JOURNAL OF VIROLOGY, May 1991, p. 2666–2675
0022-538X/91/052666-10$02.00/0

Vol. 65, No. 5

Potential Role for Herpes Simplex Virus ICP8 DNA Replication Protein in Stimulation of Late Gene Expression

MIN GAO AND DAVID M. KNIPE*

Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115

Received 16 November 1990/Accepted 20 February 1991

We have identified a *trans*-dominant mutant form of the herpes simplex virus (HSV) DNA-binding protein ICP8 which inhibits viral replication. When expressed by the V2.6 cell line, the mutant gene product inhibited wild-type HSV production by 50- to 150-fold when the multiplicity of infection was less than 5. Production of HSV types 1 and 2 but not production of pseudorabies virus was inhibited in V2.6 cells. The inhibitory effect was not due solely to the high levels of expression, because the levels of expression were comparable to those in the permissive wild-type ICP8-expressing S-2 cell line. Experiments designed to define the block in viral production in V2.6 cells demonstrated (i) that viral α and β gene expression was comparable in the different cell lines, (ii) that viral DNA replication proceeded but was reduced to approximately 20% of the control cell level, and (iii) that late gene expression was similar to that in cells in which viral DNA replication was completely blocked. Genetic experiments indicated that the mutant gene product inhibits normal functions of ICP8. Thus, ICP8 may play distinct roles in replication of viral DNA and in stimulation of late gene expression. The dual roles of ICP8 in these two processes could provide a mechanism for controlling the transition from viral DNA synthesis to late gene expression during the viral growth cycle.

The major DNA-binding protein of herpes simplex virus (HSV), ICP8, is expressed as a β or delayed early gene product during productive infection. ICP8 is one of the seven virus-encoded proteins that are required for the replication of the HSV type 1 (HSV-1) genome (2, 4, 31, 44, 45). The functions and activities performed by ICP8 have not been completely established. Some known properties of ICP8 include (i) the ability to localize to the cell nucleus independent of other viral proteins, (ii) the ability to bind DNA nonspecifically in vitro and in vivo, (iii) the ability to down-regulate the expression of ICP4 under certain conditions (12), and (iv) the ability to promote assembly of DNA replication structures in the infected cell nucleus.

To study the functional domains of ICP8, we have constructed a variety of ICP8 gene mutations (9a, 10). Phenotypic analysis of ICP8 gene mutants indicated that several regions of ICP8 are required for its nuclear localization (9b). However, analysis of ICP8-pyruvate kinase fusion proteins showed that the carboxyl-terminal 28 residues of ICP8 comprise the only portion of ICP8 that can function alone as a nuclear localization signal.

ICP8 binds to single-stranded DNA (ssDNA) or double-stranded DNA in vitro (1, 26, 34, 39), with at least a fivefold preference for ssDNA (28, 40), and can be isolated in DNA-protein complexes from infected cells (27, 29). The ICP8 sequences required for ssDNA binding have been mapped between residues 564 and 849, on the basis of the studies of a variety of ICP8 mutant viruses, of in vitro transcription-translation products of ICP8, and of partial protease digestion of purified ICP8 (10, 30, 43).

The intranuclear location of ICP8 is determined, at least in part, by the status of viral DNA replication (5, 35). In infected cells, before viral DNA synthesis ICP8 localizes to nuclear framework-associated structures called prereplicative sites (5, 35, 36). As viral DNA replication occurs, ICP8 migrates to replication compartments (5, 35), where it is bound to progeny and replicating viral DNA (27, 29). Viruses expressing an altered ICP8 molecule fail to assemble prereplicative sites. Therefore, ICP8 is required for the assembly of prereplicative sites (5).

To further study the functional domains of ICP8, we have introduced various mutated ICP8 gene sequences into the viral genome (9a, 10). However, we were unable to introduce certain mutations from ICP8 gene plasmids into the viral genome by recombination. One of these mutants has a small deletion between the sequences encoding the DNA-binding region and the nuclear localization signal of ICP8. This report describes that this mutant ICP8 protein exhibits a *trans*-dominant phenotype and can significantly inhibit the production of wild-type (wt) HSV-1. Analysis of this mutant phenotype suggests that wt ICP8 plays a role in the stimulation of late gene expression.

MATERIALS AND METHODS

Plasmids. The plasmid pSV8 and the nucleotide numbering system for the ICP8 gene were described previously (9, 20). The plasmid pSV8 was constructed by inserting the ICP8 coding sequences (map units 0.374 to 0.409) downstream of the simian virus 40 early promoter in plasmid RL18:PK12 (24). The plasmid pSV8.3 was derived from pSV8 by deleting the polylinker region and the *Sac*I-*Bgl*II fragment of the pyruvate kinase gene. The plasmid pSVd105 was constructed by deletion of a *Bgl*II fragment of pSV8.3 after the conversion of *Pvu*II (nucleotide 3853) and *Nae*I (nucleotide 4111) sites to *Bgl*II sites. Thus, pSVd105 lacks codons 1083 to 1168 of the ICP8 coding sequence but encodes four additional amino acids, Gly-Arg-Ser-Ser, in the *Bgl*II linker sequence. The plasmid pSG28 was provided by M. Levine (University of Michigan).

Cells and viruses. Vero cells were grown and maintained as described previously (25). The growth medium for the neomycin-resistant cell lines S-2 (10) and V2.6 (see below)

* Corresponding author.

included 200 μg of the antibiotic G418 per ml during the first passage of the cells after thawing or 500 μg of G418 per ml of medium every five passages.

The HSV-1 wt strain KOS1.1 was propagated and assayed as described previously (25, 27). The mutant viruses *d*301 and *n*10 were grown and propagated in the ICP8-expressing S-2 cell line (10). Pseudorabies virus (PRV) was provided by E. Sinn and R. Roeder (Rockefeller University).

For infections at a multiplicity of infection (MOI) of 2 PFU per cell for biochemical analysis, cell numbers were determined by cell counts of a culture flask of each cell line being used prior to the infection.

Isolation of d105 ICP8-expressing cell line. Vero cells were transformed with the plasmids pSVd105 and pSVneo (Fig. 1) (42) as described previously (6, 10). After growth in medium containing the antibiotic G418 (a neomycin analog), drug-resistant colonies were isolated, grown into cultures, and screened by indirect immunofluorescence (5) using the 10E-3 anti-ICP8 monoclonal antibody (38) for the ability to express the mutant form of ICP8 upon *n*10 infection. The 10E-3 antibody recognizes d105 ICP8 but not *n*10 ICP8 (9b).

ssDNA cellulose chromatography. ssDNA cellulose chromatography of infected cell extracts was performed as described previously (10, 26).

Analysis of viral proteins and viral DNA replication. Cell monolayer cultures were infected with KOS1.1 or *d*301 virus and then labeled with [^{35}S]methionine and harvested as indicated below. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of infected cell lysates was performed as described previously (25). After electrophoresis, the gels were fixed, dried, and exposed to Kodak SB5 film, or the proteins were transferred by electrophoresis to nitrocellulose filters for Western immunoblot analysis. Immune complexes were detected on blots by a procedure involving a color reaction for alkaline phosphatase activity conducted as specified by the manufacturer (Promega Biotec, Madison, Wis.). The rabbit polyclonal serum PP5 (46) and the mouse monoclonal antibody 10E-3 (38) were used to detect HSV-1 DNA polymerase and ICP8, respectively.

