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Mutants Defective in Herpes Simplex Virus Type 2 ICP4: Isolation and Preliminary Characterization

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Vero cells were biochemically transformed with the gene encoding ICP4 of herpes simplex virus type 2 (HSV-2). These cells were used as permissive hosts to isolate and propagate HSV-2 mutants defective in this gene. Two mutants, designated *hr*259 and *hr*79, were isolated. Neither mutant grew in nontransformed Vero cells, but both grew to near wild-type levels in HSV-2 ICP4-expressing cells. *hr*259 contains a deletion of about 0.6 kilobases which eliminates the mRNA start site of the ICP4 gene. *hr*79 contains a mutation which maps by marker rescue to the portion of the ICP4 gene encoding the carboxy-terminal half of the protein. Although *hr*259 failed to generate any detectable ICP4 mRNA in nontransformed Vero cells, *hr*79 encoded an ICP4 mRNA which is wild type with respect to size. In nontransformed Vero cells infected with *hr*259, only ICP0, ICP6, ICP22, and ICP27 were readily detectable. In the case of *hr*79, a truncated form of ICP4 appeared to be made in addition to ICP0, ICP6, ICP22, and ICP27. Both *hr*259 and *hr*79 grew efficiently on cell lines transformed with the ICP4 gene of HSV-1 as evidenced by plating efficiencies and single-burst experiments. Similarly, cells transformed with the ICP4 gene of HSV-2 served as efficient hosts for the growth of *d*120, HSV-1 ICP4 deletion mutant.

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) have many similarities. Their genomes are colinear (4), their transcriptional programs appear similar (7), and many of their proteins are functionally interchangeable (8). Despite these similarities, the two viruses exhibit significant differences. For example, they exhibit host range differences (12), HSV-2 shuts off host protein synthesis more rapidly than HSV-1 does (9), and HSV-2 reduces the cell surface expression of class 1 H-2 antigens to a greater extent (13). It is reasonable to expect that further analysis will reveal additional differences.

In this report, we describe our initial efforts to determine whether the corresponding immediate-early proteins of HSV-1 and HSV-2 exhibit differences with respect to function. The immediate-early proteins include ICP4, ICP0, ICP22, ICP27, and ICP47 and are operationally defined as products whose genes are transcribed in the absence of prior viral protein synthesis. A variety of HSV-1 mutants with mutations in these genes have been isolated and characterized (6, 14, 20, 22; W. Sacks, personal communication). As yet, no analogous HSV-2 mutants have been reported. In this study, we describe the derivation and characterization of two distinct HSV-2 mutants defective in the gene encoding ICP4. We found that these mutants have the same basic phenotype exhibited by analogous HSV-1 mutants, indicating that the ICP4s of these two viruses perform similar roles during productive infection.

MATERIALS AND METHODS

Cells and viruses. Vero and CV-1 cells were propagated and maintained as previously described (26). E5 cells contain the gene encoding HSV-1 ICP4 and express complementing levels of this protein upon infection with ICP4 deletion mutants (N. DeLuca, manuscript in preparation). HSV-1 strain KOS and HSV-2 strain 186 were used as wild-type viruses in this study. *d*120 is an HSV-1 (KOS) mutant harboring a deletion in the gene encoding ICP4 (6).

