A Dominant Mutant Form of the Herpes Simplex Virus ICP8 Protein Decreases Viral Late Gene Transcription

YU-MEI CHEN and DAVID M. KNIPE

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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The herpes simplex virus (HSV) infected cell protein 8 (ICP8) is required for viral DNA replication and normal viral gene expression. Previous work in our laboratory has shown that ICP8 may play a role in stimulating late gene expression. In V2.6 cells which express the d105 mutant form of ICP8, synthesis of late proteins and accumulation of late gC mRNA are reduced during HSV infection (Gao, M., and Knipe, D. M., J. Virol. 65, 2666 – 2675, 1991). To determine if the negative effect of d105 ICP8 on late gene expression was exerted at the transcriptional level, we measured the levels of mRNAs and transcription from three late genes, gC, UL47, and gD, in V2.6 cells and Vero cells infected with the HSV-1 wild-type virus. In infected V2.6 cells, the levels of late gC and UL47 mRNA were 7- to 12-fold lower than those of infected Vero cells under conditions where the levels of viral DNA replication in these two cell types were similar. The transcription levels of these two late genes in infected V2.6 cells were reduced to similar extents (9- to 14-fold). The levels of accumulated mRNA and transcription of the early–late gD gene also showed parallel reductions in infected V2.6 cells (about 6-fold). In contrast, transcription of the β pol gene was reduced only slightly (about 2-fold) by d105 ICP8. These results demonstrate that the d105 ICP8 inhibits expression of three viral late genes at the transcriptional level, and in general, the effect of d105 ICP8 on viral gene expression appears to correlate with the extent to which expression of the gene is stimulated by viral DNA synthesis.

INTRODUCTION

Herpes simplex virus 1 (HSV-1) is a double-stranded DNA virus whose genome encodes more than 70 genes. These genes are divided into three classes: immediate-early or α, early or β, and late or γ genes, based on the kinetics and requirements for their expression (Hones and Roizman, 1974). After viral entry, the viral DNA enters the nucleus of host cells leading to the expression of the first set of viral genes. The transcription of α genes is activated by the virion protein VP16 and cellular transcription factors. The products of the α genes transactivate the expression of the next set of viral genes, the β genes, whose products are required for viral DNA replication. After the onset of viral DNA replication, the expression of late genes greatly increases. Late genes, which encode primarily viral structural proteins, are further divided into two subclasses, early–late (γ1) and late (γ2) genes, based on the stringency of their dependence on viral DNA replication for expression. The expression of γ1 genes is readily detectable prior to viral DNA replication; however, maximal expression is attained only after viral DNA replication. The expression of γ2 genes absolutely requires viral DNA replication (Roizman and Sears, 1996). In addition to a dependence on viral DNA replication, late gene expression is also regulated by viral proteins; the products of two α genes, ICP4 (Preston, 1979; DeLuca and Schaffer, 1985; Godowski and Knipe, 1986) and ICP27 (Sacks et al., 1985; Rice and Knipe, 1990; Jean et al., manuscript in preparation), are required for late gene expression. Previous studies in our laboratory have shown that infected cell protein 8 (ICP8), the product of a β gene, may also play a role in stimulating late gene expression (Gao and Knipe, 1991).

ICP8, the major viral DNA-binding protein, consists of 1196 amino acid residues (Quinn and McGeoch, 1985; Gao et al., 1988) and is located in the nucleus of the infected cells (Fenwick et al., 1978; Knipe and Spang, 1982; Quinlan and Knipe, 1983). ICP8 has been identified as an essential gene product for viral replication (Conley et al., 1981) and is one of the seven viral β proteins necessary and sufficient for viral DNA synthesis in transfected cells (Challberg, 1986; Wu et al., 1988). Genetic studies have shown that ICP8 is a multifunctional protein, playing a variety of other roles during the HSV lytic cycle. It is required for the organization of viral replication proteins and cellular proteins in prereplicative sites in the nucleus where viral DNA replication is believed to initiate (de Bruyn Kops and Knipe, 1988; Bush et al., 1991), and it is also involved in the regulation of viral gene expression. ICP8 exerts a negative effect on transcription from the parental viral genome (Godowski and Knipe, 1983, 1985, 1986), while it appears to exert a positive effect on late gene expression from the progeny genome (Gao and Knipe, 1991).
1991). The relationships between these multiple functions remain to be determined.

