JOURNAL OF VIROLOGY, Apr. 1987, p. 1136–1146
0022-538X/87/041136-11$02.00/0

Vol. 61, No. 4

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

PAULO K. ORBERG AND PRISCILLA A. SCHAFFER*

Laboratory of Tumor Virus Genetics, Dana-Farber Cancer Institute, and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received 16 September 1986/Accepted 11 December 1986

To minimize the contribution of residual activity associated with the temperature-sensitive (*ts*) form of ICP8 specified by available *ts* mutants, deletion mutations in this gene were constructed. Cells permissive for the generation and propagation of ICP8 deletion mutants were first obtained. Vero cells were cotransfected with pKEF-P4, which contains the gene for ICP8, and pSV2neo or a hybrid plasmid containing the G418 resistance gene linked to pKEF-P4. Of the 48 G418-resistant cell lines, 21 complemented ICP8 *ts* mutants in plaque asssays at the nonpermissive temperature. Four of these were examined by Southern blot analysis and shown to contain 1 to 3 copies of the ICP8 gene per haploid genome equivalent. Cell line U-47 was used as the permissive host for construction of ICP8 deletion mutants. In addition to cell lines which complemented *ts* mutants, two lines, U-27 and U-35, significantly inhibited plaque formation by wild-type virus, contained 30 and 100 copies of the ICP8 gene per haploid genome equivalent, respectively, and expressed large amounts of ICP8 after infection with wild-type virus. At low but not high multiplicities of infection, this inhibition was accompanied by underproduction of viral polypeptides of the early, delayed-early, and late kinetic classes. For construction of deletion mutants, a 780-base-pair *Xho*I fragment was deleted from pSG18-*Sal*IA, a plasmid which contains the gene for ICP8, to yield pDX. U-47 cells were then cotransfected with pDX and infectious wild-type DNA. Mutant *d*61, isolated from the progeny of cotransfection, was found to contain both the engineered deletion in the ICP8 gene and an ori_L-associated deletion of approximately 55 base pairs. Because *d*61 contained two mutations, a second mutant, *d*21, which carried the engineered ICP8 deletion but an intact ori_L, was constructed by cotransfection of U-47 cells with wild-type DNA and an *Sal*I-*Kpn*I fragment purified from pDX. Phenotypic analysis of *d*21 and *d*61 revealed that they were similar in all properties examined: both exhibited efficient growth in U-47 cells but not in Vero cells; both induced the synthesis of an ICP8 polypeptide which was smaller than the wild-type form of the protein and which, unlike the wild-type protein, was found in the cytoplasm and not the nucleus of infected Vero cells; and nonpermissive Vero cells infected with either mutant failed to express late viral polypeptides.

The major herpes simplex virus type 1 (HSV-1) DNA-binding protein ICP8 is the product of an early, or β, gene located between map coordinates 0.380 and 0.410 on the viral genome (Fig. 1) (16, 27, 39). This gene is transcribed primarily as a 4.2-kilobase (kb) mRNA species containing a coding sequence of 3,591 bases (27). A much less abundant ICP8-specific mRNA, of approximately 10 kb, has also been detected (16). The major DNA-binding protein of HSV-1 is composed of 1,196 amino acids of molecular weight 128,341 (27).

During infection, ICP8 is transported to the nucleus (9, 18), where it associates with the nuclear matrix (26). Purified ICP8 is able to destabilize a poly(dA:dt) helix (24) and hold single-stranded DNA in an extended conformation (29). The observation that temperature-sensitive (*ts*) mutants of ICP8 are phenotypically DNA negative at the nonpermissive temperature demonstrates that ICP8 is required for viral DNA synthesis (39). Indirect evidence that ICP8 interacts with viral DNA polymerase to form a DNA synthetic complex (38) includes the following: (i) antibodies to ICP8 have been reported to inhibit DNA polymerase activity in chromatin isolated from HSV-infected cells (24); (ii) viral DNA polymerase and alkaline nuclease activities were found to be reduced in extracts prepared from cells infected with an ICP8 *ts* mutant at the nonpermissive temperature (21), and addition of purified ICP8 restored the ability of such extracts to synthesize DNA; (iii) purified ICP8 has been reported to stimulate DNA polymerase activity in vitro (30); and (iv) ICP8 *ts* mutants may exhibit altered sensitivity to DNA polymerase inhibitors (3).

In addition to its involvement in viral DNA synthesis, ICP8 plays an essential role in inhibiting the expression of viral genes of all kinetic classes, as shown by the fact that ICP8 *ts* mutants overproduce certain α, β, γ_1, and γ_2 mRNAs and proteins at the nonpermissive temperature (10–12).