Plasmids. The viral DNA sequences in the plasmids used in this study are illustrated in Fig. 1. pB6 contains the indicated HSV-2 *Bam*HI-*Eco*RI fragment cloned into pBR325 (DeLuca, personal communication). p*Pst*, a derivative of pB6, contains the indicated *Pst*I fragment cloned into pUC8 such that the *Pst*I site located at about map unit 0.86 is adjacent to the unique *Hin*dIII site of the polylinker. pBal4 was generated by cleaving p*Pst* with *Hin*dIII, digesting it with Bal 31 nuclease, and ligating it with *Hin*dIII linkers. pBal4 lacks ori_S, as determined by DNA sequencing (data not shown). p2-Bal3 was constructed by cleaving pBal4 with *Sph*I, digesting it with Bal 31 nuclease, and ligating it with *Eco*RI linkers. The deletion in pdlBal4 was constructed by cleaving pBal4 at the *Nru*I site located at +252 with respect to the ICP4 mRNA initiation site and digesting it with Bal 31 nuclease until about 0.6 kilobases (kb) had been removed. The resulting DNA was ligated with *Bgl*II linkers. pdlBal4 lacks the mRNA initiation site, as demonstrated by restriction enzyme analysis (data not shown). Plasmids pR, pM, pg, pNB, and pNH are subclones of p2-Bal3 inserted as *Hin*dIII fragments into pUC8. pSV2neo harbors the bacterial gene encoding G418 resistance under the transcriptional control of the simian virus 40 early promoter (24).

Nucleic acid isolation. Cytoplasmic RNA and plasmid DNA were isolated as previously described (15). Infectious HSV-2 DNA was purified by previously described procedures (11).

Biochemical transformation of Vero cells with plasmid DNA. Vero cells were cotransfected with pSV2neo and pBal4, and G418-resistant colonies were isolated by the procedure described by DeLuca et al. (6). The resulting cell lines were screened by plaque assay for the ability to complement *d*120. One cell line, designated n-33, was chosen for use as the permissive host cell.

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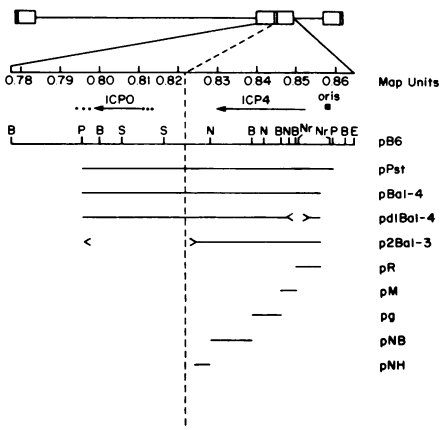

FIG. 1. Plasmids used in this study. Restriction enzyme cleavage sites and subclones of the region of the HSV-2 genome between map units 0.773 and 0.865 are shown beneath the arrows indicating the locations of the ICP0 and ICP4 mRNAs. The black box designates ori_S. The precise location of HSV-2 ICP0 mRNA has not been determined (dots at the 3′ and 5′ ends of the ICP0 transcript). The dashed line represents the L-S junction. Restriction sites shown are *Bam*HI (B), *Pst*I (P), *Eco*RI (E), *Nco*I (N), *Nru*I (Nr), and *Sph*I (S). By virtue of their orientation in pUC8, the HSV DNA inserts in pBal4, pdlBal4, and p2-Bal3 terminate on the right at a *Hin*dIII site. The HSV DNA inserts in pPst, pBal4, and pdlBal4 terminate on the left at the *Pst*I site, whereas p2-Bal3 terminates at an inserted *Eco*RI site. Plasmids pR, pM, pg, pNB, and pNH are subclones of p2-Bal3 inserted as *Hin*dIII fragments into pUC8. See Materials and Methods for details of these constructions.

Marker rescue test. Marker rescue tests were performed essentially as described previously (18). The subclones of p2-Bal3 described in Fig. 1 were linearized with *Pst*I and separately cotransfected with the appropriate infectious mutant virus DNA into n-33 cells. Transfection progeny were assayed simultaneously on Vero and n-33 cells.

Southern and Northern blot analyses. Southern and Northern blot analyses were conducted as described previously (15).

Analysis of infected-cell polypeptides. Labeling with [^{35}S]methionine was carried out as described previously (5). Labeling with $^{32}P_i$ involved incubating cell monolayers in phosphate-free Dulbecco modified Eagle medium for 6 h before infection and maintaining them in this medium until the time of harvest. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of labeled cell extracts was conducted by previously described procedures (5).