The effect of ICP8 on late gene expression from the progeny genome was defined in studies with a mutant form of ICP8, d105, which lacks residues 1083–1166. This mutant form of ICP8 protein can still properly localize to the nucleus and can bind to ssDNA and dsDNA in vitro; however, it possesses a dominant mutant phenotype in interfering with viral DNA replication and inhibiting late gene expression of wild-type virus, presumably due to competition between the wild-type and mutant forms of ICP8. Comparison of viral gene expression in infected V2.6 cells which express d105 ICP8 with infected control Vero cells showed approximately normal levels of viral α and β gene products but decreased levels of γ proteins and gC mRNA (Gao and Knipe, 1991).

To determine the level at which d105 ICP8 affects late gene expression, we analyzed the accumulation of mRNA and transcription from two γ2 genes, gC and UL47, and a γ1 gene, gD, in V2.6 cells and in Vero cells infected with wild-type virus under conditions in which viral DNA replication was at the same level in the two cell types. We demonstrate that the effect of d105 ICP8 on late gene expression is at the transcriptional level.

MATERIALS AND METHODS

Cells and viruses

Vero cells were grown and maintained as described previously (Knipe et al., 1982). S-2 and V2.6 cell lines containing the wild-type ICP8 gene and the d105 ICP8 gene, respectively, were derived from Vero cells (Gao and Knipe, 1989). For maintenance of these two cell lines, the antibiotic G418 (GIBCO Laboratories) was added to a final concentration of 500 μg/ml in the growth medium for the first three passages after thawing and every fifth passage thereafter. The HSV-1 wild-type strain KOS1.1 was propagated and titered as described previously (Knipe et al., 1982; Lee and Knipe, 1983), and the ICP8 null mutant strain HD-2 was propagated on S-2 cells (Gao and Knipe, 1989).

Infections

Infections were carried out at a multiplicity of infection (m.o.i.) of 2 plaque-forming units per cell. To ensure that an equivalent m.o.i. was established in all cell lines, the number of cells in one flask of each cell line was determined prior to infection. Sodium phosphonoacetate (PAA) was added to the medium to inhibit viral DNA synthesis as indicated. A stock solution of PAA was made by neutralizing phosphonoacetic acid (Sigma) with sodium hydroxide to pH 7.0. When PAA was used, 20 mM HEPES buffer (pH 7.6) was included in all media either with or without PAA to maintain all cultures at the same pH, and cells were also incubated with PAA during the viral absorption period and labeling period.

Analysis of viral DNA replication

Two methods were used to analyze viral DNA replication. One was a [3H]thymidine labeling method (Gao and Knipe, 1989), and the other was a hybridization method (Rice and Knipe, 1990). For the labeling method, cultures of ~2 × 10⁵ mock-infected or HSV-infected cells in 25-cm² flasks were labeled from 7 to 10 hr postinfection (hpi) with 2 ml of medium containing 50 μCi [3H]thymidine (New England Nuclear, 88 Ci/mmol), and total infected cell DNA was isolated and purified. Four micrograms of each DNA sample was cleaved with EcoRI and XbaI and then subjected to electrophoresis in a 0.9% agarose gel.

After electrophoresis, the gel was permeated with a fluorography-enhancing agent (Entensify, DuPont Corp.), dried, and exposed to X-ray film at −70°C with an intensifying screen. The intensities of labeled DNA bands on the autoradiogram were quantified by densitometry scanning (LKB Densitometer). For the hybridization method, DNA was isolated from the infected cells at 10 hpi. Five-fold serial dilutions of each DNA sample (ranging from 8 to 1000 μg DNA) were immobilized onto a nylon membrane using a slot-blot apparatus (Schleicher and Schuell). The filter was then hybridized with 32P-labeled pSG28 plasmid DNA, which contains the ~21-kb EcoRI EK fragment of HSV-1 DNA (Goldin et al., 1981). The intensity of the hybridized viral DNA was quantified with a PhosphorImager (Molecular Dynamics).

Northern blot analysis

Cytoplasmic RNA was prepared as described previously (Sambrook et al., 1989). The RNA preparation was treated with 30 U/ml RNase-free DNase (Boehringer Mannheim Biochemicals) at 37°C for 20 min. Ten micrograms of each RNA sample was separated in a 1.5% agarose–6% formaldehyde gel and then transferred to a nylon membrane. The membrane was hybridized with DNA probes labeled with [α-32P]dCTP by the random priming method. The DNA probe for each gene was identical to the internal DNA fragment of the gene which was used as probe in the transcription assays (see below). The relative amount of the radioactivity of each hybridized mRNA was quantified by phosphoimage analysis.

