Intragenic Complementation of Herpes Simplex Virus ICP8 DNA-Binding Protein Mutants

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The major DNA-binding protein, or infected-cell protein 8 (ICP8), of herpes simplex virus is required for viral DNA synthesis and normal regulation of viral gene expression. Previous genetic analysis has indicated that the carboxyl-terminal 28 residues are the only portion of ICP8 capable of acting independently as a nuclear localization signal. In this study, we constructed a mutant virus (η 11SV) in which the carboxyl-terminal 28 residues of ICP8 were replaced by the simian virus 40 large-T-antigen nuclear localization signal. The η 11SV ICP8 localized into the nucleus and bound to single-stranded DNA in vitro as tightly as wild-type ICP8 did but was defective for viral DNA synthesis and viral growth in Vero cells. Two mutant ICP8 proteins (TL4 and TL5) containing amino-terminal alterations could complement the η 11SV mutant but not ICP8 gene deletion mutants. Cell lines expressing TL4 and TL5 ICP8 were isolated, and in these cells, complementation of η 11SV was observed at the levels of both viral DNA replication and viral growth. Therefore, complementation between η 11SV ICP8 and TL4 or TL5 ICP8 reconstituted wild-type ICP8 functions. Our results demonstrate that (i) the carboxyl-terminal 28 residues of ICP8 are required for a function(s) involved in viral DNA replication, (ii) this function can be supplied in *trans* by another mutant ICP8, and (iii) ICP8 has multiple domains possessing different functions, and at least some of these functions can complement in *trans*.

The major DNA-binding protein of herpes simplex virus (HSV), infected-cell polypeptide 8, or ICP8, is one of seven virus-encoded proteins required for replication of the HSV genome (3, 4, 20, 24, 39, 40). ICP8, a 130-kDa polypeptide, is expressed as a β or delayed-early-gene product (20, 24). From studies of the phenotypes of virus strains containing temperature-sensitive (4, 39), nonsense, deletion, or site-specific mutations (5, 8–10, 12, 23) in the ICP8 gene, ICP8 has been shown to have the ability to (i) bind to DNA in vitro and in vivo (1, 16, 18, 25, 31), (ii) localize to the cell nucleus (10), (iii) down-regulate the expression of viral genes from parental genomes (12), (iv) stimulate late-gene expression from progeny templates (9), and (v) promote organization of nuclear structures involved in viral and cellular DNA replication proteins (5).

ICP8 binds preferentially to single-stranded DNA (ssDNA) in vitro (1, 16, 18, 25, 32, 33), and the interaction of ICP8 with ssDNA is cooperative (18, 22, 31). On the basis of studies of ICP8 molecules expressed by mutant viruses, of in vitro transcription-translation products of ICP8, and of partial protease digestion of purified ICP8, the ICP8 sequences required for ssDNA-binding activity have been mapped between residues 564 and 849 (8, 19, 38). Genetic evidence indicates that ICP8 specifies other nuclear functions in addition to DNA binding (8, 9).

The intranuclear localization of ICP8 to specific structures appears to be required for ICP8 to exert its nuclear functions. In the absence of viral DNA replication, ICP8 localizes to nuclear framework-associated structures called pre-replicative sites (5, 26, 27). These structures have been hypothesized to be the sites of viral and cellular protein

complexes poised to initiate viral DNA synthesis. As viral DNA replication occurs, progeny viral DNA-ICP8 complexes and additional viral and cellular proteins migrate to large globular replication compartments (5, 26). HSV polymerase colocalizes with ICP8 in both replicative sites and replication compartments. However, localization of HSV polymerase to these structures is dependent on functional ICP8 molecules. Therefore, ICP8 is required for the assembly of prereplicative sites (5).

Genetic analysis of ICP8 gene mutants and ICP8-pyruvate kinase fusion proteins demonstrated that the carboxyl-terminal 28 residues of ICP8 constitute the only portion of ICP8 that can function alone as a nuclear localization signal (NLS) (10). The carboxyl-terminal 28 residues of ICP8 are not needed for ssDNA-binding activity (10).

To study the functional domains of ICP8 further, we have constructed a mutant virus (η 11SV) in which the carboxyl-terminal 28 residues of ICP8 were replaced by the nuclear localization signal of simian virus 40 (SV40) T antigen. Despite the fact that η 11SV was unable to promote viral DNA synthesis, η 11SV ICP8 still retains some physical and functional properties of wild-type (wt) ICP8. This paper reports that the carboxyl-terminal 28 residues of ICP8 are required for a function(s) involved in viral DNA replication and that the function(s) missing in η 11SV can be supplied in *trans* by another mutant ICP8 molecule.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown and maintained as described previously (14). The growth medium for the neomycin-resistant S-2 cell line included 200 μ g of the antibiotic G418 per ml during the first passage of the cells after thawing or 500 μ g of G418 per ml of medium every five passages.