RESULTS

It was our goal to generate HSV-2 mutants harboring deletions in the gene encoding ICP4. For isolation of such mutants, we assumed that HSV-2 requires ICP4 to grow, as in the case of HSV-1. Accordingly, we used the approach described by DeLuca et al. for the derivation of HSV-1 ICP4 mutants (6). This approach involved (i) generating a cell line expressing complementing levels of HSV-2 ICP4, (ii) constructing an appropriate deletion in the plasmid-borne copy of the HSV-2 ICP4 gene, (iii) cotransfecting the complementing cell line with this deletion plasmid and infectious wild-type HSV-2 DNA, (iv) screening the transfection progeny for plaque isolates unable to grow on nontransformed Vero cells, and (v) determining whether these isolates had acquired the engineered deletion.

Generation of cell lines expressing HSV-2 ICP4. To generate cell lines able to express complementing levels of HSV-2 ICP4, we cotransfected Vero cells with pBal4 (Fig. 1) and pSV2neo. pSV2neo contains the gene specifying G418 resistance (24). G418-resistant cell lines were derived and screened for the ability to support the growth of *d*120, an HSV-1 ICP4 deletion mutant (6). This screening procedure was chosen because previous studies indicated that HSV-2 is able to complement HSV-1 mutants defective in ICP4 (8). Of the 19 G148-resistant cell lines tested, 4 complemented *d*120. Of these four, cell line 33 (referred to hereafter as n-33) complemented *d*120 most efficiently and was therefore further characterized by Southern blotting (Fig. 2). n-33 cells harbor approximately one intact copy of the HSV-2 ICP4 gene per haploid equivalent. ICP4 sequences smaller and larger than the intact gene were also evident, suggesting that deletions and rearrangements of ICP4 sequences had occurred. Hybridization of pBal4 to Vero cell DNA (lane 1) was not surprising in view of the extremely high G+C content of the HSV-1 ICP4 gene (16, 21).

Derivation of HSV-2 ICP4 mutants. pdlBal4 contains an engineered 0.6-kb deletion that eliminates the transcriptional initiation site of the HSV-2 ICP4 gene (Fig. 1). As anticipated, pdlBal4 did not stimulate the expression of chloramphenicol acetyltransferase (CAT) when cotransfected into CV-1 cells with a plasmid containing the gene encoding CAT under the transcriptional control of the HSV-1 thymidine kinase promoter, a promoter of the early kinetic class responsive to the ICP4 products of HSV-1 and HSV-2 (data not shown). Likewise, pdlBal4 failed to complement the growth of *d*120 in a transient assay (data not shown). Therefore, it was expected that HSV-2 mutants containing this constructed deletion in both copies of the ICP4 gene would not be viable. Accordingly, n-33 cells were cotransfected with pdlBal4 and wild-type HSV-2 DNA, and the progeny were plated on n-33 cells. Of 500 plaque isolates tested for the ability to grow on n-33 cells versus Vero cells,

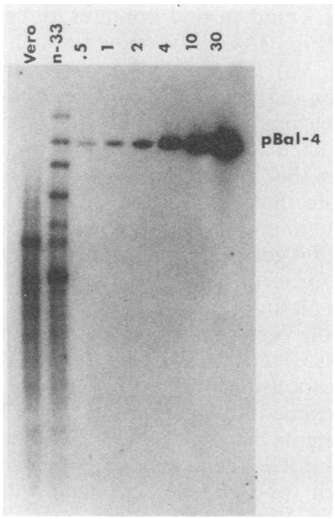

FIG. 2. Southern blot analysis of HSV-2 DNA in n-33 cells. DNA from n-33 cells and Vero cells was cleaved with *Pst*I and *Hin*dIII and subjected to Southern blot analysis with, as probe, the HSV-2 DNA insert of pBal4 (Fig. 1). pBal4 cleaved with *Pst*I and *Hin*dIII was included to visualize 0.5 (31 pg), 1 (62 pg), 2 (124 pg), 4 (248 pg), 10 (620 pg), and 30 (1.86 ng) copies of viral DNA per 3 × 10^9 base pairs of cellular DNA. A 10-μg portion of cellular DNA was analyzed.