Measurement of viral gene transcription

The levels of transcription of viral genes were measured by a modification (Jean et al., manuscript in preparation) of a previously published method for an in vivo [3H]uridine labeling method (Zhang et al., 1987). Cultures of ~1.5 × 10⁷ cells in 150-cm² flasks were mock-infected or infected with KOS1.1 virus. At the indicated time after infection, cells were pulse labeled with 1 mCi of [3H]uridine (New England Nuclear, 35–50 Ci/mmol) in 5 ml
of medium at 37°C for 30 min except as otherwise indicated. Total RNA was prepared from infected cells by the guanidinium–CsCl step gradient method (Frederick et al., 1991). The RNA preparations were then treated with 30 U/ml RNase-free DNase at 37°C for 20 min. From one 150-cm² flask, we generally isolated 150–250 µg of total RNA containing 1–5 × 10⁷ cpm of incorporated label. An equal portion of each labeled RNA sample (usually 100 µg) was incubated with filters containing 10 µg of each single-stranded complementary or anticomplementary DNA probe which had been immobilized on a nylon membrane using a slot-blot apparatus. For each sample the M13 DNA vector was included as the control. The background level for each sample was a blank filter incubated with the sample. Hybridization was carried out at 68°C for 40 hr, after which the filter was washed, and each slot was cut out. The radioactivity of each slot was quantified by liquid scintillation counting. The level of transcription was expressed as cpm of [³H]uridine incorporated mRNA hybridized to the complementary probe subtracting cpm of the background. The probes for all genes contained a viral DNA fragment internal to the HSV gene cloned into a bacteriophage M13 vector in either of two orientations. Single-stranded DNA probes complementary or anticomplementary to the mRNA of each gene were prepared from virions of the appropriate M13 recombinant bacteriophage. The HSV sequences within each of the probes were as follows: gC (UL44) gene—a 918-bp EcoRI–XbaI fragment (nt 96,751–97,669) (Godowski and Knipe, 1986; McGeoch et al., 1988); UL47 gene—a 870-bp PstI–PstI fragment (nt 101,485–102,355) (McGeoch et al., 1988; LeVan and Knipe, unpublished data); gd (US6) gene—a 1007-bp HindIII–NarI fragment (nt 138,344–139,351) (McGeoch et al., 1985; LeVan and Knipe, unpublished data); pol (UL30) gene—a 649-bp BglII–EcoRI fragment (nt 63,472–64,121) (Weinheimer and McKnight, 1987; McGeoch et al., 1988); tk (UL23) gene—a 1079-bp BglII–HindIII fragment (nt 46,776–47,855) (McKnight, 1980; Weinheimer and McKnight, 1987; McGeoch et al., 1988).

Analysis of viral proteins

Cultures of ~2 × 10⁶ cells in 25-cm² flasks were mock-infected or infected and labeled with [³⁵S]methionine (ICN, 1280 Ci/mmol) for the times indicated. The labeled infected cell lysates were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described previously (Knipe et al., 1982). After electrophoresis, the gel was fixed, dried, and exposed to Kodak SB5 film. The labeled wild-type or d105 ICP8 bands were quantified by phosphoimage analysis.

RESULTS

Time course of gC gene transcription

The late gC gene is transcribed at an undetectable level during the early stages of infection, and its transcription reaches a maximum at about 6 hpi when cells are infected at an m.o.i. of 10–20 (Godowski and Knipe, 1986; Weinheimer and McKnight, 1987; Zhang et al., 1987). When cells were infected at such an m.o.i., the trans-dominant effect of d105 ICP8 was partially overcome by the presence of the greater amount of the wild-type ICP8 (Gao and Knipe, 1991), so the experiments designed in this study to test the mechanism of the effect of the d105 ICP8 protein were carried out at a lower m.o.i. (m.o.i. 2), where a dominant mutant effect had been observed (Gao and Knipe, 1991). To determine the kinetics of gC transcription in cells infected at an m.o.i. of 2, we examined gC transcription at 2-hr intervals from 2 to 12 hpi. Because expression of early genes is independent of viral DNA replication and therefore insensitive to viral DNA synthesis inhibitors (Holland et al., 1984; Rafield and Knipe, 1984; Weinheimer and McKnight, 1987), we wanted to use early genes as controls to ensure that any observed effects of d105 ICP8 on late gene expression were specific. Therefore, two early genes of HSV-1, DNA polymerase (pol) and thymidine kinase (tk), were also included in this experiment. Transcription was measured by in vivo [³H]uridine labeling. When Vero cells were infected at an m.o.i. of 2, transcription of the gC gene was undetectable at early times of infection (2–4 hpi). Transcription started by 6 hpi and then increased rapidly. The level of gC transcription reached its maximum at 12 hpi and declined thereafter (Fig. 1 and results not shown). Transcription of the pol gene was low but detectable at 4 hpi, then increased rapidly, and reached
its plateau at 8-10 hpi. Transcription of another early gene, tk, was turned on earlier compared to that of the pol gene. There was a significant level of tk transcription at 4 hpi, and it reached its maximal level at 6 hpi and declined rapidly afterwards. At 8 hpi, tk gene transcription was nearly undetectable. The hybridization assay appeared to detect authentic viral transcripts, because in all cases the hybridization was specific for the complementary DNA probe. Furthermore, the cpm values from labeled mock-infected cells were similar to the control levels, which were obtained from hybridization with the M13 probe (data not shown). Based on this kinetic study, we chose 10 hpi as the time at which to measure the transcription of late genes and the pol gene as a control early gene.