Analysis of viral DNA amplification during the course of infection was performed as described previously (37). The probes used were the plasmids pBR3441 (30) and pSG28 (13). To quantify the data, the slots were cut out and radioactivity was measured by liquid scintillation counting.

Northern (RNA) blot analysis. Total cytoplasmic RNA for Northern blots was isolated and analyzed as described previously (37). The plasmid used for the probe was pEcoRI-BamHI-I-I (gC gene probe [8]). Band intensities on the autoradiogram were determined with an Ultrascan laser densitometer and an on-line integrator (LKB Instruments, Inc., Rockville, Md.).

RESULTS

The mutant d105 exhibits a *trans*-dominant phenotype. When we attempted to introduce various mutant ICP8 genes into the viral genome by recombination, we observed that viruses containing certain mutant alleles could not be isolated. One of these was the d105 mutation (Fig. 1). The mutant d105 ICP8 gene has a deletion of 86 codons between the sequence encoding the DNA-binding domain and the sequence encoding the nuclear localization signal. One possible reason for the failure of introduction of the d105 mutation into the viral genome was that the d105 gene product exhibited a *trans*-dominant mutant phenotype. To test this, we cotransfected various amounts of mutant d105 plasmid with 1 μg of infectious HSV-1 DNA into Vero cells; we observed that plaque numbers were dramatically decreased compared with those obtained when the wt ICP8 plasmid was cotransfected (Table 1). Even when the molar ratio of the amounts of ICP8 gene sequences in d105 plasmid to those in the infectious viral genome was only 2:1 (0.1 μg of d105 plasmid), significant inhibition of plaque formation was observed. Thus, the d105 mutant showed a *trans*-dominant phenotype. In contrast, many other ICP8 mutants did not cause an inhibition of viral growth (data not shown).

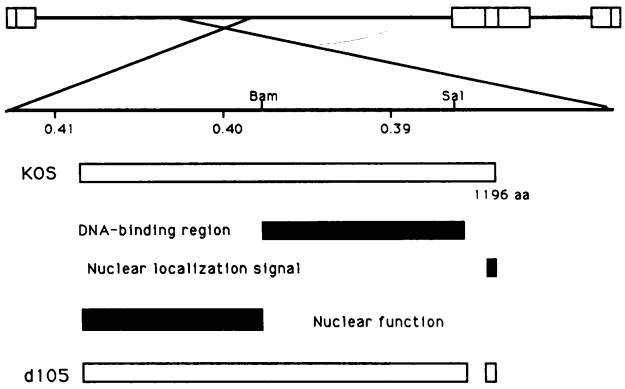

FIG. 1. Functional domains of ICP8. Analysis of a variety of ICP8 mutants indicated that the DNA-binding region of ICP8 is located within residues 564 to 1081 (10) and that the C-terminal 28 residues of ICP8 can function as a nuclear localization signal (9b). Mutant *d*101 ICP8 which is missing residues 17 to 563 localizes to the nucleus and binds to ssDNA but fails to promote viral DNA replication (10). This indicates that the N-terminal half of ICP8 has a nuclear function other than DNA binding. The d105 mutant ICP8 has a deletion of residues 1082 to 1169 and exhibits a *trans*-dominant negative phenotype.

The d105 gene product was able to localize into the nucleus when it was expressed in Vero cells transfected with the pSVd105 plasmid (Fig. 2). To study the phenotype of the d105 gene product in more detail, we isolated a cell line named V2.6 which expressed d105 ICP8 upon HSV infection. The V2.6 cell line was a poor host for wt virus growth, despite the presence of wt ICP8 expressed from the viral genome. The plating efficiency of wt virus on V2.6 cells was 100- to 1,000-fold lower than that on Vero cells or on the wt ICP8-expressing S-2 cell line, and the size of the plaques was

TABLE 1. Inhibition of HSV-1 plaque formation by *trans*-dominant mutant ICP8[a]

Type of ICP8 DNA and amt (μg)	No. of plaques		
	Expt 1	Expt 2	Expt 3
wt			
0.1	85	81	
0.5	86	83	107
1.0	64	73	192
Mutant			
0.1	25	12	
0.5	0	2	0
1.0	0	2	0

[a] Infectious KOS1.1 DNA (1 μg) was cotransfected with various amounts of either wt ICP8 plasmid (pSV8.3) or d105 mutant plasmid (pSVd105) by the calcium phosphate precipitation procedure (9, 14). Plaques were counted 3 to 4 days after transfection.

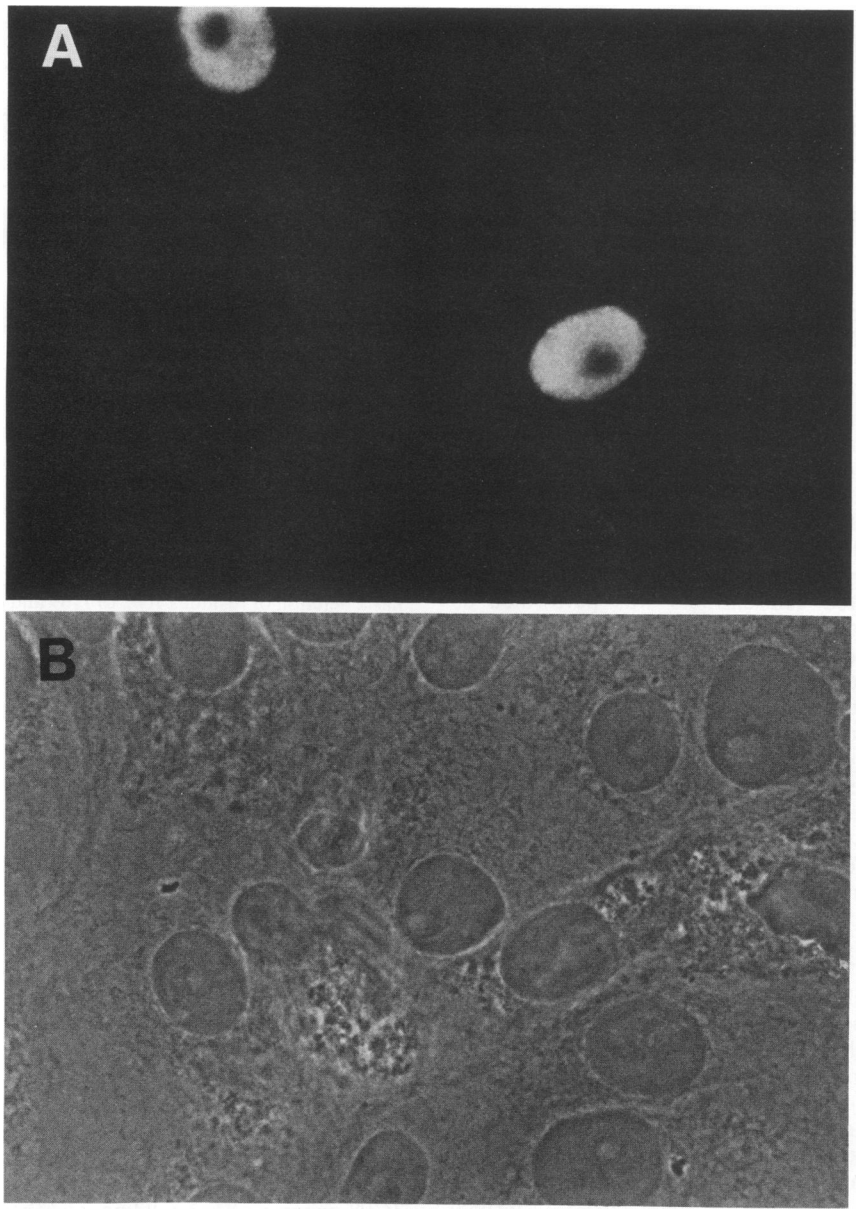

FIG. 2. Nuclear localization of mutant d105 ICP8 in transfected cells. Vero cells were transfected with pSVd105 and processed for immunofluorescence by using the 10E-3 antibody. (A) Immunofluorescence micrograph, (B) corresponding phase-contrast micrograph.

