less active than the gD*lac*ZA virus, 5-fold less active than the gC*lac*Z virus and 6-fold less active than the ICP4B virus.

3.1.7. Mechanism of action studies with the experimental antiviral E3925

Vero cells were treated with E3925 (Blasecki and Domaille, 1991; Blasecki and Mayer-Mihalski, 1993; Blasecki, 1994) using 4 different protocols designed to shed light on its mechanism of action. In the first experiment (a), cells in 96-well microtiter plates were incubated at 37°C with E3925 in complete medium (25λ) at twice the final concentration. After 75 min, the volume was doubled with the addition of ICP4*lac*ZB (m.o.i. 5). In a second experiment (b), virus and E3925 were premixed then added to

Table 4
Correlation of the inhibitory concentrations of E3925 [a]

Virus	Assay time (h)	Calculated IC_{50} (μg/ml)	Calculated IC_{90} (μg/ml)
ICP4*lac*ZB [b]	21.5	3	10
gD*lac*Z [b]	28	1	8
HSV-1 [c]	48	1.3	2.1

[a] Vero cells were infected with ICP4*lac*ZB and gD*lac*Z at m.o.i. 0.005, treated with various concentrations of E3925 at 2 h p.i., then assayed, as described in Materials and methods.
[b] As measured by β-galactosidase assay.
[c] As measured by plaque assay.

cells (m.o.i. 5). In a third experiment (c), cells were infected with ICP4*lac*ZB (m.o.i. 5) for 2 h prior to adding E3925. Assays were performed 5 h p.i. to assess expression of β-galactosidase within the first infectious cycle. E3925 inhibited viral growth so long as it was present before or at the time of infection (a and b, Fig. 9). It was completely ineffective in suppressing this first cycle expression when added to cells which had been previously infected (c), indicating that E3925 acts by inhibiting viral entry, uncoating, or both. In a fourth experiment (d), using the conditions from Expt. c but with infection at m.o.i. 0.005 (where there would only be a barely detectable β-galactosidase signal at 5 h p.i.), viral growth was fully inhibited when assayed at 21.5 h p.i. This indicates E3925 must have blocked the re-entry of virus particles released after the first round of replication, thereby preventing reinfection. It should be noted that IC_{50}s for inhibition by E3925 determined in Expt. d at m.o.i. 0.005 or against gD*lac*Z are in good agreement with those obtained by standard plaque assays for this compound (Table 4). Thus, use of this marker virus quantitatively reproduced the results from a standard plaque assay while providing valuable information on the mechanism of action of this compound against HSV-1 in vitro.

3.1.8. Evaluation of inhibition of HSV by IFN-γ

IFN-γ has been reported to inhibit the expression of HSV IE genes (Raniero De Stasio and Taylor, 1990). Vero cells in 96-well microtiter plates were preincubated for 15 h with 1×10^4 U/ml human recombinant IFN-γ and then infected with ICP4*lac*ZB at m.o.i. 0.2, 1 and 5. β-Galactosidase activity at 6 h p.i. was reduced 80, 72 and 56%, respectively, indicating that inhibition was occurring prior to or at the level of IE gene expression. That the degree of inhibition was inversely proportional to the viral load suggests that higher levels of virus were able to overcome the inhibitory effects to some extent.

In contrast, when cells were first treated with IFN-γ, then immediately infected, IE expression at 6 h p.i. was not inhibited. This indicates that the antiherpes effects of IFN-γ must have a secondary effect on IE expression, requiring cellular changes, rather than the result of any direct action on the virus. Thus, direct inhibition of entry or uncoating could be ruled out.