Analysis of viral DNA replication in Vero and V2.6 cells

Expression of viral late genes increases after the onset of viral DNA replication and continues as replication proceeds; therefore, if viral DNA replication was reduced, late gene expression would also be reduced. In V2.6 cells, one effect of d105 ICP8 observed previously was a partial reduction in viral DNA replication (Gao and Knipe, 1991), which could account for some of the reduction in late gene expression. To analyze transcription of late genes in V2.6 and Vero cells under conditions of the same levels of viral DNA replication, we used PAA, a specific inhibitor of the HSV-1 viral DNA polymerase (Mao and Robishaw, 1975), to reduce the level of viral DNA replication in infected Vero cells. To determine the proper concentration of PAA needed to inhibit viral DNA replication in Vero cells to the same level as in V2.6 cells, we measured viral DNA synthesis in infected V2.6 and Vero cells in the absence or presence of various amounts of PAA by two different methods. One method was a [3H]thymidine labeling method (Gao and Knipe, 1989), and the other was a hybridization method (Rice and Knipe, 1990).

Figure 2A shows the results of an experiment using the labeling method. In mock-infected Vero cells (lane 1), a smear of bands of labeled cellular DNA was seen. In infected Vero cells (lanes 2-4) or V2.6 cells (lane 5), there was diminished label in cellular DNA, but instead there were heavily labeled bands of 15-21 kb and several smaller bands which corresponded to the predicted restriction pattern of HSV-1 DNA digested with EcoRI and XbaI. Results from three separate experiments showed that in infected V2.6 cells the amount of viral DNA synthesized ranged from 15 to 22% of that in infected Vero cells in the absence of PAA. In infected Vero cells the addition of 25 and 50 μg/ml PAA reduced the amount of the viral DNA synthesized to 24 and 8%, respectively, of that synthesized without the addition of PAA (lanes 3 and 4). Therefore, viral DNA replication in infected V2.6 cells was at a level comparable to that in Vero cells infected in the presence of 25-50 μg/ml PAA (Fig. 2B). The hybridization method was also used, and comparable results were found (Fig. 2B), confirming this observation.