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FIG. 1. Locations of ICP8 gene mutations used in this study. The location of the ICP8 coding region on the HSV genome is shown at the top. Restriction sites shown are *Bam*HI and *Sal*I. The hatched bar in mutant virus *n11SV* represents the SV40 T-antigen NLS (see Materials and Methods). L, long component; S, short component of the viral genome.

TL4 and TL5 cell lines were isolated by procedures described previously (6, 8). Vero cells were transformed with the plasmid TL4 or TL5 and pSVneo (Fig. 1) in the presence of the antibiotic G418 (36). Drug-resistant colonies were isolated, grown into cultures, and screened by indirect immunofluorescence (27) with anti-ICP8 monoclonal antibody 10E-3 (30) for the ability to express mutant forms of ICP8 upon infection by *n10* mutant virus. The 10E-3 antibody recognizes TL4 ICP8 and TL5 ICP8 but not *n10* ICP8 (10).

HSV type 1 wt strain KOS1.1 was propagated and assayed as described previously (16, 17). Mutant viruses were grown in ICP8-expressing S-2 cells (8).

Plasmids. Plasmids p8B-S, pICP8, pSV8, pSV8.2, and pSVd101 as well as the nucleotide-numbering system for the ICP8 gene were described previously (7-9). The plasmid p8B-S contains the ICP8 gene, including its own promoter. The plasmid pICP8 contains the ICP8 gene coding sequences without its promoter. The plasmid pn9 was generated by linearization of the plasmid pICP8 (which was achieved by partial digestion with *Sma*I) and subsequent insertion of a 14-nucleotide *Xba*I linker (New England BioLabs, Inc., Beverly, Mass.) containing stop codons in all three reading frames. Thus, pn9 encodes the first 833 amino acid residues of ICP8. The plasmid pSV8 was constructed by inserting a 5.5-kbp *Sma*I-*Sac*I fragment (map units 0.374 to 0.409) downstream of the SV40 early promoter. The plasmid pSV8.2 was derived from pSV8 by deleting the polylinker region and the *Sac*I-*Bgl*III fragment of the pyruvate kinase

gene. The plasmid pSVd101 was generated as described previously (8); it lacks codons for residues 17 to 563 but has an insertion of one Arg codon encoded by the *Bgl*III linker sequence. The plasmids p8S/3583 and p8E/3583 were derived from pICP8 by deleting the *Nae*I (nucleotide 3583)-*Sac*I (nucleotide 6075) and the *Sma*I (nucleotide 436)-*Nae*I (nucleotide 3583) fragments, respectively. The plasmids TL4, TL5, and TL16 were constructed as described previously (37). They contain eight, six, or two codon insertions after residues 204, 207, or 732, respectively, of ICP8.

The plasmid p8N/12B-1 was constructed by inserting a 12-nucleotide *Bgl*III linker at an *Nae*I site (nucleotide 4111). The plasmid pSVn11 was generated by linearization of the plasmid p8N/12B-1 and subsequent insertion of an *Xba*I linker containing stop codons in all three reading frames. Thus, pSVn11 encodes the first 1,169 amino acid residues of ICP8 as well as 4 additional amino acids (Thr-Ser-Leu-Asp) encoded by the *Xba*I linker sequence. The plasmid pSVn11SV was generated by linearization of the plasmid pSVn11 with *Xba*I and subsequent insertion of a 27-nucleotide linker (5'-CTAGCACCAAAAAAGAAGAGAAAGGTA-3') encoding the SV40 large-T-antigen NLS. The correct orientation of the insertion was confirmed by DNA sequencing. Thus, pSVn11SV encodes the first 1,169 amino acid residues of ICP8 as well as the additional amino acids Thr-Ser-Leu-Ala-Pro-Lys-Lys-Lys-Arg-Lys-Val-Leu-Asp. The underlined amino acid sequences represent the SV40 NLS (13).

Isolation of *n11* and *n11SV* mutant viruses. The strategy for isolation of *n11* and *n11SV* mutant viruses was as described previously (8). The HD-2 mutant virus contains an in-frame insertion of the *lacZ* gene in the ICP8 coding sequence and forms blue plaques in the presence of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). This virus served as a recipient in marker transfer experiments to introduce the *n9*, *n11*, and *n11SV* mutations in the ICP8 gene into the viral genome.

Indirect immunofluorescence. Indirect immunofluorescence was performed as described previously (27) with anti-ICP8 monoclonal antibody 10E-3 or 793 (1:50 dilution; 30) and rhodamine-conjugated goat anti-mouse antibody (1:100 dilution).