Although *ts* mutants have proven extremely useful for the functional analysis of HSV, they revert and may exhibit residual wild-type activity (leak). Additionally, studies of *ts* mutants of ICP4, an immediate-early (α) transcriptional regulatory protein, have shown that some *ts* forms of the protein exhibit distinct activities not characteristic of residual wild-type activity (8). By contrast, ICP4 deletion mutants exhibited a true null phenotype and were more efficiently complemented in ICP4-expressing cells than was an ICP4 *ts* mutant (7). This observation indicated that the *ts* polypeptide had an inhibitory effect on the activity of the wild-type protein supplied by the cells. Because deletion mutants neither leak nor revert, they better reflect the null phenotype than *ts* mutants and therefore constitute useful tools for

* Corresponding author.

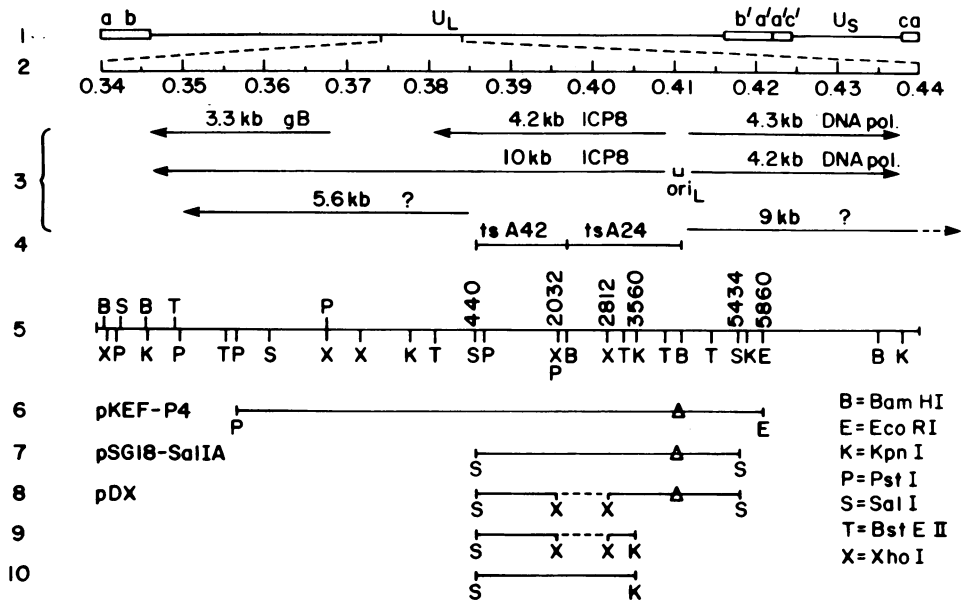

FIG. 1. Genomic location of the ICP8 gene and plasmids and DNA fragments used in this study. The prototype arrangement of the HSV-1 genome is shown in line 1. The map units of the expanded, central portion of the long unique region (U_L) are shown in line 2. Transcripts known to map to this region of the genome (all of the β kinetic class [16]) and the location of a viral origin of DNA replication, ori_L (14, 40), are shown in line 3. The locations of the mutations in two ICP8 *ts* mutants used in this study, *ts*A24 and *ts*A42, appear in line 4 (39). Relevant restriction sites and the corresponding nucleotide numbers (27) are depicted in line 5. In lines 6 to 8 the symbol Δ represents deletions in ori_L of ~55 bp which occur during cloning in bacteria. The fragment shown in line 9 was generated from pDX and used to construct *d*21 by marker transfer (see text). The fragment shown in line 10 was derived from pSG18-*Sal*IA and used as a probe for Southern blot analysis of cellular DNAs (Fig. 3).

studies of the functional roles of specific viral genes in the life cycle of the virus.

In this paper we report the derivation and characterization of Vero cell lines that express ICP8 and the use of these cells as permissive hosts for the construction and characterization of HSV-1 mutants carrying deletions in the gene for ICP8. In addition to the cell lines used to generate deletion mutants, a second class of ICP8-expressing cells was isolated and shown to inhibit wild-type viral plaque formation and late viral gene expression.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) and human embryonic lung (HEL) cells were propagated and maintained as described previously (39). In some tests, G418-resistant (Nero) cells were used (7). Nero cells were derived by G418 selection of Vero cells transfected with pSV2neo.

The KOS strain of HSV-1 was used as the wild-type virus from which *ts* (*ts*A24 and *ts*A42 [39]) and deletion mutants were derived. All viruses were propagated and assayed as described previously (33).