extremely small (data not shown). Single-cycle growth experiments showed that the yield of wt virus on the V2.6 cell line was 50- to 150-fold lower than that on Vero cells or on the wt ICP8-expressing S-2 cell line when the MOI was less than 5 (Table 2). At an MOI of 0.1, the V2.6 cell line exhibited a yield of HSV-1 KOS1.1 reduced approximately 150-fold from that on Vero cells or on the wt ICP8-expressing S-2 cell line. The inhibition of growth on V2.6 cells was overcome at an MOI of 10. Thus, although the d105 gene product could inhibit viral replication in the presence of wt ICP8 at low MOIs, the *trans*-dominant effect appeared to be due to competitive inhibition because the inhibition was decreased at a high MOI. Because significant inhibition was obtained at an MOI of 2.5 and most cells were infected, we chose an MOI of 2 to 2.5 PFU per cell for the biochemical studies described below.

To examine the specificity of resistance of the V2.6 cell line to HSV-1, we also tested the ability of the V2.6 cell line to restrict the growth of HSV-2 and of a closely related herpesvirus, PRV. An inhibitory effect of similar magnitude was observed for HSV-2 on the V2.6 cell line (data not shown). However, no decrease of virus yields was observed when PRV was plated on the V2.6 cell line (Table 3). The induction of the mutant form of ICP8 in the V2.6 cell line by PRV was evidenced by Western blot analysis by using an antibody specific for ICP8 (data not shown). These results indicated that the inhibitory effect of mutant d105 was HSV specific.

To determine whether this inhibitory effect was exerted on ICP8, the mutant d105 plasmid was cotransfected with increasing amounts of wt ICP8 plasmid. When this was done, the inhibition of the production of wt virus was

TABLE 2. Growth of wt HSV-1 on different cell lines

Cell line	Expt	Yield (PFU per cell) at the following MOI[a]				
		0.1	1.0	2.5	5.0	10.0
Vero	1	260 (153[b])	380 (91)			420 (3.8)
Neo[r]	2		950 (158)	660 (66)	410 (8.2)	
S-2	1	260 (153)	310 (74)			230 (2.1)
	2		560 (92)	450 (45)	420 (8.4)	
V2.6	1	1.7	4.2			110
	2		6.1	10	50	

[a] Vero cells, the wt ICP8-expressing S-2 cell line, and the V2.6 cell line expressing d105 ICP8 were infected with KOS wt virus at the indicated MOI and were incubated at 37°C. Cells were harvested 24 h after infection, and titers of the progeny viruses were determined on Vero cells.
[b] Numbers in parentheses represent the fold greater yield on these cells relative to the yield on V2.6 cells.

partially relieved (Table 4). These results demonstrated that the inhibitory effect caused by the d105 gene product was a *trans*-dominant effect mediated on wt ICP8.

The mutant d105 ICP8 binds to ssDNA. For many DNA-binding proteins, such as yeast GCN4 (22), *trans*-dominant mutant forms retain their DNA-binding ability but lose transactivation activity or their ability to interact with certain other proteins (reviewed in reference 18). Therefore, to determine whether the inhibitory gene product retained the ability to bind DNA, we infected V2.6 cells with a DNA-binding-negative ICP8 mutant *d*301 (10). Extract from infected cells was passed over a ssDNA cellulose column, and ICP8 was eluted stepwise with buffer containing increasing salt concentrations. Polypeptides in the various fractions were analyzed by PAGE (data not shown) or Western blotting (Fig. 3). In V2.6 cells infected with *d*301, a 120-kDa form of ICP8 was detected (Fig. 3, lanes 12 and 13), in agreement with the predicted size of the d105 gene product (9). Mutant *d*301 ICP8 bound poorly to ssDNA cellulose (Fig. 3, lanes 12 through 15) and was found in the flow-through fractions (lanes 7 and 8). In contrast, d105 ICP8 bound to ssDNA (Fig. 3, lanes 12 through 15), and the majority of it was eluted with 0.5 M NaCl (lane 13). In other comparisons, d105 ICP8 bound to ssDNA with an efficiency similar to that of wt ICP8 (data not shown).

Partial inhibition of viral DNA replication in V2.6 cells. To determine whether the decreased viral yield in the V2.6 cell line was due to a block in viral DNA synthesis, we examined viral DNA replication (Fig. 4). The *d*301 mutant virus, which contains a large deletion in the ICP8 gene and is unable to replicate its DNA (10), was included in the experiment as a negative control. Total DNA was isolated from mock- or wt virus-infected Vero cells, from the wt ICP8-expressing S-2 cell line, and from the inhibitory V2.6 cell line at 1 or 16 h postinfection. Fivefold serial dilutions of the DNA were bound to a nitrocellulose filter, which was then hybridized with ^{32}P-labeled HSV-1 VP16 gene DNA as a probe (32). As expected, mutant *d*301-infected Vero cells showed no amplification of viral DNA during the course of infection, consistent with previous results (10). In contrast, wt virus-infected Vero, S-2, and V2.6 cells showed substantial amplification of viral DNA during the infection. To quantitate these results, the amount of radioactivity hybridized to each slot was measured by scintillation counting, and the relative amounts of HSV-1 DNA in each sample were determined. In this particular experiment, the amount of viral DNA synthesized in the V2.6 cells was about 33% of the level in Vero cells and 19% of that in the S-2 cells. In several separate experiments, viral DNA replication in V2.6 cells was reduced to an average of 17% of the Vero cell level or S-2 cell level. Similar results were obtained when the *Eco*RI joint fragment (pSG28) was used as a probe, indicating that the full length of the viral genome was probably amplified. Thus, substantial viral DNA replication occurred in the V2.6 cells.

Block in late gene expression in V2.6 cells. To determine whether the inhibition of the growth of wt HSV-1 in V2.6 cells was due to the inhibition of α or β gene expression, we measured the amounts of DNA polymerase expressed by wt virus in V2.6 cells, Vero cells, or the wt ICP8-expressing S-2 cells. Western blots revealed that the quantity of DNA polymerase (Fig. 5A) in V2.6 cells was only slightly reduced relative to that in Vero cells or the wt ICP8-expressing S-2 cell line (Fig. 5A, compare lane 9 and lane 7 or 8 at 6 h

TABLE 3. Growth of PRV on different cell lines

Cell line	Yield (PFU per cell) at the following MOI[a]	
	0.1	2.0
Vero	180 (3.6[b])	340 (1.8)
S-2	100 (2.0)	330 (1.8)
V2.6	50	188

[a] Vero cells, the wt ICP8-expressing S-2 cell line, and the V2.6 cell line expressing d105 ICP8 were infected with PRV at an MOI of 0.1 or 2 and were incubated at 37°C. Cells were harvested 24 h after infection, and the titers of the progeny viruses were determined on rabbit kidney cells.
[b] Numbers in parentheses represent fold greater yield on these cells relative to the yield on V2.6 cells.