Marker rescue and complementation experiments. Marker rescue experiments were performed by cotransfection of S-2 cells with 1.0 μ g each of infectious mutant virus DNA and linearized wt ICP8 gene plasmid DNA (Fig. 1) as previously described (15). Transfected cultures were incubated at 37°C for 2 to 3 days, and progeny virus was assayed in S-2 and Vero cells.

Complementation of the growth of *n9*, *d301*, and *n11SV* mutant viruses by prior transfection of the plasmid TL4, TL5, TL16, or pSVd101 was carried out as described previously (28).

Analysis of viral proteins and viral DNA replication. Vero cell monolayer cultures were infected with KOS1.1, *n11*, *n11R*, *n11SV*, or *n11SVR* and labeled with [³⁵S]methionine for 30 min before harvest at 9 h postinfection (hpi). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of infected-cell lysates was performed as described previously (17). After electrophoresis, the gels were fixed, dried, and exposed to Kodak SB5 film.

Analysis of viral DNA amplification during the course of infection was performed as described previously (9, 29). The probe used was the plasmid pBR3441 (21).

FIG. 2. Subcellular localization of ICP8 encoded by mutant viruses *n11* and *n11SV*. Vero cells were infected with KOS1.1 or ICP8 mutant viruses. At 5 hpi, the cells were fixed, permeabilized, and incubated with anti-ICP8 monoclonal antibody 793 and rhodamine-conjugated goat anti-mouse immunoglobulin. Immunofluorescence micrographs are of KOS1.1-infected cells (A), KOS1.1-infected cells in the presence of PAA (B), *n11*-infected cells (C), and *n11SV*-infected cells (D).

ssDNA cellulose chromatography. ssDNA cellulose chromatography of infected-cell extracts was performed as described elsewhere (8, 14).

RESULTS

Phenotype of mutant viruses *n11* and *n11SV*. Our previous genetic analysis demonstrated that the mutant *n10* ICP8, which lacks the carboxyl-terminal 36 residues and localizes to the cytoplasm, does not support viral growth in Vero cells even though it binds ssDNA in vitro as tightly as wt ICP8 does (8). Further study indicated that the carboxyl-terminal 28 residues are the only portion of ICP8 capable of acting independently as an NLS (10). Thus, the failure of mutant *n10* ICP8 to support viral DNA synthesis may have been solely a consequence of its inability to localize to the cell nucleus. If this were the case, replacement of ICP8 NLS with another known NLS should restore the full functionality of ICP8. Therefore, we constructed two ICP8 mutant viruses. One, *n11*, lacks the carboxyl-terminal 28 residues of ICP8, and the other (*n11SV*) has the SV40 T-antigen NLS (NH₂-Pro-Lys-Lys-Lys-Arg-Lys-Val-COOH) replacing the carboxyl-terminal 28 residues of ICP8 (Fig. 1).

(i) **Subcellular locations of *n11* ICP8 and *n11SV* ICP8.** We first used immunofluorescence microscopy to examine the intracellular localization of the *n11* and *n11SV* ICP8 polypeptides when they were expressed from plasmids in transfected Vero cells. The *n11* ICP8 polypeptide remained within the cytoplasm, while the *n11SV* ICP8 polypeptide localized in the nucleus (11). We next determined the intracellular distribution of mutant ICP8 molecules when they were

expressed from recombinant viruses (Fig. 2). At 5 hpi, infected Vero cells were fixed and processed for immunofluorescence. Cells infected with wt virus showed that ICP8 localized in the nucleus in characteristic replication compartments in the absence of phosphonoacetic acid (PAA) (Fig. 2A) and at prereplicative sites in the presence of PAA (Fig. 2B; 26, 27). As expected, *n11* ICP8 localized in the cytoplasm (Fig. 2C) because of lack of the carboxyl-terminal 28 residues. In most cells, the *n11SV* ICP8 localized throughout the cell nucleus (Fig. 2D) and did not appear to localize specifically either in replication compartments or at prereplicative sites. Therefore, replacement of the ICP8 NLS with the SV40 T-antigen NLS restored the capacity of mutant ICP8 to localize into the nucleus but not to the same sites as wt ICP8.

(ii) **Growth properties of *n11* and *n11SV*.** Plaque assays were performed to determine whether the mutant viruses were able to grow in Vero cells. Both mutant viruses failed to grow in Vero cells but grew to titers near those of wt on the ICP8-expressing S-2 cell line (Table 1). Thus, although replacement of the ICP8 NLS with the SV40 T-antigen NLS restored the ability of ICP8 to localize to the nucleus, the substitution did not restore complete functionality of ICP8.