Plasmids and cloning. The map locations of viral DNA inserts in plasmids used in this study are shown in Fig. 1. pKEF-P4 (5) and a modified form of pSV2 neo (36) (see below) were generously provided by N. DeLuca. pSG18-*Sal*IA (19) was the gift of S. K. Weller (University of Connecticut Health Center, Farmington).

Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, Mass.) and used as suggested by the manufacturer.

Viral DNA isolation. Infected-cell DNA was prepared as described previously (6). After proteinase K digestion, viral DNA was separated from cellular DNA by centrifugation in CsCl gradients (13). KOS DNA was isolated from infected HEL cells, and deletion mutant DNA (*d*21 or *d*61) was isolated from infected U-47 cells (see below).

Isolation of DNA fragments. DNA fragments for use in cotransfection experiments or as probes in Southern blot hybridizations were isolated after restriction enzyme digestion of plasmid DNA and electrophoresis through a 0.4% agarose gel. After being stained with ethidium bromide, the bands of interest were excised and submitted to three cycles of freezing and thawing (34). Agarose was separated from the DNA solution by centrifugation. The DNA in the supernatant fluid was purified by passage through an Elutip-d column (Schleicher & Schuell, Keene, N.H.) and ethanol precipitation.

Blot hybridization. Specific DNA sequences in digests of viral or cellular DNA were detected by the method of Southern (22, 35). Probes were labeled with [^{32}P]dCTP and [^{32}P]dGTP (Amersham Corp. Arlington Heights, Ill.) by nick-translation (22).

Transfection of Vero cells. Cells lines containing the gene for HSV-1 ICP8 were generated from Vero cells essentially as described for ICP4-containing cell lines (7), except that either pSV2neo (0.5 μg) was coprecipitated and transfected with 1.5 μg of pKEF-P4 or, alternatively, the hybrid plasmid pKEF-P4-G418 (2.0 μg) was used (Fig. 2). The concentration of G418 (Geneticin Sulfate; Gibco Laboratories, Chagrin Falls, Ohio) used for selection of ICP8-containing cells was 0.5 mg/ml. G418-resistant cell lines were propagated in this level of drug at alternate passages to maintain cells carrying the selectable marker and ICP8 DNA sequences.

Marker transfer. Cotransfection of U-47 and Nero cells with infectious KOS DNA and plasmid DNA for construc-

tion of deletion mutants was carried out as described previously for marker rescue (31).

Analysis of infected-cell polypeptides. [^{35}S]methionine labeling of polypeptides produced in infected cells between 5 and 18 h postinfection was conducted as described previously (31).

Western blot analysis. Immunological detection of ICP8-related polypeptides among infected cell polypeptides separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (2) was performed as described (7) with 0.2 ml of polyclonal rabbit antiserum to ICP8 (diluted 1:40 with Blotto [17]), which was the generous gift of Richard Courtney (Louisiana State University, Shreveport).

Immunofluorescence. Demonstration of the intracellular location of wild-type and mutant ICP8 polyeptides was performed essentially as described by Quinlan et al. (26). Cells grown on cover slips were infected at a multiplicity of 1 PFU/cell and incubated at 37°C for 12 h before fixation. The ICP8 antiserum was the same as that used in the Western blot analysis described above, but it was used at a dilution of 1:5. Indirect staining was carried out with rhodamine-conjugated goat anti-rabbit immunoglobulins (Cooper Biomedical, Malvern, Pa.) at a 1:30 dilution.

RESULTS

Derivation of cell lines containing the gene for HSV-1 ICP8. Plasmid pKEF-P4 (5) contains the viral DNA fragment with map coordinates 0.357 to 0.421. The only intact gene specified by this fragment is the gene for the major DNA-binding protein, ICP8, under control of its own promoter (Fig. 1 and 2). Plasmid pSV2neo (Fig. 2) contains the gene which specifies resistance to G418 under control of the simian virus 40 (SV40) early promoter (36). pSV2neo was modified by addition of an *Eco*RI linker at its single *Nde*I site (N. DeLuca and A. McCarthy, personal communication) (Fig. 2). The G418 resistance moiety of *Eco*RI-cleaved pSV2neo was cloned into the unique *Eco*RI site of pKEF-P4 to generate pKEF-P4-G418 (Fig. 2).

Vero cells were transfected in suspension (7) with either the hybrid plasmid pKEF-P4-G418 (linked transfection) or a mixture of pKEF-P4 and pSV2neo (unlinked cotransfection). The resulting G418-resistant colonies were designated L for colonies derived from linked transfection or U for colonies derived from unlinked cotransfection.