TABLE 4. Relief of d105 ICP8 inhibition by wt ICP8

Transfection with:			No. of plaques	
d105 DNA (μg)	wt ICP8 DNA (μg)	pUC18 DNA (μg)	Expt 1	Expt 2
			172	171
0.2			11	31
0.2		0.2	8	26
0.2		1.0	9	18
0.2	0.2		32	168
0.2	1.0		33	69
	0.2		138	
	1.0		76	

[a] Infectious KOS1.1 DNA (0.2 μg) was cotransfected with various amounts of wt ICP8 plasmid (pSV8.3), d105 mutant plasmid (pSVd105), and pUC18 plasmid by the calcium phosphate precipitation procedure (9, 14). Plaques were counted 3 days after transfection.

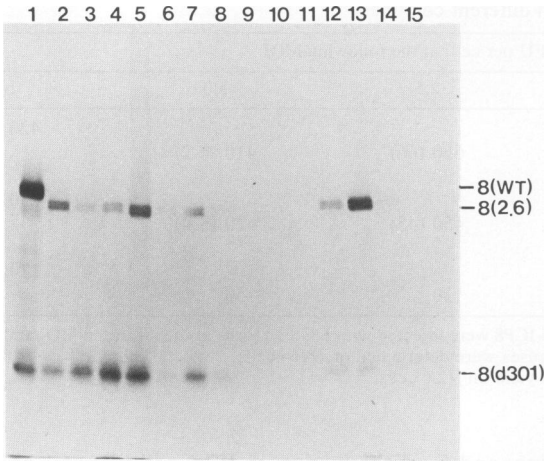

FIG. 3. DNA binding of the d105 mutant gene product. V2.6 cells were infected with the DNA-binding-negative ICP8 mutant *d*301 at an MOI of 10 PFU per cell and labeled with [^{35}S]methionine from 6 to 8 h postinfection. The various protein fractions resolved on a ssDNA cellulose column were subjected to SDS-PAGE and electroblotted onto a nitrocellulose filter. The filter was then probed with a monoclonal antibody specific for ICP8 (10E-3). Lanes: 1, purified wt ICP8 as a size marker; 2, total cellular lysate; 3, pellet from high-salt DNase extraction; 4, pellet after dialysis; 5, extract put on ssDNA column; 6 to 11, flowthrough and wash; 12, 0.3 M NaCl elute; 13, 0.5 M NaCl elute; 14, 1.0 M NaCl elute; 15, 4.0 M NaCl elute. The positions of wt ICP8, the d105 form of ICP8 expressed from V2.6 cells, and *d*301 ICP8 are indicated on the right.

postinfection, or lane 12 and lane 10 or 11 at 12 h postinfection).

We also examined the amounts of ICP8 expression in the different cell types. Western blots (Fig. 5B) revealed that the levels of wt ICP8 expression were equivalent in Vero (Fig. 5B, lanes 1 and 2) and V2.6 cells (Fig. 5B, lanes 5 and 6). This blot also shows that the amount of d105 ICP8 expressed in V2.6 cells (Fig. 5B, lanes 5 and 6) was only slightly greater than the amount of wt ICP8 expressed in the permissive S-2 cells (Fig. 5B, lanes 3 and 4). Other Western blots demonstrated no apparent reduction of ICP4 expression. These results demonstrated that the major part of the inhibition of the growth of wt HSV-1 in the V2.6 cell line was not due to the inhibition of α or β gene expression.

To further examine viral gene expression in V2.6 cells, we infected Vero cells, wt ICP8-expressing S-2 cells, and V2.6 cells with wt HSV-1 in the absence or presence of sodium phosphonoacetate (PAA), a specific inhibitor of HSV-1 DNA synthesis, and we pulse-labeled the cells with [^{35}S]methionine at 3, 6, or 12 h postinfection. The labeled proteins were separated by SDS-PAGE and visualized by autoradiography (Fig. 6). The mutant form of ICP8 expressed from V2.6 cells, like wt ICP8 expressed from S-2 cells, was not constitutively expressed but was induced upon virus infection. The patterns of viral protein synthesis at 3 or 6 h postinfection were similar in the 3 different cell lines, except that significant amounts of the mutant form of ICP8 expressed from the V2.6 cell line and of wt ICP8 expressed from the S-2 cell line were observed. The amount of the mutant form of ICP8 produced by the V2.6 cell line was greater than that of wt ICP8 in Vero cells (Fig. 6, lanes 21 and 19) but was comparable to that produced by the wt ICP8-expressing S-2 cell line (Fig. 6, lanes 21 and 20) at 12 h postinfection. Although slightly greater amounts of d105 ICP8 than of S-2 ICP8 were expressed at 6 h postinfection in this experiment (Fig. 5B and 6, lanes 14 and 15), this was not observed in other experiments (data not shown). Because S-2 cells were as permissive for HSV as Vero cells (Table 2), the inhibition of wt virus growth on the V2.6 cell line was not

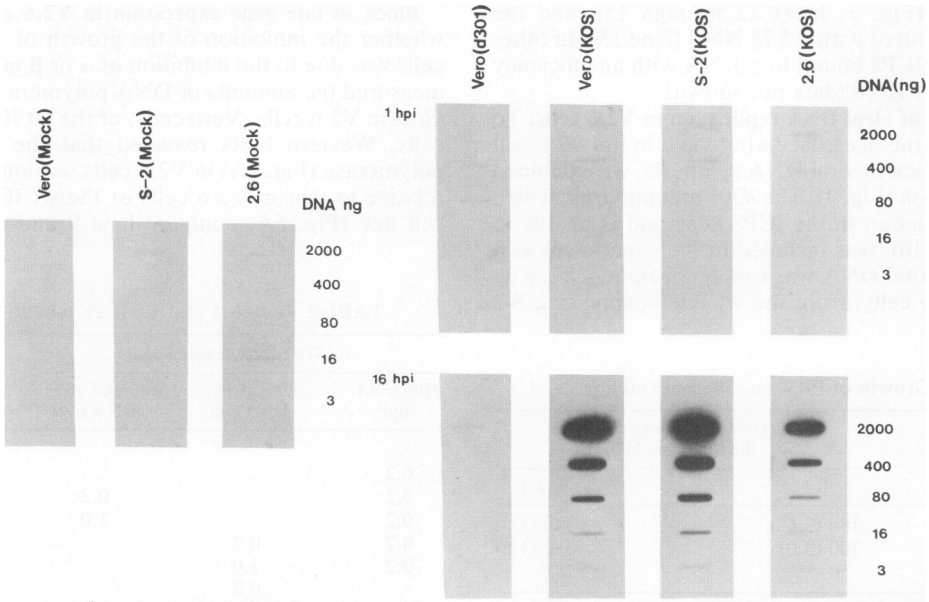

FIG. 4. HSV-1 DNA replication in different cell lines. Vero cells, the wt ICP8-expressing S-2 cell line, and the inhibitory V2.6 cell line were mock infected or infected with the ICP8 mutant *d*301 or wt HSV-1. Total cellular DNA was prepared immediately after viral adsorption (1 h) or near the end of the infection cycle (16 h). Equal amounts of each DNA were subjected to fivefold serial dilutions, and the DNAs were bound to a nitrocellulose filter, which was probed with ^{32}P-labeled DNA specific for the VP16 gene. An autoradiograph of the blot is shown. hpi, Hours postinfection.