(iii) **Marker rescue of mutant viruses *n11* and *n11SV*.** To verify that the phenotypes of mutant viruses *n11* and *n11SV* were due to mutations in the ICP8 gene, we performed marker rescue experiments (Fig. 3). Homologous recombination between *n11* DNA or *n11SV* DNA and a fragment containing a wt ICP8 DNA sequence (p8S/3583) resulted in viral progeny possessing the ability to grow in Vero cells, thereby rescuing the growth deficiencies of *n11* and *n11SV*.

TABLE 1. Growth of *n11* and *n11SV*^a

Virus	Titer (PFU/ml)	
	S-2 cells	Vero cells
KOS1.1	1.7×10^9	1.8×10^9
<i>n11</i>	2.3×10^9	2.0×10^3
<i>n11SV</i>	2.8×10^8	2.0×10^3

^a Cultures of ICP8-expressing cells (S-2) and Vero cells were infected with each virus and incubated at 37°C. Plaques were counted at 3 days.

The high percentage of rescue (1.5 to 8.3%) indicated that rescue was likely due to a recombinational event involving the transfected plasmids and not additional events. The titers of rescued viruses *n11R* and *n11SVR* were approximately 5 orders of magnitude greater than those of the parental viruses *n11* and *n11SV* on Vero cells and roughly equal to their titers in S-2 cells (compare Tables 1 and 2). We conclude that the phenotype of mutant viruses *n11* and *n11SV* was a result of mutations within the ICP8 gene, most likely the engineered mutations.

(iv) **DNA-binding properties of mutant viruses *n11* and *n11SV*.** To attempt to define the nature of the defective functions of the mutant ICP8 molecules, we examined the ssDNA-binding properties of mutant *n11* ICP8 and *n11SV* ICP8. Vero cells were infected with *n11* or *n11SV*, the extract from infected cells was passed over an ssDNA cellulose column, and ICP8 was eluted stepwise with buffer containing increasing salt concentrations. Polypeptides in the various fractions were analyzed by SDS-PAGE (Fig. 4). The salt elution patterns for *n11* ICP8 (Fig. 4B) and *n11SV*

FIG. 3. Marker rescue of mutant viruses *n11* and *n11SV*. Black bars indicate coding regions of ICP8 genes. Relative nucleotide positions of the recombinant plasmids are as follows: p8E/3583, nucleotides 432 to 3583; p8S/3583, nucleotides 3583 to approximately 5900; and pSV8.2, nucleotides 432 to approximately 5900. Percentage of wt recombinants = (virus titer in Vero cells/virus titer in S-2 cells) $\times$ 100%.

TABLE 2. Growth properties of *n11R* and *n11SVR*^a

Virus	Titer (10^8 PFU/ml)	
	S-2 cells	Vero cells
<i>n11R</i>	3.8	2.8
<i>n11SVR</i>	1.3	2.0

^a Cultures of ICP8-expressing cells (S-2) and Vero cells were infected with each virus and incubated at 37°C. Plaques were counted at 3 days.

(Fig. 4C) ICP8 were very similar to that for wt ICP8 (Fig. 4A), and the majority of wt or mutant ICP8 was eluted with 0.5 M NaCl (Fig. 4, lane 12). Thus, *n11* and *n11SV* ICP8 bound to ssDNA in vitro as tightly as wt ICP8 did. Therefore, the inability of the *n11SV* mutant to replicate its DNA in Vero cells was likely not a result of an inability to bind to ssDNA.

Intragenic complementation of ICP8 mutants. Despite its failure to promote viral DNA synthesis, the mutant *n11SV* ICP8 retained several of the physical and functional properties of wt ICP8: it localized to the nucleus, bound to ssDNA in vitro, and was recognized by the 39S anti-ICP8 monoclonal antibody (not shown), recognition by which is very sensitive to conformational changes in ICP8. These results suggest that the mutant virus *n11SV* may be altered in only a single functional domain of ICP8 which is involved in viral DNA replication. We therefore screened a series of ICP8 linker insertion mutant plasmids to determine whether they could complement the *n11SV* defect by providing certain functions of ICP8 in *trans*.