In companion experiments, cells were treated for 1 h with ICP4I*lac*ZB at low m.o.i. (0.4). The inoculum was removed and replaced with medium containing IFN-γ at 10 through 10^5 U/ml. Assays 24 h p.i. showed an 88% reduction in β-galactosidase

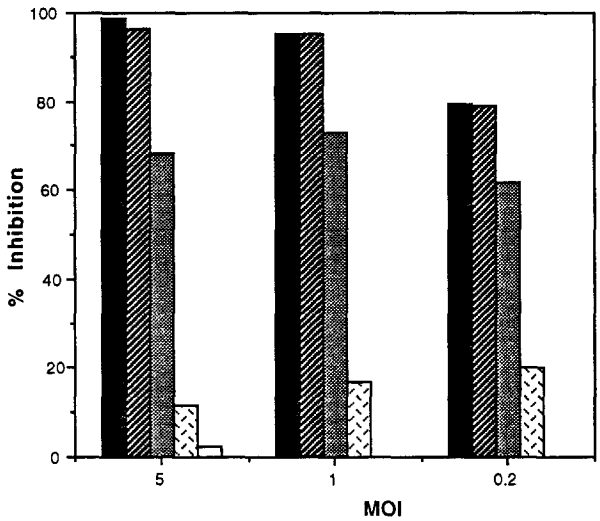

Fig. 10. Inhibitory effect of DUP55306 on growth of ICP4*lac*ZB. Monolayers of Vero cells (3×10^4 cells) in microtiter dishes were incubated for 1 h at 37°C with 8 (■), 2.66 (▩), 0.8 (▩), 0.26 (▨) and 0.08 (□) μg/ml DUP55306 before infection with virus at m.o.i. 0.2, 1 and 5. β-Galactosidase activity was measured 6 h p.i., as described in Materials and methods.

activity at 10^4 U/ml IFN-γ (IC_{50} 550 U/ml), indicating an inhibitory effect of IFN-γ on the IE expression of virus released after the first round of replication.

3.1.9. Inhibition of ICP4lacZB by MAb DUP55306

The gD protein of HSV plays a vital role in mediating entry of HSV into cells (Watson et al., 1982; Johnson and Ligas, 1988). We probed the effects of the neutralizing anti-gD monoclonal antibody, DUP55306, against ICP4*lac*ZB. Virus was preincubated with DUP55306 at concentrations from 0.08 to 8 μg/ml. Mixtures were applied to Vero cells in 96-well plates and β-galactosidase activity was measured 6 h p.i. (Fig. 10). Inhibition was insensitive to viral load (IC_{50} 0.61 μg/ml, IC_{90} 2.2 μg/ml) for multiplicities from 0.2 to 5. These values compare favorably with those obtained by standard plaque assays (IC_{50} 0.81 μg/ml, IC_{90} 3.1 μg/ml (Dr. C. Heilman, personal communication). In a companion experiment, cells were infected for 2 h with ICP4*lac*ZB prior to the addition of DUP55306. Assays at 6 h p.i. showed no inhibition of β-galactosidase activity, in accordance with the proposed mechanism of action (inhibition of entry).

4. Discussion

HSV-1 recombinants were constructed with the regulatory regions of the ICP4, TK and gD genes controlling the expression of β-galactosidase. This was achieved by

recombining the *lac*Z gene, under the control of these HSV regulatory regions, into the HSV TK locus and selecting for resistance to the nucleoside analog, AraT. The level of β-galactosidase activity expressed by the recombinant viruses was extremely high and followed the kinetics expected from the identity of each regulatory region. The recombinant viruses were highly resistant to AraT and acyclovir, as expected for TK^- viruses and Northern analysis of Vero cells infected with gD*lac*Z or ICP4*lac*Z showed high levels of the *lac*Z transcript.

We used transient transfection of plasmids containing the HSV promoter sequences driving *lac*Z as a guide to the kind of β-galactosidase expression that can be expected from ICP4 recombinant viruses derived from these plasmids. We found that expression levels from the recombinants followed the rank order of expression observed transiently in BHK21 cells for this series. When the fold-induction was greater than 3 (the case for pHSV*lac*ZA), we were successful in creating a recombinant virus; for levels below this value (pICP4*lac*Z), recombinant viruses could not be isolated.

Transfection/superinfection studies done on the ICP4, TK and gD constructions gave results in accordance with the temporal character of expression expected for these promoters and served as a guide for the construction of reporter viruses.

It should be noted that this approach can never fully reproduce exactly the viral kinetic program; the best that can be hoped for is an approximation of the viral kinetics which is faithful enough to render verdicts concerning mechanisms of action of antiherpes agents of unknown mechanism.