Comparison of the accumulation of late mRNAs in infected Vero and V2.6 cells

Before analyzing late gene transcription, we wanted to confirm the effect of d105 ICP8 on the accumulation of late mRNA at 10 hpi by examining other late genes in addition to the gC gene, which had been examined in the previous study (Gao and Knipe, 1991). We chose another γ2 gene, UL47, and a γ1 gene, gD. Two early genes, pol and tk, were used as controls. Infections were carried out in both V2.6 and Vero cells at an m.o.i. of 2. In infected Vero cells, PAA was added at 0, 25, or 50 μg/ml. Cytoplasmic RNA was isolated at 10 hpi and subjected to Northern blot analysis. Figure 3A shows the autoradiogram of one of the resulting Northern blots. The relative amount of the radioactivity of each hybridized mRNA was quantified by phosphoimage analysis and expressed as a percentage of the mRNA level in infected Vero cells in the absence of PAA (Fig. 3B). There was a high level of accumulated gC, UL47, and gD mRNA in control infected Vero cells, and the level of gC and UL47 mRNA was decreased by 25–58% by the addition of 25–50 μg/ml PAA. In V2.6 cells, however, the level of gC and UL47 mRNA was further decreased by 7- to 12-fold and 7- to 10-fold, respectively, relative to the level in Vero cells in the presence of 25–50 μg/ml PAA. The level of gD mRNA was decreased by 30–52% with the addition of 25–50 μg/ml of PAA, and in V2.6 cells, the level of gD mRNA was further decreased by 6- to 9-fold relative to the level in Vero cells when the levels of viral DNA replication were equal. The levels of accumulated tk mRNA in all samples were similar in that neither PAA nor d105 ICP8 altered the accumulation of tk mRNA. However, the presence of PAA did lead to a moderate decrease in the accumulated level of pol mRNA; 25 and 50 μg/ml PAA reduced pol mRNA by 17 and 29%, respectively. In infected V2.6 cells, the accumulation of pol mRNA was further reduced by 1.7- to 2.1-fold relative to the level in Vero cells in the presence of 25–50 μg/ml PAA. Although the pol gene has been classified as a β gene by its requirement for α proteins and its relative independence of viral DNA replication for its expression (Weinheimer and McKnight, 1987), our observation of the reduction of pol mRNA accumulation by inhibiting viral DNA replication was consistent with a previous report (Weinheimer and McKnight, 1987) and a recent publication (Wobbe et al., 1993), indicating that the pol gene has both early and late characteristics. These results showed that the mutant form of the d105 ICP8, which had little effect on the level of accumulated tk mRNA, did significantly reduce the level of γ2 transcripts, gC and UL47, by 7- to 12-fold and a γ1 transcript, gD, by 6- to 9-fold. In contrast,
V2.6 cells showed only a slight reduction (1.7- to 2.1-fold) in the accumulated level of pol transcripts.

Levels of transcription of late genes in infected Vero and V2.6 cells

To determine if the reduction in the accumulation of \( \gamma_2 \) and \( \gamma_1 \) mRNA in V2.6 cells was quantitatively reflected in changes in the transcription of these genes, we measured gC, UL47, and gD gene transcription using in vivo pulse-labeling of the RNAs. Although pol mRNA was decreased slightly by PAA and by d105 ICP8, we still used it as the early gene control, because transcription of the tk gene was barely measurable at 10 hpi. Figure 4 shows the results from a representative experiment of three separate experiments, where transcription was measured at 10 hpi with infections at an m.o.i. of 2. The levels of transcription from the gC gene in Vero cells were reduced when viral DNA replication was partially blocked by PAA. The addition of 25 and 50 \( \mu \)g/ml PAA to the infected Vero cells resulted in a \( \sim 30 \) and \( \sim 45\% \) decrease, respectively, in the level of gC transcription. In infected V2.6 cells, gC transcription was further reduced by 9- to 11-fold compared with that in infected Vero cells in the presence of 25 to 50 \( \mu \)g/ml PAA. The pattern of UL47 transcription showed a similar reduction in infected V2.6 cells (10- to 14-fold). Transcription of the \( \gamma_1 \) gD gene was reduced to a lesser extent (5- to 6-fold) in infected V2.6 cells compared with the reduction in the \( \gamma_2 \) genes, gC or UL47. Transcription of the pol gene was reduced in infected Vero cells by 25 and 29% with the addition of 25 and 50 \( \mu \)g/ml PAA, respectively, and in infected V2.6 cells, pol gene transcription was further reduced by about 2.5-fold. Therefore, in infected V2.6 cells the fold reduction in the transcription level of each gene paralleled the fold reduction in the mRNA level, indicating that d105 ICP8 has an inhibitory effect on late gene transcription.

d105 ICP8 did not affect the stability of gC mRNA

It was possible that the decrease in late gene mRNA synthesis in V2.6 cells was due to an alteration in mRNA stability and that the reduced rate of late transcription observed in V2.6 cells was actually caused by the rapid degradation of late transcripts during the labeling period. If that were the case, the ratio of the amount of [\( ^3 \)H]uridine incorporated into late transcripts from Vero cells versus V2.6 cells would become higher for longer labeling periods. To test this hypothesis, we pulse-labeled infected Vero cells and V2.6 cells for various periods of times and measured the level of gC transcription. Similar ratios for the levels of gC transcription in Vero versus V2.6 cells were observed for labeling times of 15, 30, 60, or 90 min (Table 1), demonstrating that d105 ICP8 did not affect the stability of the newly synthesized gC mRNA and providing further evidence that d105 ICP8 exerted its effect on late gene expression at the level of transcription.