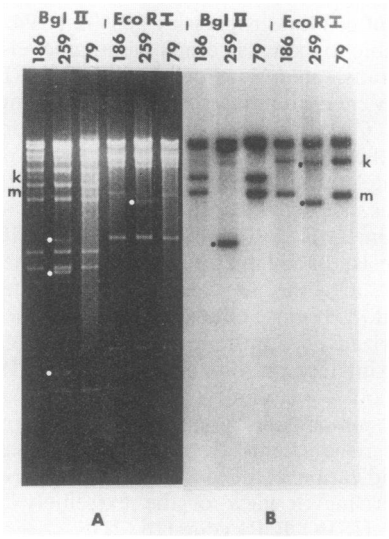

FIG. 3. Restriction analysis of *hr*259, *hr*79, and wild-type DNAs. On the left, k and m refer to fragments in the *Bgl*II digest of HSV-2 (186) DNA. On the right, k and m refer to fragments in the *Eco*RI digest of strain 186 DNA. Dots designate detectable fragments resulting from the incorporation of the deletion contained in pdlBal4. (A) *Bgl*II and *Eco*RI digests stained with ethidium bromide. (B) Same DNA digests as shown in panel A, but blotted and probed with plasmid pg (Fig. 1).

3, designated *hr*259, *hr*79, and *hr*12, exhibited the desired host range. *hr*12 exhibited a plating efficiency of 1.1×10^{-2} (calculated as PFU per milliliter on Vero cells divided by PFU per milliliter on n-33 cells). Upon reassay on the two cell types, *hr*12 plaques arising on Vero cells exhibited a plating efficiency of unity. Because *hr*12 exhibited a high frequency of reversion, it was not analyzed further. *hr*259 exhibited a plating efficiency of less than 5×10^{-6}. *hr*259 harbors the deletion engineered in pdlBal4 in both copies of the ICP4 gene (Fig. 3). It is evident that *hr*259 DNA lacks *Bgl*II fragments k and m and contains three smaller fragments instead (Fig. 3A), only one of which hybridizes to plasmid pg (Fig. 3B). Since k and m each contain one copy of the ICP4 gene (25) and since pdlBal4 contains a unique *Bgl*II site, it is reasonable to conclude that *hr*259 contains the deletion constructed in pdlBal4 in both copies of the ICP4 gene. To confirm this, *Eco*RI digests were conducted. These tests demonstrate that *hr*259 possesses shortened forms of *Eco*RI fragments m and k (Fig. 3A), each of which contains a copy of the ICP4 gene (25). As expected, pg hybridizes to both shortened forms of *Eco*RI fragments m and k (Fig. 3B). *hr*79 exhibited a plating efficiency of 3×10^{-4}, and, like *hr*12, plaques arising on Vero cells exhibited a plating efficiency of unity upon reassay on the two cells types. The location of the mutation responsible for the host range phenotype of *hr*79 was determined by marker rescue with the cloned restriction fragments listed in Table 1. The mutation maps to the portion of the ICP4 gene encoding the carboxy-terminal half of the protein (pNB; Table 1). Rescue with pNB yielded similar results in three independent tests. *hr*79 does not appear to contain any obvious deletions in the ICP4 gene, as evidenced by the analysis in Fig. 3 and by the fact that pNB hybridizes to the same-sized fragment in *Nco*I-*Bam*HI-digested *hr*79 DNA as it does in similarly digested wild-type 186 DNA (data not shown).