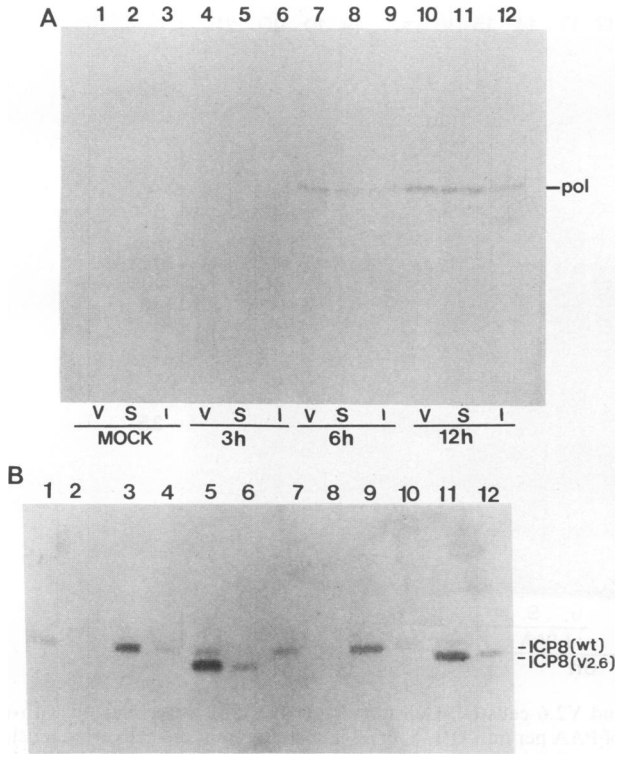

FIG. 5. Western blot analysis of the amounts of HSV DNA polymerase and ICP8 expressed in different cell lines. (A) Vero cells (V), the wt ICP8-expressing cell line (S), and the inhibitory cell line (I) were mock infected or infected with wt HSV-1 at an MOI of 2 PFU per cell and harvested at 3, 6, or 12 h postinfection. Proteins in the cell extracts were separated by SDS-PAGE and electroblotted onto nitrocellulose. The filter was then probed with the PP5 polyclonal antisera against HSV-1 DNA polymerase (provided by D. Coen). The position of DNA polymerase (pol) is indicated to the right of the filter. (B) Western blot for ICP8, obtained by using the 10E-3 monoclonal antibody specific for ICP8. Pairs of panels contain threefold dilutions of extract obtained from cells at 6 h postinfection. Lanes 1 and 2, Vero cells; lanes 3 and 4, S-2 cells; lanes 5 and 6, V2.6 cells; lanes 7 and 8, Vero cells with 400 μg of PAA per ml; lanes 9 and 10, S-2 cells with 400 μg of PAA per ml; lanes 11 and 12, V2.6 cells with 400 μg of PAA per ml.

simply due to overexpression of the mutant form of ICP8. In addition, the amount of wt ICP8 produced by wt virus infection on the V2.6 cell line was only slightly reduced compared with that on Vero cells (Fig. 6, lanes 19 and 21). This result was consistent with those in Fig. 5, suggesting that the inhibition of wt virus growth on the V2.6 cell line was not a consequence of the mutant form of ICP8 attenuating the expression of α and β genes.

In contrast, the synthesis of viral polypeptides of γ1 (ICP5 and ICP25) and γ2 (ICP1-2 and ICP15) classes was markedly reduced in V2.6 cells (Fig. 6, lane 18) compared with that in Vero cells (lane 16) or the wt ICP8-expressing S-2 cell line (lane 17). In fact, the pattern of viral polypeptide synthesis at 12 h postinfection was almost identical to that observed in the absence of viral DNA synthesis (Fig. 6, compare lane 18 and lane 19, 20, or 21). Thus, viral late protein synthesis was reduced in V2.6 cells.

Separation of defects in viral DNA synthesis and late gene expression. It was conceivable that the decreased viral DNA replication in V2.6 cells was the sole cause of the decreased late gene expression. To determine whether this was the case, we used various concentrations of PAA to attempt to reduce viral DNA synthesis in Vero cells or in the wt ICP8-expressing S-2 cell line to levels similar to that of the V2.6 cell line in the absence of PAA. Thus, if the level of DNA synthesis was the sole determinant of the levels of late gene expression, late gene expression would then be similar in the different cell lines. Cells were infected with wt virus and harvested at 16 h postinfection for analysis of viral DNA and protein synthesis (Fig. 7). With increasing concentrations of PAA, decreasing amounts of viral DNA amplification and late gene expression were observed in Vero cells or in S-2 cells. At a PAA concentration of 400 μg per ml, viral DNA synthesis was completely inhibited, and no γ2 viral polypeptide synthesis (e.g., ICP15) was observed. At PAA concentrations between 80 and 160 μg per ml, the amounts of viral DNA synthesized in Vero cells or in S-2 cells were similar to that synthesized in V2.6 cells infected without PAA. However, under these conditions, synthesis of viral polypeptides of γ1 (ICP5 and ICP25) and γ2 (ICP15) classes in the V2.6 cells was markedly reduced compared with that in Vero cells or in S-2 cells. Quantitation of ICP5 synthesis by densitometry indicated that in V2.6 cells the rate of synthesis of ICP5 was approximately eightfold lower than in Vero cells or S-2 cells. Therefore, we conclude that the lack of late gene expression in the inhibitory V2.6 cell line was not due entirely to the decreased amount of viral DNA synthesis.

To further define the level at which late gene expression was decreased, we performed Northern blot analysis to measure the steady-state level of γ2 gC mRNA (Fig. 8) in different cells. Infection of Vero cells or V2.6 cells was carried out in the absence or in the presence of 100 μg of PAA per ml. Total cytoplasmic RNA was isolated from infected cells at 16 h postinfection. Equal amounts of total cellular RNA were probed on a Northern blot with a ^{32}P-labeled DNA fragment for the gC gene. No gC mRNA was detected in mock-infected V2.6 cells (Fig. 8). As expected, partial inhibition of viral DNA synthesis by PAA reduced the amounts of gC mRNA in Vero cells or in S-2 cells. At a PAA concentration of 100 μg per ml, the level of gC mRNA in V2.6 cells was at least sixfold less than in the other cells. Therefore, the block in late gene expression in V2.6 cells was due, at least in part, to a decrease in mRNA accumulation.

DISCUSSION

We have expressed a *trans*-dominant mutant form of the HSV ICP8 DNA-binding protein and observed that it confers significant resistance to HSV infection. In cells expressing the mutant gene product, viral DNA synthesis and late gene expression are decreased. Our results indicate that the decreased late gene expression is not due to decreased DNA replication. This suggests a new role for ICP8 in viral replication, i.e., stimulation of late gene expression.