Levels of wild-type and d105 ICP8 in infected Vero and V2.6 cells

It had been reported previously that overexpression of ICP8 may cause decreased viral DNA replication and
viral gene expression (Orberg and Schaffer, 1987). To address this possibility in our previous study (Gao and Knipe, 1991), we had measured the levels of protein synthesis of wt ICP8 and d105 ICP8 in infected Vero, S-2 (cell line expressing wt ICP8), and V2.6 cells by pulse-labeling infected cells with [35S]methionine for 30 min at 3, 6, and 12 hpi. Those results showed that the levels of wt ICP8 synthesis in infected Vero or V2.6 cells were similar and that the level of d105 ICP8 synthesis in infected V2.6 cells was slightly greater than that of the wt ICP8 in infected S-2 cells. We had also shown that the presence of the greater amount of wt ICP8 in infected S-2 cells did not have any influence on virus lytic infection as judged by normal virus plaque size and plating efficiency. To further confirm that the decreased late gene transcription in V2.6 cells was not simply due to overexpression of the d105 ICP8 protein, we measured the accumulated level of ICP8 at times when we were measuring transcription and compared the levels of late gene transcription in infected Vero and S-2 cells. Infected Vero, V2.6, and S-2 cells were labeled with [35S]methionine from 4 to 10.5 hpi. The labeled proteins were separated by SDS-PAGE and the levels of ICP8 proteins in each sample were quantified by phosphoimage analysis (Ta-

![Fig. 3](image1)

**Fig. 3.** Levels of gC, UL47, gD, pol, and tk mRNA in mock-infected or infected Vero or V2.6 cells in the absence or presence of 25 or 50 μg/ml PAA. Cells were infected at an m.o.i. of 2. Cytoplasmic RNA was isolated at 10 hpi and subjected to Northern blot analysis. (A) An autoradiogram of the Northern blots. Lanes: 1, Vero cells infected in the absence of PAA; 2, Vero cells infected in the presence of 25 μg/ml PAA; 3, Vero cells infected in the presence of 50 μg/ml PAA; 4, V2.6 cells infected in the absence of PAA; 5, mock-infected Vero cells. The major transcripts from the gC, UL47, gD, pol, and tk genes are indicated to the right of each blot. (B) Quantitative comparison of levels of gC, UL47, gD, pol, and tk mRNAs in Vero or V2.6 cells infected in the absence or presence of 25 or 50 μg/ml PAA. The amount of radioactivity of each mRNA was quantified by phosphoimage analysis of the Northern blot and expressed as a percentage of the value for Vero cells infected in the absence of PAA.

![Fig. 4](image2)

**Fig. 4.** Levels of transcription of gC, UL47, gD, and pol in Vero or V2.6 cells infected in the absence or presence of 25 or 50 μg/ml PAA. Infections were carried at an m.o.i. of 2. Transcription was measured by in vivo pulse labeling with [3H]uridine at 10 hpi and hybridization to ssDNA probes. The values are expressed as cpm of [3H]uridine incorporated mRNA hybridized to the specific ssDNA probe for each gene. ssDNA probes: -c, complementary to a specific mRNA; -a, anti-complementary to a specific mRNA; M13, single-stranded DNA of M13 vector.

<table>
<thead>
<tr>
<th>Labeling period (min)</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
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<tbody>
<tr>
<td>15</td>
<td>9.5</td>
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<td>14.9</td>
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<td>12.4</td>
<td>11.4</td>
</tr>
<tr>
<td>90</td>
<td>N.D.</td>
<td>N.D.</td>
<td>13.0</td>
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</tbody>
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*Not determined.

**Table 1**

Labeling of gC Gene Transcripts in Vero versus V2.6 Cells

<table>
<thead>
<tr>
<th>Labeling period (min)</th>
<th>Expt 1</th>
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<tr>
<td>90</td>
<td>N.D.</td>
<td>N.D.</td>
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TABLE 2

Comparison of ICP8 and d105 ICP8 Protein Levels in Infected Vero, S-2, or V2.6 Cells*

<table>
<thead>
<tr>
<th>Cell line infected</th>
<th>wt ICP8</th>
<th>d105 ICP8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>1</td>
<td>N.A.</td>
</tr>
<tr>
<td>S-2</td>
<td>2.8</td>
<td>N.A.</td>
</tr>
<tr>
<td>V2.6</td>
<td>1</td>
<td>6.8</td>
</tr>
</tbody>
</table>

* Vero, S-2, or V2.6 cells were infected with KOS1.1 at an m.o.i. of 2 and labeled with [35S]methionine from 4 to 10.5 hpi. Labeled proteins were separated by SDS – PAGE and quantified by phosphorimager analysis. The level of ICP8 protein in infected Vero cells was defined as 1. 