(i) **Intragenic complementation between mutant virus *n11SV* and mutant plasmid TL4 or TL5.** We performed complementation experiments to determine whether expression of mutant ICP8 polypeptides from linker insertion mutant plasmids could complement the growth of *n11SV* mutant virus in Vero cells. All of the linker insertion mutant forms of ICP8 used in this study were unable to complement the ICP8 gene deletion mutant virus *d301* and appeared to localize into the nucleus in transfected cells (37). The plasmid pSVd101 containing a large deletion in the ICP8 gene was used as a negative control, and TL16 was used a linker insertion mutant control (Table 3). Neither of these mutants complemented the growth of either *d301* or *n11SV*. In contrast, cotransfection of the wt ICP8 plasmid (p8B-S) increased the yields of mutant virus *d301* 4,400- to 10,000-fold and of *n11SV* 1,500- to 3,000-fold. Two linker insertion mutants, TL4 and TL5, which could complement growth of the mutant virus *n11SV* in Vero cells, were identified (Table 3). Neither of these mutant plasmids was able to complement *d301*, but both plasmids complemented *n11SV* significantly. For plasmid TL4, complementation ranged from 7 to 23% of the level of wt ICP8 plasmid (Table 3). The plasmids TL4 and TL5 contain 8- and 6-amino-acid insertions after residues 204 and 207 of ICP8, respectively (Fig. 1). Because the insertions in TL4 ICP8 and TL5 ICP8 are only three residues apart, the same functional domain(s) of ICP8 may be affected.

By looking for the ability of mutants to form plaques on Vero cells (Table 4), we also examined infected-cell lysates from the complementation experiments shown in Table 3 for wt recombinants. The plasmid pSVd101 was used as a negative control because deletions in the ICP8 genes of mutants pSVd101 and *d301* are overlapping; thus, no recombination between these two mutants should occur. Recom-

FIG. 4. ICP8 binding to ssDNA-cellulose. Vero cells were infected with KOS1.1 (A), *n11* (B), or *n11SV* (C) in the presence of PAA and labeled with [³⁵S]methionine from 4 to 6 hpi. Various protein fractions resolved on ssDNA cellulose columns were subjected to SDS-PAGE. Lanes: 1, pellet from high-salt DNase extraction; 2, pellet after dialysis; 3, extract put on ssDNA column; 4 through 10, flowthrough and wash; 11, 0.3 M NaCl eluate; 12, 0.5 M NaCl eluate; 13, 1.0 M NaCl eluate. Positions of ICP8 are indicated at the right.

bination between the transfected wt ICP8 plasmid and infected mutant viruses did occur at low frequency. However, wt virus frequencies were almost the same for *d301* (where no complementation occurred) and for TL4 or TL5 (where

TABLE 4. Recombination between ICP8 mutants^a

Plasmid transfected	Virus superinfected	Titer (PFU/ml)	
		Expt 1	Expt 2
pSVd101	<i>d301</i>	ND	ND
pSVd101	<i>n11SV</i>	10	70
TL16	<i>d301</i>	ND	ND
TL16	<i>n11SV</i>	ND	50
p8B-S (wt)	<i>d301</i>	590	300
p8B-S (wt)	<i>n11SV</i>	1,300	800
TL4	<i>d301</i>	40	ND
TL4	<i>n11SV</i>	10	60
TL5	<i>d301</i>	10	ND
TL5	<i>n11SV</i>	40	80

^a Infected-cell lysates from the experiments whose results are shown in Table 3 were assayed for wt recombinants by assaying for the ability to form plaques on Vero cells. ND = titer of <10 PFU/ml.

complementation occurred). Thus, the enhancement of growth of mutant virus *n11SV* by TL4 or TL5 was not due to recombination. We conclude that the function(s) required for the mutant virus *n11SV* DNA replication could be supplied in *trans* by partially functional TL4 or TL5 ICP8.

(ii) **Phenotype of TL4 and TL5 mutants.** To study the mechanism of intragenic complementation by ICP8 mutants, we examined the properties of the TL4 and TL5 ICP8 molecules. Our results indicated that both TL4 and TL5 mutant gene products exhibited a *trans*-dominant phenotype which prevented the introduction of these mutations into virus. We therefore isolated two cell lines, named TL4.6 and TL5.2, which expressed TL4 ICP8 and TL5 ICP8, respectively, upon HSV infection (11).

Mutant ICP4 molecules containing alterations in the trans-activation domain can form a heterodimer with another mutant ICP4 molecule containing an alteration in the DNA-binding domain and can complement each other in *trans* (34, 35). To test whether this interpretation could apply to the mutant ICP8 also, we examined the DNA-binding ability of TL4 ICP8 expressed from the cell line. We concluded that TL4 ICP8, like *n11SV* ICP8, binds ssDNA in vitro as tightly as wt ICP8 does (11). These results suggested that the mechanism of intragenic complementation between *n11SV* and TL4 ICP8 mutants is different from that of the ICP4 mutants, because both mutant ICP8 molecules can bind DNA.