Inhibition of viral replication by a mutant viral gene product. Viral replication was inhibited significantly by the mutant gene product either in cotransfected cells or in a cell line expressing the mutant gene product. Orberg and Schaffer (33) previously isolated a cell line, U35, by transformation of large amounts of wt ICP8 gene DNA which showed reduced permissiveness for HSV. The U35 cell line expresses high levels of ICP8 upon viral infection, and it is believed that overexpression of wt ICP8 is the cause of decreased viral replication. This cell line also contained a significant number

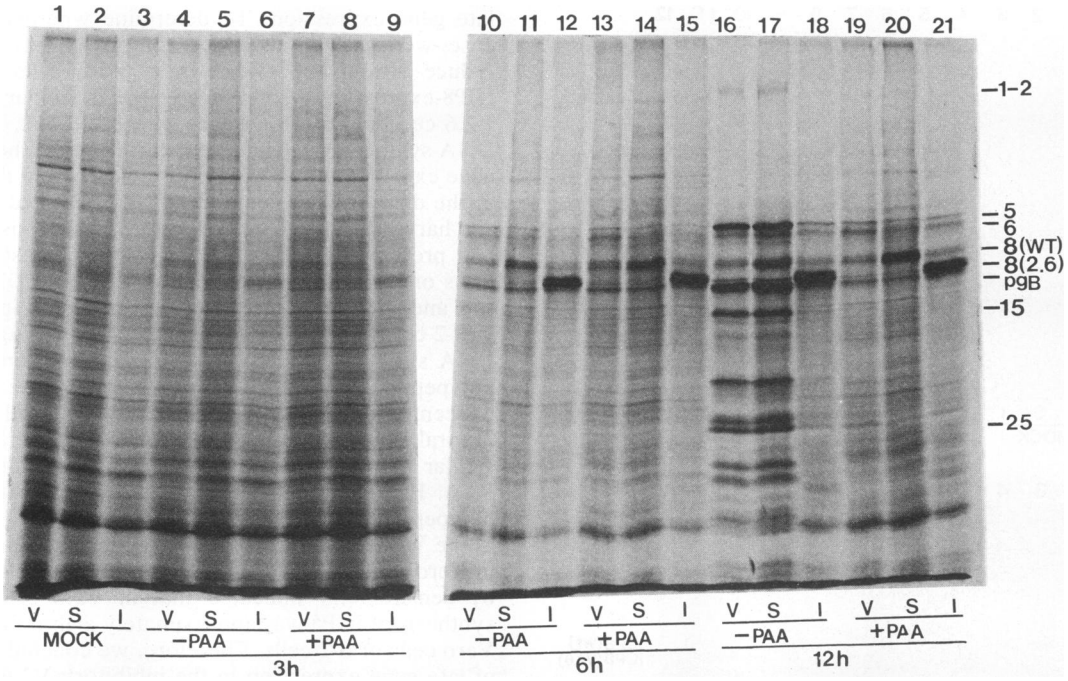

FIG. 6. Polypeptide profile of wt HSV-1-infected Vero (V), S-2 (S), and V2.6 cells (I). Cell monolayer cultures were infected with wt KOS1.1 at an MOI of 2 PFU per cell in the absence or presence of 400 μg of PAA per ml. At 3, 6, or 12 h postinfection, the cells were labeled with [^{35}S]methionine for 30 min and then harvested. Equal fractions of each cell lysate were subjected to SDS-PAGE and autoradiography. Shown to the right of the gel are the positions of several HSV-1 proteins.

of rearranged and deleted ICP8 genes (33). Thus, defective gene products could be expressed in these cells. Two lines of evidence argue that the d105 defective gene product, not overexpression of ICP8, is responsible for the inhibition of viral replication observed in this work. First, parallel transfections with equal amounts of d105 and wt ICP8 gene DNAs led to greatly reduced numbers of plaques in the presence of d105 DNA. Second, although S-2 cells and V2.6 cells express approximately equal amounts of the two forms of ICP8, infection of V2.6 cells gives 150-fold less virus at a low MOI. Therefore, there is some unique feature of the defective gene product which causes an inhibition of viral replication.

Because cotransfection of wt ICP8 gene DNA with the d105 DNA relieved the inhibitory effect of the d105 gene, we concluded that the d105 gene product exhibited a dominant inhibitory phenotype by acting as a competitive inhibitor of wt ICP8 function. The d105 protein retains the ssDNA-binding properties of wt ICP8, and it seems likely that it could compete with wt ICP8 for DNA-binding sites. Infection of V2.6 cells at a high MOI also overcame the inhibitory effect. These results indicate that the mutant gene product has not acquired a new activity but instead acts as a competitive inhibitor of the normal functions of wt ICP8.

Two HSV-1 *trans*-dominant mutant gene products have been reported (7, 41). It was hypothesized that these mutant gene products interfered with wt viral functions either by formation of nonfunctional dimers (41) or by tying up cell factors in inactive complexes (7). The reduction in titer of wt virus stock observed on these different inhibitory cell lines varied from mutant to mutant, but in most cases it was in the range of 10- to 40-fold. The V2.6 cell line showed a much greater inhibitory effect on the production of wt virus because the reduction in the yield of wt virus produced in a single lytic infectious cycle was as much as 150-fold.

A potential role for ICP8 in stimulation of late gene expression. Seven viral gene products, including ICP8, are required for HSV DNA replication (2). Because HSV DNA replication is absolutely required for true late (γ2) gene expression during HSV infection (19, 21, 23), all DNA replication proteins are indirectly required for late gene expression. It has been difficult to demonstrate a direct role for any of these seven genes in late gene expression because these two events are tightly coupled. ICP8 mutant viruses cannot activate late gene expression, apparently because these mutants could not promote viral DNA synthesis (10). V2.6 cells offer a unique situation to examine a possible regulatory role of ICP8 in late gene expression.

In V2.6 cells, wt virus synthesized amounts of viral DNA sufficient to support late gene expression, but virtually no γ2 gene expression was observed. This was in contrast to the results in which significant amounts of γ2 genes were expressed when viral DNA replication was partially inhibited by PAA to a level similar to that on V2.6 cells. These results suggested that late gene expression could be partially separated from DNA replication and that ICP8 has a distinct role in stimulation of late gene expression.

The ability of a ssDNA-binding protein to promote late gene transcription would not be unique to ICP8. The adenovirus DNA-binding protein, the gene 32 protein of bacteriophage T4, and the SSB protein of *Escherichia coli* exhibited similar roles in activation of transcription (3, 11, 15). The adenovirus DNA-binding protein can enhance the expression of a reporter gene controlled by several different promoters within transfected cells (3). The adenovirus major late promoter showed a greater response to the DNA-

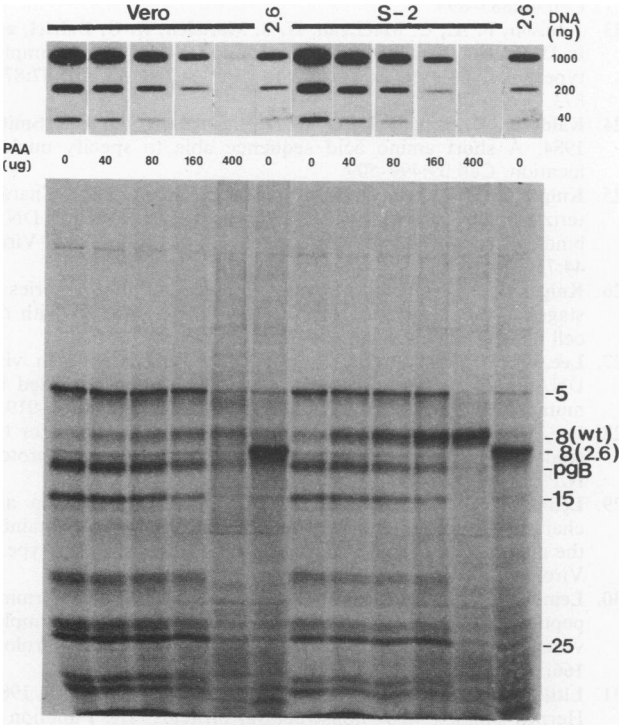

FIG. 7. The block in late gene expression in V2.6 cells is independent of decreased viral DNA replication. Vero, S-2, and V2.6 cells were infected with wt HSV-1 at an MOI of 2 PFU per cell with the indicated concentrations of PAA and were harvested at 16 h postinfection for the analysis of viral DNA and protein synthesis as described in the legends to Fig. 4 and 5.