The accumulated levels of wt ICP8 protein were the same in infected Vero cells and V2.6 cells, and the amount of wt ICP8 protein expressed from infected S-2 cells was 2.8-fold greater than that in infected Vero cells, whereas the amount of d105 ICP8 protein expressed from infected V2.6 cells was 2.4-fold greater than that of the wt ICP8 protein in infected S-2 cells. We then compared the levels of gC and UL47 transcription in infected S-2 and Vero cells. As shown in Fig. 5, the levels of gC and UL47 transcription in infected S-2 and Vero cells were similar, and in both cell lines the addition of 25-50 μg/ml PAA reduced gC and UL47 transcription to the same extent, confirming that the greater amount of ICP8 protein had no effect on late gene expression. Because the amount of d105 ICP8 protein was only 2.4-fold greater than the amount of wt ICP8 in infected S-2 cells, we reasoned that the 10-fold decreased level of late gene transcription in infected V2.6 cells was due to a novel effect of the d105 mutant form of ICP8 and not simply to its overexpression. Nevertheless, the presence of the greater amount of d105 ICP8 was apparently necessary for the dominant effect on late gene expression, because at a higher m.o.i. the inhibitory effect of d105 ICP8 was not observed (Gao and Knipe, 1991; data not shown).

Effect of d105 ICP8 in the absence of wt ICP8

Two alternative explanations for the phenotypic effects of d105 ICP8 were a dominant negative mutant effect or a “gain of function” effect conferred by the mutation. A dominant mutant phenotype of d105 ICP8 would likely be exerted through interactions with or effects on wt ICP8 while a new function present in d105 ICP8 might be exerted in the absence of wt ICP8. In an attempt to distinguish between these two alternatives, we examined the effect of d105 ICP8 on viral gene expression in cells infected with the ICP8 null mutant HD-2 (Gao and Knipe, 1989). In Vero cells and V2.6 cells infected with HD-2 virus, equal levels of the γ1 ICP5 protein were observed while in cells infected with wt virus, sevenfold more ICP5 protein was expressed in Vero cells compared to V2.6 cells expressing d105 ICP8 (Table 3). Thus, the inhibitory effect of d105 ICP8 was not exerted in HD-2-infected cells, i.e., in the absence of wt ICP8 and viral DNA replication, demonstrating that one or both of these were required for the inhibitory effect of d105 ICP8. This result is more consistent with the inhibitory effect of d105 ICP8 being a dominant mutant effect than a new function introduced by the mutation.

DISCUSSION

Previous studies have shown that the d105 mutant form of ICP8 inhibits HSV infection and that in d105 ICP8-expressing V2.6 cells, synthesis of late proteins is greatly

TABLE 3

Effects of d105 ICP8 on ICP5 Expression with or without wt ICP8

<table>
<thead>
<tr>
<th>Infection*</th>
<th>Units of ICP5 expressed (% of control)</th>
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<tbody>
<tr>
<td>HD-2 (ICP8^-) virus in Vero cells</td>
<td>209 (100)</td>
</tr>
<tr>
<td>HD-2 (ICP8^-) virus in V2.6 cells</td>
<td>219 (105)</td>
</tr>
<tr>
<td>wt virus in Vero cells + 30 μg/ml PAA^c</td>
<td>843 (100)</td>
</tr>
<tr>
<td>wt virus in V2.6 cells</td>
<td>116 (14)</td>
</tr>
</tbody>
</table>

* Cell was infected with the indicated virus at m.o.i. 2. Infected cells were labeled with [35S]methionine from 9.5 to 10 hpi and then harvested. Cells lysates were resolved by SDS – PAGE, and the label in the ICP5 band was quantified by phosphoimage analysis.