TABLE 3. Intragenic complementation between ICP8 mutants^a

Plasmid transfected	Virus superinfected	Virus yield (PFU/ml) ^b		Complementation index ^c	
		Expt 1	Expt 2	Expt 1	Expt 2
pSVd101	<i>d301</i>	9.0×10^1	3.4×10^2	1.0	1.0
pSVd101	<i>n11SV</i>	3.0×10^2	1.1×10^3	1.0	1.0
TL16	<i>d301</i>	1.3×10^2	6.0×10^2	1.4	2.0
TL16	<i>n11SV</i>	2.3×10^2	1.1×10^3	0.8	1.0
p8B-S (wt)	<i>d301</i>	9.2×10^5	1.5×10^6	10,000	4,412
p8B-S (wt)	<i>n11SV</i>	9.0×10^5	1.6×10^6	3,000	1,455
TL4	<i>d301</i>	1.1×10^2	2.8×10^3	1.2	8
TL4	<i>n11SV</i>	6.4×10^4	3.6×10^5	210	327
TL5	<i>d301</i>	6.2×10^2	3.3×10^3	6.9	10
TL5	<i>n11SV</i>	1.2×10^4	1.8×10^5	40	164

^a Vero cells were transfected with the plasmids indicated. At 20 h posttransfection, cells were superinfected with 3 PFU of either *d301* or *n11SV* per cell and incubated for a further 24 h at 37°C before being harvested.

^b Determined by plaque assay on S-2 cells.

^c Expressed as virus yield relative to yield after transfection with pSVd101 DNA.

FIG. 5. Formation of replication compartments in *n11SV*-infected TL4.6 cells. Vero and TL4.6 cells, respectively, were infected with *n9* (A and B), *n11SV* (C and D), or KOS1.1 (E and F). At 5 hpi, the cells were fixed, permeabilized, and incubated with monoclonal antibody 10E-3 and rhodamine-conjugated goat anti-mouse immunoglobulin.

(iii) **Formation of replication compartments in *n11SV*-infected TL4 and TL5 cells.** Because the plasmid TL4 resulted in more-significant complementation of the growth of *n11SV* in Vero cells than TL5 did (Table 3), we used TL4.6 cells for most of our further studies. To examine the localization properties of TL4 ICP8, we infected Vero and TL4.6 cells with *n9*, *n11SV*, or wt KOS1.1 for 5 h and then processed the cells for immunofluorescence by using monoclonal antibody 10E-3 as the primary antibody (Fig. 5). This antibody recognizes the carboxyl-terminal 28 residues of ICP8 (10) and therefore recognizes only the ICP8 expressed from TL4.6 cells (Fig. 5B and D) and not that expressed by *n9* (Fig. 5A) or *n11SV* (Fig. 5C). The antibody, of course, also recognizes wt ICP8. KOS1.1-infected Vero cells were used for controls of the antibody and formation of replication compartments. Replication compartments were observed in *n11SV*-infected

(Fig. 5D) but not *n9*-infected (Fig. 5B) TL4.6 cells. Thus, we conclude that TL4 ICP8 could localize into replication compartments in the presence of *n11SV* ICP8.

(iv) **DNA replication in *n11SV*-infected TL4.6 cells.** To determine whether the mutant virus *n11SV* replicates its DNA in TL4.6 cells, we examined viral DNA accumulation in these cells. Total viral DNA was isolated from mock-, wt-virus-, *d301*-, or *n11SV*-infected Vero cells, wt-ICP8-expressing S-2 cell line, and TL4.6 cells at 1 or 16 hpi. wt-virus-infected Vero cells in the presence or absence of PAA were used as positive and negative controls of viral DNA synthesis, respectively (Fig. 6). As expected, mutant *d301*-infected TL4.6 cells, like *d301*-infected Vero cells, showed no amplification of viral DNA during the course of infection, indicating that the TL4 ICP8 was unable to promote synthesis of mutant viral DNA. In contrast, *n11SV*-

FIG. 6. Mutant *n11SV* DNA replication in TL4.6 cells. Vero, wt-ICP8-expressing S-2, and TL4.6 cells were infected with KOS1.1, *d301*, or *n11SV*. Total cellular DNA was prepared immediately after viral absorption (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.

infected TL4 cells showed substantial amplification of viral DNA during the infection. To quantitate these results, the amount of radioactive probe hybridized to each slot was measured, and the relative amounts of replicated HSV type 1 DNA in each sample were determined. In this particular experiment, the amount of *n11SV* viral DNA synthesized in TL4 cells was about 42% of the level of DNA synthesized in wt virus-infected Vero cells. We conclude that the function(s) which is required for *n11SV* to replicate its DNA can be supplied *trans* by the mutant TL4 ICP8.