Phenotypic analysis of *hr*259 and *hr*79. Cytoplasmic RNA was extracted at 5 h postinfection from anisomycin-treated Vero cells infected with *hr*259, *hr*79, or HSV-2 (186) and subjected to Northern blot analysis with pBal4 as the probe (Fig. 4). pBal4 is able to detect both ICP4 and ICP0 mRNAs (Fig. 1). Both the 4.7-kb ICP4 mRNA and the 3.4-kb ICP0 mRNA were produced during infection with *hr*79 and HSV-2 (186). As expected, the pBal4 probe detected only ICP0 mRNA in extracts of *hr*259-infected cells.

SDS-PAGE analysis of extracts of cells infected with *hr*79, *hr*259, or HSV-2 (186) and labeled with [^{35}S]methionine from 3 to 15 h postinfection revealed that the mutant viruses exhibited identical polypeptide profiles in nonpermissive Vero cells (Fig. 5). ICP6, ICP0, and ICP27 were synthesized in relatively small amounts, and the shutoff of host-specific translation was efficient but not complete. The status of the three other immediate-early proteins, ICP4, ICP22, and ICP47, was not clear in this experiment. SDS-PAGE analysis of extracts of infected n-33 cells revealed that these cells complemented *hr*259 and *hr*79 very efficiently and that *hr*79 apparently does not specify a full-length thymidine kinase

TABLE 1. Marker rescue of the host-range mutation in *hr*79[a]

Plasmid	Percent rescue
pUC8	<0.04%
p2-Bal3	2.60%
pR	<0.04%
pM	<0.07%
pg	<0.04%
pNB	0.30%
pNH	<0.09%

[a] n-33 cells were cotransfected with *hr*79 DNA and the designated plasmids linearized with *Pst*I. Titers of the progeny of the cotransfection were determined simultaneously on n-33 and Vero cells. Percent rescue was calculated as: (PFU per milliliter on Vero cells/PFU per milliliter on n-33 cells) × 100.

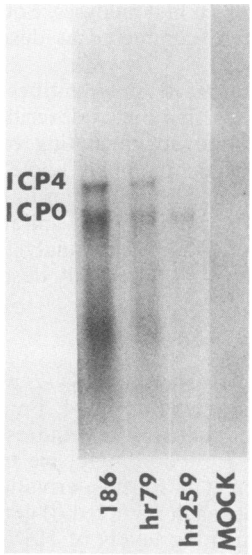

FIG. 4. Northern blot analysis of cytoplasmic RNA extracted at 5 h postinfection from anisomycin-treated Vero cells infected at a multiplicity of infection of 20 PFU per cell with *hr*259, *hr*79, or HSV-2 (186). pBal4 (Fig. 1) was used as probe. Vero cell monolayers were incubated in 100 μM anisomycin for 1 h before infection and were maintained in this concentration of drug until the time of harvest.

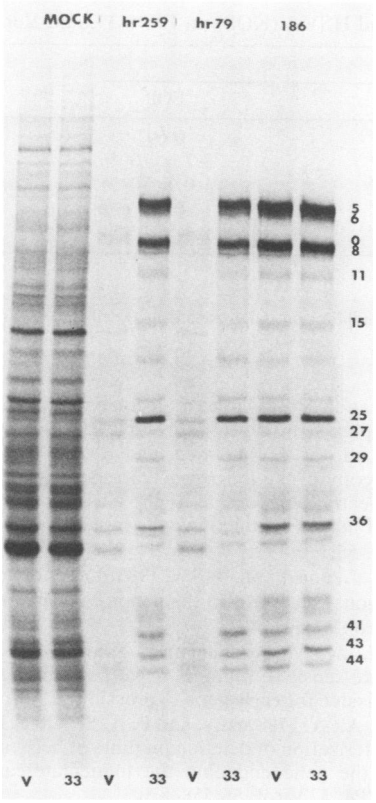

FIG. 5. SDS-PAGE analysis of extracts of Vero or n-33 cells infected at a multiplicity of infection of 20 PFU per cell with *hr*259, *hr*79, or HSV-2 (186) and labeled with [^{35}S]methionine from 3 to 15 h postinfection. V designates Vero cells; 33 designates n-33 cells.