^c 30 μg/ml PAA added to make viral DNA synthesis equivalent to V2.6 cells infected with wt virus.
diminished, while synthesis of α and β proteins is not changed significantly (Gao and Knipe, 1991). The effect on γ gene expression could be exerted at one or more levels of gene regulation, such as transcription, RNA processing, RNA transport, or translation. To address the mechanism by which d105 ICP8 regulates HSV late gene expression, we analyzed the levels of accumulated mRNA and transcription of selected genes from the β, γ1, and γ2 classes of HSV-1 genes. We also showed that when compared with the control Vero cells at the same level of viral DNA replication, the reduction in late gene transcription in infected V2.6 cells correlated closely with the reduction in the accumulated level of late mRNA. Thus, the reduction in the steady-state late mRNA level in infected V2.6 cells can be accounted for by the reduction in the transcription of late genes. Moreover, we showed that the stability of gC transcripts was a dominant negative mutant phenotype based on our observation of competition between wt and d105 ICP8 and the lack of an effect of d105 ICP8 in the absence of wt ICP8. In the latter model, ICP8 might bind to replicating viral DNA or progeny genomes, activating late gene transcription by interacting with or promoting the binding of other viral or cellular proteins to the late promoter regions. The mechanism(s) underlying the inhibition of late gene expression by d105 ICP8 is not yet clear. The decreased late gene transcription observed in infected V2.6 cells could be due to a new function caused by the mutation or an effect of d105 ICP8 competing away a normal function of wt ICP8. We favor the latter model involving a dominant negative mutant phenotype based on our observation of competition between wt and d105 ICP8 and the lack of an effect of d105 ICP8 in the absence of wt ICP8. In the latter model, ICP8 might bind to replicating viral DNA or progeny genomes, activating late gene transcription by interacting with or promoting the binding of other viral or cellular proteins to the late promoter regions. The d105 ICP8 may be altered so that it only partially blocks viral DNA synthesis but is greatly decreased in its ability to interact with other viral or cellular proteins that are needed for late gene transcription. Nevertheless, d105 ICP8 retains its ability to bind to wt ICP8 binding sites and therefore prevents wt ICP8 from binding and performing its normal function(s). It is not known if ICP8 can function to stimulate late gene expression independently of viral DNA synthesis, but the isolation of ICP8 mutant viruses in which the two functions could be separated would help us to understand how ICP8 is involved in activating viral DNA replication and regulating late gene transcription.

In addition to the positive effect of ICP8 on late gene transcription from viral progeny genomes discussed here, we have also provided evidence that ICP8 exerts a negative role on immediate–early, early, and late gene transcription from parental viral genomes (Godowski and Knipe, 1985, 1986). In the latter studies, a negative effect of ICP8 on gene expression from parental viral genomes was demonstrated by analyzing cells infected with an ICP8 temperature-sensitive mutant virus, which expressed increased levels of late proteins and mRNAs at the nonpermissive temperature under conditions in which viral DNA replication was inhibited (Godowski and Knipe, 1985). This is also supported by our recent studies of an ICP8 null mutant virus, in which the level of late mRNA was increased while the level of β gene mRNA was not affected (Chen and Knipe, unpublished data). Thus, ICP8 appears to exert different effects on late gene transcription from parental versus progeny viral genomes. These two effects would effectively provide a replication-dependent control on late gene transcription.
in that transcription from parental viral genomes is prevented until replication occurs, whereas upon DNA replication, late viral gene transcription is promoted by the ICP8 protein. These effects could be exerted by ICP8 itself or as part of a DNA replication protein complex which appears to be assembled at prereplicative sites within the host cell nucleus (Quinlan and Knipe, 1983; de Bruyn Kops and Knipe, 1988; Liptak et al., 1996).

The two opposing effects of ICP8 on late gene expression may be explained by different conformations of the viral DNA before and after viral DNA replication. Prior to viral DNA replication the parental viral genomes are circular (Poffenberger and Roizman, 1985), and thus, rotation of the strands would be restricted. ICP8 or a complex of viral DNA replication proteins involving ICP8 may bind to the circular genomic molecule and inhibit transcription by preventing the binding of viral or cellular transcription factors. Alternatively, the complex of DNA replication proteins may pull the parental viral DNA molecules to sites within the nucleus where DNA replication takes place but transcription of the viral genome cannot take place. After viral DNA replication, the viral DNA templates are linear and thus the binding of ICP8 or other viral proteins may be able to unwind the strands or alter their conformation. Thus, as viral DNA synthesis proceeds, transcription from late promoters could be initiated. ICP8 may play a role either in stabilizing the promoter region in an unwound, single-stranded form or by binding to the unwound strands, thereby stimulating protein–protein interactions or protein–DNA interactions at the late promoter regions. Further studies on the dual effect of ICP8 on transcription from parental and progeny viral DNA molecules should contribute to our understanding of the processes that control the utilization of DNA molecules for transcription versus DNA replication and the factors involved in replication-dependent transcription of DNA molecules.

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