(v) **Viral gene expression in *n11SV*-infected TL4 cells.** ICP8 is not only required for viral DNA replication but is also involved in stimulation of late-gene expression (9). We therefore examined viral late-gene expression in *n11SV*-infected TL4.6 cells. Vero cells, wt-ICP8-expressing S-2 cells, and TL4.6 cells were infected with KOS, *d301*, or *n11SV* and labeled with [^{35}S]methionine from 9.5 to 10 hpi, and labeled proteins were analyzed by SDS-PAGE (Fig. 7). Vero cells infected with wt virus in the presence or absence of PAA were used as positive and negative controls for the expression of $\gamma 2$ genes. When examined in Vero cells, the *n11SV* mutation resulted in a phenotype similar to that of the negative control with respect to late-gene expression. Detectable levels of $\gamma 2$ viral polypeptides (such as ICP1-2 and ICP15) were not expressed. Similar results were observed in *d301*-infected Vero cells. These results demonstrated that both *n11SV* ICP8 and TL4 ICP8 are unable to exert wt ICP8 functions for expression of late genes. However, the defects for mutant *n11SV* viral late-protein synthesis were corrected by expression of the TL4 ICP8 in TL4.6 cells. The pattern of viral polypeptides synthesized in *n11SV*-infected TL4.6 cells was indistinguishable from that observed in *n11SV*-infected wt-ICP8-expressing S-2 cells or in wt-infected Vero cells (Fig. 6).

To further define the level at which late-gene expression was restored, we performed Northern (RNA) blot analysis to measure the steady-state level of $\gamma 2$ glycoprotein C mRNA. These results indicated that the intragenic complementation

between *n11SV* ICP8 and TL4 ICP8 completely restored viral late mRNA accumulation (results not shown), although the complementation restored viral DNA replication to only 42% of the normal level (Fig. 6).

FIG. 7. Protein synthesis in *n11SV*-infected TL4.6 cells. Vero, wt-ICP8-expressing S-2, or TL4.6 cells were infected with KOS1.1, *d301*, or *n11SV*. At 8.5 hpi, cells were labeled for 30 min with [^{35}S] methionine and then harvested. Equal fractions of each cell lysate were subjected to SDS-PAGE. An autoradiograph of the resulting gel is shown. At the right of each panel are the migration positions of several HSV type 1 proteins. Lane M, mock-infected cell lysate.

TABLE 5. Growth of wt and mutant viruses in different cell lines^a

Virus and cell type	Yield (PFU/cell) at MOI of:	
	1.0	10.0
KOS1.1		
Vero	137	393
S-2	110	306
TL4.6	39	245
TL5.2	4	NT
d301		
Vero	0.0006	0.0079
S-2	275	361
TL4.6	0.02	0.33
TL5.2	0.008	NT
n11SV		
Vero	0.0003	0.0071
S-2	225	119
TL4.6	44	50
TL5.2	0.9	NT

^a Cells were infected with different viruses, incubated at 37°C for 24 h, and harvested. Titers of progeny viruses were determined in S-2 cells. NT, not tested.

(vi) **Growth of n11SV in TL4.6 and TL5.2 cells.** Because the plaques of n11SV in TL4.6 or TL5.2 cells were very small, we performed single-cycle growth experiments to examine the growth of n11SV in TL4.6 and TL5.2 cells. The yields of wt virus in TL4.6 and TL5.2 cells were 3- to 30-fold lower than those in Vero cells or in the wt-ICP8-expressing S-2 cells at a multiplicity of infection (MOI) of 1.0 (Table 5), suggesting that the TL4 and TL5 mutant gene products have some *trans*-dominant negative phenotype. The inhibition of growth of wt virus in TL4.6 cells was overcome at an MOI of 10. Thus, the *trans*-dominant effect appeared to be due to competition between wt ICP8 and TL4 ICP8. The yields of n11SV in TL4.6 cells were 7 to 32% of those of wt virus in Vero cells at an MOI of 1 and 10, respectively. Because only mutant virus n11SV but not d301 could grow in TL4.6 and TL5.2 cells, we conclude that the complementation between n11SV ICP8 and TL4 or TL5 ICP8 could reconstitute wt ICP8 activities.

DISCUSSION

Previous genetic and biochemical evidence suggests that the functions of the HSV DNA replication protein require different portions of the molecule: the carboxyl-terminal portion of the molecule is required for ssDNA-binding, nuclear localization, and stimulation of late-gene expression, and the amino-terminal portion of the molecule has an additional nuclear function(s) (8). In this study, we have demonstrated that these functions are discrete, because two partially functional ICP8 molecules can complement each other in *trans*.