(ICP36). Whether or not *hr*79 specified thymidine kinase activity has not been determined.

To gain further insight into the mutant phenotype exhibited by these viruses, SDS-PAGE analysis was conducted with extracts of Vero cells infected with *hr*79, *hr*259, or HSV-2 (186) and labeled with $^{32}P_i$ from 1.5 to 5 h postinfection (Fig. 6). In this experiment, ICP4 was detectable in extracts of HSV-2 (186)-infected cells but not *hr*259-infected cells. In the case of *hr*79, a set of novel proteins was detectable in the 110- to 120-kilodalton range. Marker rescue data (Table 1) are consistent with the implication that these proteins may represent truncated forms of ICP4. It is also evident from this experiment that both *hr*259 and *hr*79 synthesized ICP0, ICP6, ICP22, and ICP27 under noncomplementing conditions. The status of ICP47 was not evident in this experiment. It is unclear why neither ICP22 nor ICP0 was detectable in the wild-type extract, but this may reflect the fact that immediate-early transcription is down-regulated by ICP8, a protein not specified by *hr*259 or *hr*79 in Vero cells (Fig. 5) (10).

Functional interchangeability of the HSV-1 and HSV-2 ICP4 products. Although earlier studies demonstrated that HSV-2 ICP4 can substitute for HSV-1 ICP4 to support the growth of HSV-1, it has not been determined whether the converse is true (8). To address this question, we performed the plating-efficiency assays and single-burst studies shown in Tables 2 and 3. The plating efficiencies of *hr*79 and *hr*259 were similar to that of the HSV-1 ICP4 mutant *d*120 on HSV-1 ICP4-expressing E5 cells (Table 2). Likewise, *d*120 plated as well as the HSV-2 ICP4 mutants on HSV-2 ICP4-expressing n-33 cells. Moreover, complementation yields of all three mutants in both E5 and n-33 cells was at least 3 orders of magnitude more efficient than in Vero cells as assessed by single-burst experiments (Table 3). Hence we conclude that the two ICP4s are functionally interchangeable during productive infection in cell culture.

DISCUSSION

In this report, we describe two distinct HSV-2 mutants defective in the gene encoding ICP4. Our principal motivation for generating these mutants was to determine whether HSV-2 ICP4 mutants behave differently from their HSV-1 counterparts under nonpermissive conditions. We found that their phenotypes are basically the same. Under nonpermissive conditions, *hr*259 induced detectable amounts of only ICP0, ICP6, ICP22, and ICP27, the same spectrum of proteins induced by *d*120, an HSV-1 mutant whose ICP4 gene is almost completely deleted (6). ICP0, ICP22, and ICP27 are products of immediate-early genes, and, as such, their synthesis does not require ICP4 or any other viral protein. Hence, that *hr*259 and *hr*79 induced the synthesis of these proteins under nonpermissive conditions was not unexpected. ICP6 is formally classified as an early, or β, protein (17). β proteins are the products of genes which require prior viral protein synthesis but not viral DNA synthesis for maximum transcription. As evidenced by the phenotypes of the mutants described in this report, the synthesis of ICP6 is apparently not as dependent on ICP4 as is the synthesis of other β proteins such as ICP36. This is not unexpected, since, unlike many other β genes, the ICP6 gene is transcribed to some extent even when cycloheximide or concavanine is present throughout infection (7, 19).