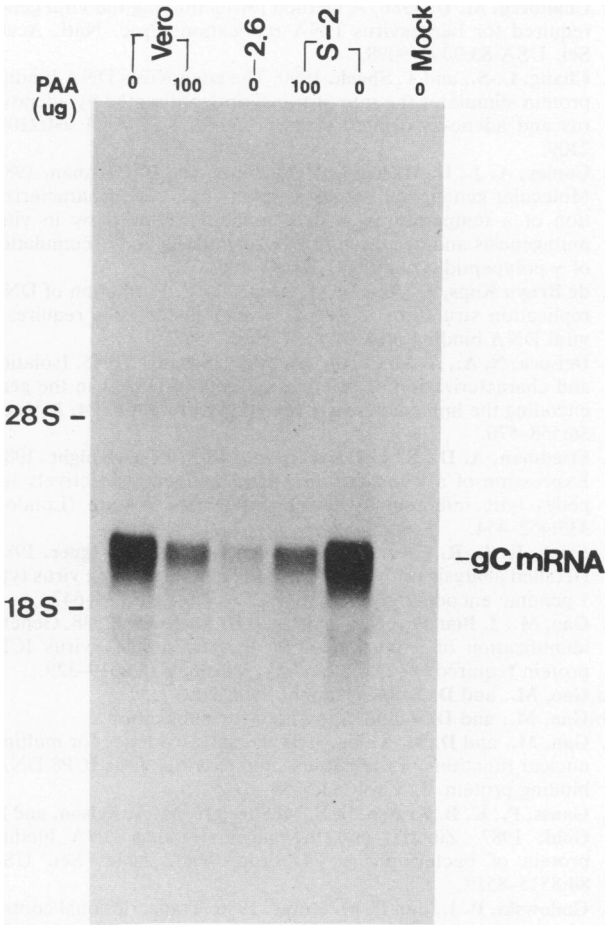

FIG. 8. Accumulation of gC mRNA in wt HSV-1-infected cells. Vero, S-2, and V2.6 cells were infected with wt virus at an MOI of 2 PFU per cell with or without 100 μg of PAA per ml. Total cytoplasmic RNA was prepared at 16 h postinfection. Equal amounts of RNA were subjected to Northern blot analysis by using ^{32}P-labeled probe specific for gC mRNA. Shown to the sides of the gel are the migration positions of rRNA and gC mRNA.

binding protein than to the E1A transactivator protein, suggesting that the DNA-binding protein plays a central role in activation of the late promoter. Several T4 bacteriophage gene 32 mutants synthesized high levels of viral DNA (30 to 55% of wt virus level) but were defective for the synthesis of late gene 23 protein, the major structural protein of the T4 capsid (11). The amounts of mRNA were also greatly reduced for at least 3 late genes in these gene 32 mutants. These results demonstrated a direct requirement for gene 32 protein in the activation of late gene expression. The *E. coli* SSB protein activates promoters transcribed by the bacteriophage N4 virion RNA polymerase and is also required to initiate transcription from specific promoter sites on double-stranded DNA (15). In addition, the DNA replication protein gp45 of bacteriophage T4 has also been demonstrated to be directly required for late gene expression (16). These three T4-encoded DNA polymerase accessory proteins stimulate the opening of T4 late promoters, and the activation of late promoters in vivo by these three proteins is effected by moving replication forks acting as mobile enhancers (16, 17). Thus, several DNA replication proteins appear to be necessary for stimulation of late viral gene expression.

Our current working model is that at late times after infection, wt ICP8 binds to newly synthesized double-stranded progeny DNA and holds it in a form on which late transcription is optimal. This could be due to ICP8 binding to ssDNA regions on progeny DNA and keeping the promoter regions open for transcription. Alternatively, ICP8 might bind to specific DNA structures in late promoters, or the specificity of stimulation of late gene expression might be achieved by the interactions between ICP8 and other viral or cellular proteins needed to recognize late gene promoters. In addition to altering the structure of viral chromatin, the binding of ICP8 to progeny DNA may target the DNA to nuclear sites such as replication compartments where late transcription can occur. The mutant form of ICP8 expressed by the inhibitory cell line V2.6 may have lost its ability to interact with other viral or cellular proteins, but it still retains its ability to compete with wt ICP8 for DNA binding. Thus, the d105 protein would behave in a *trans*-dominant defective manner.

ACKNOWLEDGMENTS

We thank D. Coen for PP5 antipolymerase serum and E. Sinn and R. Roeder for PRV.

This work was supported by Public Health Service grant CA26345 from the National Cancer Institute.

REFERENCES

1. **Bayliss, G. J., H. S. Marsden, and J. Hay.** 1975. Herpes simplex virus protein: DNA-binding proteins in infected cells and in the virus structure. Virology **68**:124–134.