Replacement of the ICP8 NLS with the SV40 T-antigen NLS restored the ability of ICP8 to localize into the nucleus, but the mutant virus n11SV exhibited a defect in viral DNA replication and late-gene expression, suggesting that the primary defect of the n11SV ICP8 is a failure to promote viral DNA synthesis or earlier events such as assembly of viral DNA replication complexes. The n11SV ICP8 defect can be overcome by supplying TL4 or TL5 ICP8 in *trans*. The functions defective in n11SV ICP8 may be carried out by the carboxyl terminus of ICP8 or other parts of the molecule; however, extensive conformational changes are

not apparent in n11SV ICP8, because it still reacts with anti-ICP8 monoclonal antibody 39S, which is very sensitive to conformational changes in ICP8 (7).

Because monoclonal antibody 10E-3 detects TL4 and TL5 ICP8 but not n11SV mutant ICP8, we do not know whether n11SV ICP8 localized alone or formed a complex with TL4 or TL5 ICP8 in the nucleus. However, both TL4 ICP8 and TL5 ICP8 localized to replication compartments after superinfection with the mutant virus n11SV but not after infection with n9, suggesting that these ICP8 molecules can localize to these sites only in the presence of n11SV ICP8. Moreover, because the monoclonal antibody recognized both wt and TL4 ICP8, we know that both ICP8 molecules localized into replication compartments after TL4.6 cells were infected with wt virus. These results suggest that n11SV ICP8 may also localize to replication compartments. Because the alteration in TL4 or TL5 ICP8, as in n11SV ICP8, is outside of the region required for ssDNA-binding, both mutant ICP8 molecules, as expected, bind to DNA. It is conceivable that the complementing polypeptides of TL4 or TL5 ICP8 and n11SV ICP8 do not interact with each other directly but that both bind DNA and that each molecule contributes some activities from different domains to achieve wt ICP8 functions.

An alternative mechanism for intragenic complementation is the formation of a dimer or multimers of n11SV ICP8 and TL4 or TL5 ICP8. It has been reported that ICP8 sediments in a glycerol gradient as a monomer (22), but the cooperative nature of the binding of ICP8 to ssDNA suggests that ICP8 may form polymers along ssDNA (22). In multimeric forms, the monomers may be weakly associated and assemble only upon interaction with DNA. Therefore, if multimerization is involved in the intragenic complementation of n11SV ICP8 and TL4 or TL5 ICP8, several models can be considered. First, the fact that n11SV ICP8 did not localize to prereplicative sites or replication compartments may indicate that the SV40 T-antigen NLS did not allow proper targeting of ICP8 to specific sites within the nucleus, where viral DNA replication is initiated. This suggests that the carboxyl-terminal 28 residues of ICP8 may also be involved in determining intranuclear localization and may interact with a component of the cell nucleus to initiate viral DNA replication. Therefore, it is possible that the n11SV ICP8 needs TL4 or TL5 ICP8 to form the multimers for targeting to replication machinery.

Second, we (8) and others (19) noted that the amino-terminal portion of ICP8 also contributes to the DNA-binding activity of the intact protein, although the DNA-binding region has been identified at the carboxyl-terminal half of ICP8 molecule (8, 19, 38). This contribution could be due to the intra- or intermolecular interactions of ICP8. Intermolecular interactions of this type could explain intragenic complementation. A second model is that TL4 or TL5 ICP8 and n11SV ICP8 bind DNA but that they can bind DNA cooperatively only when they interact with each other. If this is true, the domain which is required for formation of polymers must be disturbed in at least one mutant ICP8 molecule and the TL4 or TL5 ICP8 can form a heterodimer with n11SV ICP8 but cannot form polymers. This may explain why the efficiency of intragenic complementation of TL4 and n11SV ICP8 reached only 7 to 20% of that of wt: the two mutant ICP8 molecules can form only a dimer instead of polymers along with DNA. Further experiments to examine cooperative binding of n11SV ICP8 is needed to address this possibility.

A third model involves the interaction of ICP8 with other

viral and cellular proteins during viral DNA replication. Recently, Capson et al. demonstrated stepwise ATP-dependent assembly of bacteriophage T4 DNA replication proteins by using protein-DNA cross-linking (2). In this system, gene 32 ssDNA-binding protein but not DNA polymerase is required for assembly of DNA polymerase accessory proteins to form a complex on the primer-template molecule. After ATP hydrolysis, DNA polymerase binds to the complex to initiate DNA synthesis. It is possible that the alterations in both *n*11SV ICP8 and TL4 or TL5 ICP8 inactivate domains required for interactions with different DNA replication proteins. However, the complementing ICP8 molecules can interact with these replication proteins after they form multimers along DNA, resulting in the reconstitution of wt ICP8 activity.

In summary, our results demonstrate that ICP8 consists of separate domains which possess different functions and that some functions can complement each other in *trans*. Further analysis of intragenic complementation of ICP8 mutants may help define the interactions between ICP8 and other viral and cellular proteins which are involved in viral DNA replication.

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