In addition to describing mutants with mutations in the

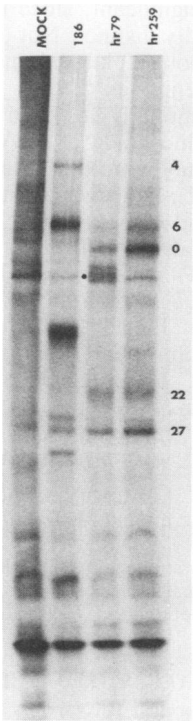

FIG. 6. SDS-PAGE analysis of Vero cells infected at a multiplicity of infection of 20 PFU per cell with *hr*259, *hr*79, or HSV-2 (186) and labeled with $^{32}P_i$ from 1.5 to 5 h postinfection. The dot designates novel polypeptides in the *hr*79-infected extract.

TABLE 2. Titers and plating efficiencies of *hr79*, *hr259*, *d120*, HSV-2 (186), and HSV-1 (KOS) on E5, n-33, and Vero cells

Virus	E5[a]		n-33		Vero
	Titer[b]	EOP[c]	Titer	EOP	Titer
HSV-2 (186)	4.4×10^8	0.66	4.5×10^8	0.64	2.9×10^8
79	1.6×10^8	2.6×10^{-4}	1.3×10^8	3.2×10^{-4}	4.1×10^4
259	8.5×10^8	$<1.2 \times 10^{-6}$	1.0×10^8	$<1.0 \times 10^{-5}$	$<1 \times 10^3$
HSV-1 (KOS)	6.5×10^8	0.92	9.5×10^8	0.63	6.0×10^8
d120[d]	3.0×10^8	$<3.3 \times 10^{-6}$	1.9×10^8	$<5.3 \times 10^{-6}$	$<1 \times 10^3$

[a] E5 cells are transformed with an HSV-1 DNA fragment encoding ICP4.
[b] Titers are expressed as PFU per milliliter.
[c] EOP, Efficiency of plating, calculated as PFU per milliliter on Vero cells divided by PFU per milliliter on ICP4-transformed cells.
[d] *d120* is an HSV-1 ICP4 deletion mutant.

TABLE 3. Yields of *d120*, HSV-1 (KOS), *hr259*, *hr79*, and HSV-2 (186) on Vero, n-33, and E5 cells

Virus	Yield (PFU/ml) on cell type[a]			Ratio (E5/n33)
	Vero	E5	n-33	
HSV-1 (KOS)	4.0×10^7	1.2×10^8	8.0×10^7	1.5
d120	$<1.0 \times 10^4$	5.4×10^6	1.7×10^4	3.2
HSV-2 (186)	1.9×10^7	1.3×10^7	3.3×10^7	0.4
hr259	$<1.0 \times 10^4$	1.2×10^7	2.4×10^7	0.5
hr79	$<1.0 \times 10^4$	5.7×10^7	2.4×10^7	2.4

[a] Cells were infected at a multiplicity of infection of 0.1 PFU per cell and harvested 18 h postinfection.

HSV-2 gene for ICP4, this report establishes that the ICP4 products encoded by HSV-1 and HSV-2 are functionally interchangeable during productive infection. Although we were the first to demonstrate complementation of HSV-1 ICP4 temperature-sensitive mutants by HSV-2 ICP4 (8), this report is the first to demonstrate the reverse. This complete interchangeability is significant, since the two proteins appear to be biochemically distinct. Not only do they differ in molecular mass by about 10 kilodaltons (19), but they also exhibit type-specific epitopes (2, 23), although one cross-reactive monoclonal antibody has been identified (1). Moreover, the first 47 amino acids of HSV-2 ICP4 have been identified and exhibit no homology with the amino terminus of the HSV-1 ICP4 (27). However, the two proteins share at least some nucleic acid homology and exhibit considerable functional colinearity as evidenced by the existence of an array of intertypic ICP4 genes whose chimeric products are fully functional (3; C. A. Smith and P. A. Schaffer, manuscript in preparation). Further study of the HSV-2 ICP4 molecule and its structural and functional homologies with its HSV-1 counterpart should help to reveal the mechanism by which these proteins function.

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