The kinetics of β-galactosidase expression indicated by the superinfection experiments is therefore only a guide to what can be expected from recombinant viruses containing these sequences, though it is likely that the actual kinetic program driven by these promoters will also be a function of the reporter gene, and the overall context in which it is placed relative to the promoter (Leary et al., 1989).

Furthermore, it should be noted that the normal mechanisms of degradation of viral gene products are not operable in this system – that is, β-galactosidase is produced by the cells and has a certain rate of degradation that does not, and should not, a priori, have any correlation with the rate of degradation of the gene products for which it is a surrogate. That being said, it is gratifying that the level of β-galactosidase from the TK virus diminishes with time after 6 h, to very low levels at 20 and 28 h p.i. We can only conclude that the rate of β-galactosidase degradation is relatively rapid, at least for the TK virus, and the correspondence of β-galactosidase production to TK expression is closer than it could otherwise be (should degradation be slow relative to expression).

Difficulty in isolating an ICP4 recombinant virus from the original ICP4 construction derived from plasmid pGal8 prompted an examination of the TI region in the parent vector used for the plasmid constructions. These studies showed that the TI sequence within the parent vector (lambda CII-derived) was the cause of the difficulty, presumably because of the poor fit to a Kozak translational initiation consensus, particularly a C residue at the −3 and a T residue at the −1 positions. In contrast, there was no difficulty in isolating a recombinant from the ICP0 regulatory region. The gD recombinant represented an intermediate case; a recombinant virus could be isolated from the original construction, but expression was increased 20-fold by replacing the TI region with the native gD sequence. A TK recombinant obtained without modification of the

construction also expressed relatively low levels of β-galactosidase. Apparently β-galactosidase expression in these recombinants is promoter-specific, indicating the importance of the TI region for proper expression of heterologous genes within the HSV context.

It should be mentioned that attempts to isolate a stable recombinant from the cotransfection of pICP4*lac*ZB into the HSV mutant, *in*-1814 (Ace et al., 1989) were unsuccessful. Small blue plaques were obtained on the first round of infection, but were unstable and could not be isolated on reinfection. Such a recombinant, which contains a non-functional copy of VP16, would be of interest in the study of immediate early gene expression by providing a 'zero' point for the basal activity of the ICP4 promoter within the viral context.

Studies of the response at relatively high m.o.i. for the ICP4*lac*ZB virus indicated a remarkable dynamic range for detection of β-galactosidase activity. Small differences in activity as a function of viral input were easily quantifiable, with the greatest differentiation at 8 h p.i. Thus, this recombinant virus can be used as a sensitive and rapid reporter of small differences in viral input and growth within only a few hours postinfection.

Differences among the reporter viruses were exploited to create a panel of these recombinants in which the activity from each virus was time-dependent. Activity from the ICP4 virus at low m.o.i. (5×10^{-4}) was barely detectable until 47 h p.i., reflecting expression from reinfection after subsequent rounds of replication. In contrast, at m.o.i. 5, this virus produced detectable β-galactosidase activity within 2.7 h p.i., the level rising through 28 h. There appeared to be an increase in activity, late in the cycle around 20 h p.i., which might have come from reinfection. Maximal activity was observed at m.o.i. 0.05 assayed at 47 h p.i. By comparison, activity from the TK*lac*Z virus increased rapidly to 50% maximal at 6 h p.i., peaking at 11 h p.i. and falling to low levels by 28 h p.i. This was a distinct difference between the ICP4B and TK*lac*Z viruses: the former continued to express activity after mid-cycle, while the latter was tightly regulated and was completely shut down in the later stages of the viral program, mirroring the situation for thymidine kinase. In fact, of the viruses studied, the activity from this virus was the most tightly regulated, noteworthy given that the TK*lac*Z virus encodes a thymidine kinase-β-galactosidase fusion protein (Hall et al., 1983) with an N-terminus derived from 128 amino acids of the N-terminus of the TK protein and 29 amino acids from CII non-coding and leader sequence. The level of β-galactosidase activity from the gD*lac*ZA virus at early times (3–6 h) was very low compared to levels at later times, but this low early level was meaningful, since it reflected the non-DNA synthesis component of gD-driven transcriptional activity. Activity from this virus rose dramatically in mid-cycle, between 5.5 and 20 h p.i., consistent with the major portion of transcription dependent on DNA synthesis (Johnson and Spear, 1984). The gC*lac*Z virus was like the gD*lac*ZA virus, except for the complete absence of β-galactosidase activity until 6 h p.i., after the start of DNA synthesis. This characteristic distinguished the gD*lac*ZA and gC*lac*Z viruses. The former showed distinct activity at relatively early times postinfection, while the latter did not. Together, the data indicate that the ICP4, TK and gD viruses are valid surrogates for reporting the transcriptional activity of their namesakes, i.e., β-galactosidase expression displayed the kinetics particular to each viral class.