2. **Challberg, M. D.** 1986. A method for identifying the viral genes required for herpesvirus DNA replication. Proc. Natl. Acad. Sci. USA **83**:9094–9098.
3. **Chang, L.-S., and T. Shenk.** 1990. The adenovirus DNA-binding protein stimulates the rate of transcription directed by adenovirus and adeno-associated virus promoters. J. Virol. **64**:2103–2109.
4. **Conley, A. J., D. M. Knipe, P. C. Jones, and B. Roizman.** 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of γ polypeptides. J. Virol. **37**:191–206.
5. **de Bruyn Kops, A., and D. M. Knipe.** 1988. Formation of DNA replication structures in herpes virus-induced cells requires a viral DNA binding protein. Cell **55**:857–868.
6. **DeLuca, N. A., A. McCarthy, and P. A. Schaffer.** 1985. Isolation and characterization of deletion mutants of HSV-1 in the gene encoding the immediate-early regulatory protein ICP4. J. Virol. **56**:558–570.
7. **Friedman, A. D., S. J. Triezenberg, and S. L. McKnight.** 1988. Expression of a truncated viral trans-activator selectively impedes lytic infection by its cognate virus. Nature (London) **335**:452–454.
8. **Frink, R. J., R. Eisenberg, G. Cohen, and E. K. Wagner.** 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. J. Virol. **45**:634–647.
9. **Gao, M., J. Bouchey, K. Curtin, and D. M. Knipe.** 1988. Genetic identification of a portion of the herpes simplex virus ICP8 protein required for DNA-binding. Virology **163**:319–329.
9a. **Gao, M., and D. Knipe.** Unpublished data.
9b. **Gao, M., and D. Knipe.** Submitted for publication.
10. **Gao, M., and D. M. Knipe.** 1989. Genetic evidence for multiple nuclear functions of the herpes simplex virus virus ICP8 DNA-binding protein. J. Virol. **63**:5258–5267.
11. **Gauss, P., K. B. Krassa, D. S. McPheeters, M. A. Nelson, and L. Gold.** 1987. Zinc(II) and the single-stranded DNA binding protein of bacteriophage T4. Proc. Natl. Acad. Sci. USA **84**:8515–8519.
12. **Godowski, P. J., and D. M. Knipe.** 1986. Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. USA **83**:256–260.
13. **Goldin, A. L., R. M. Sandri-Goldin, M. Levine, and J. C. Glorioso.** 1981. Cloning of herpes simplex virus type 1 sequences representing the whole genome. J. Virol. **38**:50–58.
14. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
15. **Haynes, L. L., and L. B. Rothman-Denes.** 1985. N4 virion RNA polymerase sites of transcription initiation. Cell **41**:597–605.
16. **Herendeen, D. R., G. A. Kassavetis, J. Barry, B. M. Alberts, and E. P. Geiduschek.** 1989. Enhancement of bacteriophage T4 late transcription by components of the T4 DNA replication apparatus. Science **245**:952–958.
17. **Herendeen, D. R., K. P. Williams, G. A. Kassavetis, and E. P. Geiduschek.** 1990. An RNA polymerase-binding protein that is required for communication between an enhancer and a promoter. Science **248**:573–578.
18. **Herskowitz, I.** 1987. Functional inactivation of genes by dominant negative mutations. Nature (London) **329**:219–222.
19. **Holland, L. E., K. P. Anderson, C. Shipman, and E. K. Wagner.** 1980. Viral DNA synthesis is required for efficient expression of specific herpes simplex virus type 1 mRNA. Virology **101**:10–24.
20. **Holland, L. E., R. M. Sandri-Goldin, A. L. Goldin, J. C. Glorioso, and M. Levine.** 1984. Transcriptional and genetic analyses of the herpes simplex virus type 1 genome: coordinates 0.29 to 0.45. J. Virol. **49**:947–959.
21. **Homa, F. L., J. C. Glorioso, and M. Levine.** 1988. A specific 15-bp TATA box promoter element is required for expression of a herpes simplex virus type 1 late gene. Genes Dev. **2**:40–53.
22. **Hope, I. A., and K. Struhl.** 1986. Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell **46**:885–894.
23. **Johnson, P. A., C. MacLean, H. S. Marsden, R. G. Dalziel, and R. D. Everett.** 1986. The product of gene US11 of herpes simplex type 1 is expressed as a true late gene. J. Gen. Virol. **67**:871–873.
24. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39**:499–509.
25. **Knipe, D. M., M. P. Quinlan, and A. E. Spang.** 1982. Characterization of two conformational forms of the major DNA-binding protein encoded by herpes simplex virus 1. J. Virol. **44**:736–741.
26. **Knipe, D. M., and A. E. Spang.** 1982. Definition of a series of stages in the association of two herpesvirus proteins with the cell nucleus. J. Virol. **43**:314–324.
27. **Lee, C. K., and D. M. Knipe.** 1983. Thermolabile in vivo DNA-binding activity associated with a protein encoded by mutants of herpes simplex virus type 1. J. Virol. **46**:909–919.
28. **Lee, C. K., and D. M. Knipe.** 1985. An immunoassay for the study of DNA-binding activities of herpes simplex virus protein ICP8. J. Virol. **54**:731–738.
29. **Leinbach, S. S., and J. F. Casto.** 1983. Identification and characterization of deoxyribonucleoprotein complex containing the major DNA-binding protein of herpes simplex virus type 1. Virology **131**:274–286.
30. **Leinbach, S. S., and L. S. Heath.** 1988. A carboxyl-terminal peptide of the DNA-binding protein ICP8 of herpes simplex virus contains a single-stranded DNA-binding site. Virology **166**:10–16.
31. **Littler, E., D. Purifoy, A. Minson, and K. L. Powell.** 1983. Herpes simplex virus nonstructural proteins. III. Function of the major DNA-binding protein. J. Gen. Virol. **64**:983–995.
32. **McKnight, J. L. C., T. M. Kristie, S. Silver, P. E. Pellett, P. Mavromara-Nazos, G. Campadelli-Fiume, M. Arsenakis, and B. Roizman.** 1986. Regulation of herpes simplex virus 1 gene expression: the effect of genomic environments and its implications for model systems. Cancer Cells **4**:163–173.
33. **Orberg, P. K., and P. A. Schaffer.** 1987. Expression of herpes simplex virus type 1 major DNA-binding protein, ICP8, in transformed cell lines: complementation of deletion mutants and inhibition of wild-type virus. J. Virol. **61**:1136–1146.
34. **Powell, K. L., and D. J. M. Purifoy.** 1976. DNA-binding proteins of cells infected by herpes simplex virus type 1 and type 2. Intervirology **7**:225–239.
35. **Quinlan, M. P., L. B. Chen, and D. M. Knipe.** 1984. The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell **36**:857–868.
36. **Quinlan, M. P., and D. M. Knipe.** 1983. Nuclear localization of herpesvirus protein: potential role for the cellular framework. Mol. Cell. Biol. **3**:315–324.
37. **Rice, S. A., and K. M. Knipe.** 1990. Genetic evidence for two distinct transactivation functions of the herpes simplex virus α protein ICP27. J. Virol. **64**:1704–1715.
38. **Rose, D. S. C., K. Shriver, D. S. Latchman, and N. B. LaThangue.** 1986. A filamentous distribution for the herpes simplex virus type 2-encoded major DNA binding protein. J. Gen. Virol. **67**:1315–1325.
39. **Ruyechan, W. T., A. Chytil, and C. M. Fisher.** 1986. In vitro characterization of a thermolabile herpes simplex virus DNA-binding protein. J. Virol. **59**:31–36.
40. **Ruyechan, W. T., and A. C. Weir.** 1984. Interaction with nucleic acids and stimulation of the viral DNA polymerase by the herpes simplex virus type 1 major DNA-binding protein. J. Virol. **52**:727–733.
41. **Shepard, A. A., P. Tolentino, and N. A. DeLuca.** 1990. *trans*-dominant inhibition of herpes simplex virus transcriptional regulatory protein ICP4 by heterodimer formation. J. Virol. **64**:3916–3926.
42. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.

43. **Wang, Y., and J. Hall.** 1990. Characterization of a major DNA-binding domain in the herpes simplex virus type 1 DNA-binding protein (ICP8). J. Virol. **64**:2082–2089.
44. **Weller, S. K., K. J. Lee, D. J. Sabourin, and P. A. Schaffer.** 1983. Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA-binding protein. J. Virol. **45**:354–366.
45. **Wu, C. A., N. J. Nelson, D. J. McGeoch, and M. D. Challberg.** 1988. Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. J. Virol. **62**:435–443.
46. **Yager, D. R., A. I. Marcy, and D. M. Coen.** 1990. Translational regulation of herpes simplex virus DNA polymerase. J. Virol. **64**:2217–2225.