The absolute expression of β-galactosidase from the viruses varied considerably: the gD*lac*ZA virus produced 1.5–3 times the activity of the gC*lac*Z virus (depending on when in the cycle it was measured), while the gC*lac*Z virus produced roughly twice the activity of the ICP4B virus (at later times of infection). The TK*lac*Z virus produced approximately 10-fold less activity than the gD*lac*ZA virus, 5-fold less activity than the gC*lac*Z virus and 6-fold less activity than the ICP4B virus. Normalizing expression from each virus to its peak level within a 22 hour period for infection at m.o.i. 5 gave a rank order of β-galactosidase expression of: gD(3) > gC(2) > ICP4(1) > TK(0.5).

We have used the ICP4*lac*ZB virus to investigate the mechanism of action of several antiherpes substances. Order of addition experiments with E3925 indicate that this compound inhibited viral growth within the first cycle, so long as it was present before or during infection. This is consistent with the conclusion that this compound inhibits viral entry, uncoating, or both. We have ruled out effects on IE expression (as might be expected, for example, of a compound capable of inhibiting VP16-mediated transactivation of α gene expression) because cells infected with a high m.o.i. challenge for 2 h, but post-treated with this compound at that time, showed no inhibition at all of the IE report at 5 h. Given that there is little IE-driven β-galactosidase expression by 2 h (see Fig. 8), inhibition of IE function should have been evident when assayed at 5 h p.i. Inhibition by E3925 extended to subsequent cycles of infection by those virus particles released after the first round of viral synthesis. Inhibitory concentrations for E3925 determined using the ICP4*lac*ZB or gD*lac*Z reporter viruses at m.o.i. 0.005 were in good agreement with those obtained by standard plaque assays for this compound, but required only 1 day of growth for this measurement. The agreement with plaque assays was best for determination of IC_{50} values, with IC_{90} values being overestimated by about 4-fold. Thus, use of this marker virus provided valuable information on the mechanism of action of this antiviral agent while quantitatively reproducing the results for IC_{50} determinations from a standard plaque assay in only 24 h.

The ICP4*lac*ZB virus was also used to verify the mechanism of action of IFN-γ, known to inhibit IE synthesis of HSV. By varying m.o.i. and order of addition, it could be confirmed that the antiherpes effects of IFN-γ are a secondary effect on IE expression requiring cellular changes, rather than the result of any direct action on the virus, such as inhibition of entry or uncoating or both. Interestingly, the degree of inhibition was inversely proportional to the viral load, i.e., higher levels of virus were able to overcome the inhibitory effects to some extent.

Lastly, we investigated the mechanism of action of the neutralizing gD monoclonal antibody, DUP55306. Inhibition of immediate early expression was insensitive to viral load, but completely dependent upon its presence before infection, similar to the action of E3925. The data support the proposed mechanism involving inhibition of viral entry.

Acknowledgements

We gratefully acknowledge the kind gifts of the gC*lac*Z virus from Dr. Jerry Weir, Walter Reed Army Institute of Research, BSC40 and BSV65 cell lines from Dr. John P. Capone, McMaster University, plasmids pSG28B/K and pSG1, from Dr. R. Sandri-

Goldin (via Dr. Annie Colberg-Poley), MAb DUP55306 from Dr. Conrad Heilman, DuPont Merck Pharmaceutical Co. and plasmid pMSVP16, from Dr. Kent Vrana, West Virginia University Health Science Center. We thank Dr. John Blasecki for the gift of the experimental antiviral, E3925. Lastly, we thank Drs. Pam Benfield, Annie Colberg-Poley and John Blasecki for critical reading of the manuscript.

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