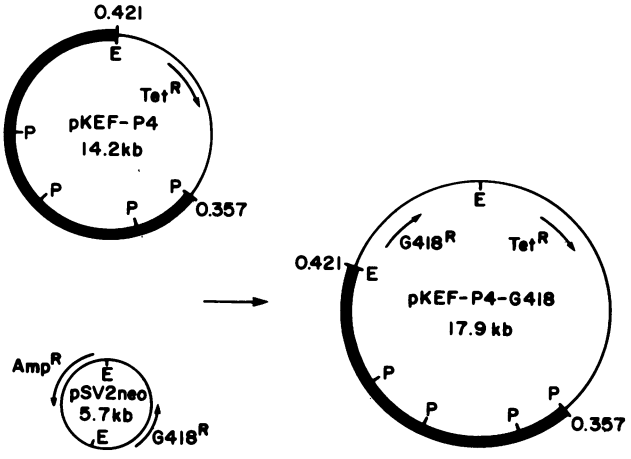

FIG. 2. Plasmids used to generate ICP8-expressing cell lines. Cell lines resistant to G418 were obtained by cotransfection with pSV2neo and pKEF-P4 (Fig. 1) or by transfection with pKEF-P4-G418 alone. Heavy lines represent HSV-1 DNA sequences. E, *Eco*RI; P, *Pst*I.

TABLE 1. Qualitative complementation tests of ICP8 *ts* mutants *ts*A24 and *ts*A42 in G418-resistant Vero cell lines[a]

Derivation of cell line	Plaque formation (%)	
	Positive	Negative
Linked transfection	7 (41)	10 (59)
Unlinked transfection	14 (45)	17 (55)
Total	21 (44)	27 (56)

[a] Cell lines obtained by linked transfection with pKEF-P4-G418 or unlinked cotransfection with pKEF-P4 and pSV2neo (Fig. 2) were tested for their ability to support plaque formation by ICP8 *ts* mutants at the nonpermissive temperature (39.4°C). Tests were scored as positive (plaques) or negative (no plaques).

After isolation and amplification of individual G418-resistant colonies, the 48 resulting cell lines were screened for the ability to complement the *ts* mutant *ts*A24 at 39.4°C. *ts*A24 is an HSV-1 mutant carrying a mutation in the gene for ICP8 (Fig. 1) (39). Complementation was assessed qualitatively by the ability of *ts*A24 to produce plaques on G418-resistant cell lines at 39.4°C relative to nontransformed Vero cells. Wild-type virus was used as a control. Cell lines which supported plaque formation by *ts*A24 at 39.4°C were then retested simultaneously with *ts*A24 and *ts*A42, a mutant whose *ts* mutation maps immediately to the left of the mutation in *ts*A24 in the ICP8 gene (Fig. 1) (39). In every case, cell lines which complemented *ts*A24 also complemented *ts*A42. The results of complementation tests with the 48 cell lines are summarized in Table 1. Despite differences in the transfection procedure used to generate G418-resistant cells, the frequency of isolation of lines able to complement ICP8 *ts* mutants at 39.4°C was quite similar: linked transfection, 41%; unlinked cotransfection, 44%.

Among the 21 cell lines which complemented the *ts* mutants, 6 exhibited efficient complementation as judged by the size and number of *ts*A24 plaques at 39.4°C. Of these, line U-47 was chosen as the permissive host for the construction and propagation of deletion mutants. Prior to the generation of deletion mutants, however, U-47 and other complementing cell lines were characterized further.

Presence of ICP8-related sequences in complementing cell lines. Cell lines were examined by Southern blot analysis to establish the presence and copy number of the ICP8 gene. For this purpose cellular DNAs were digested with *Eco*RI, electrophoresed, and transferred to nitrocellulose filters. The filter was probed with a ^{32}P-labeled *Sal*I-*Kpn*I fragment (map coordinates 0.386 to 0.406) purified from pSG18-*Sal*IA (19) (Fig. 1). This probe comprises approximately 71% of the ICP8 gene and is contained entirely within this gene (Fig. 1). The results of this analysis are shown in Fig. 3.

The Southern analysis included pKEF-P4 standards representing from 1 to 50 copies of the ICP8 gene per haploid genome equivalent and U-7 and Nero cells as negative controls. U-7 cells are G418-resistant cells selected after cotransfection of Vero cells with pSV2neo and pKEF-P4 that failed to complement *ts*A24 or *ts*A42. Cells which complemented the ICP8 *ts* mutants, such as U-42 and U-47, contained from 1 (U-42 cells) to 3 (U-47 cells) copies of the ICP8 gene per haploid genome equivalent. ICP8-associated sequences in U-47 cells appeared to be associated with cellular DNA, resulting in bands of lesser mobility than that of the viral DNA band in plasmid pKEF-P4. Additionally,

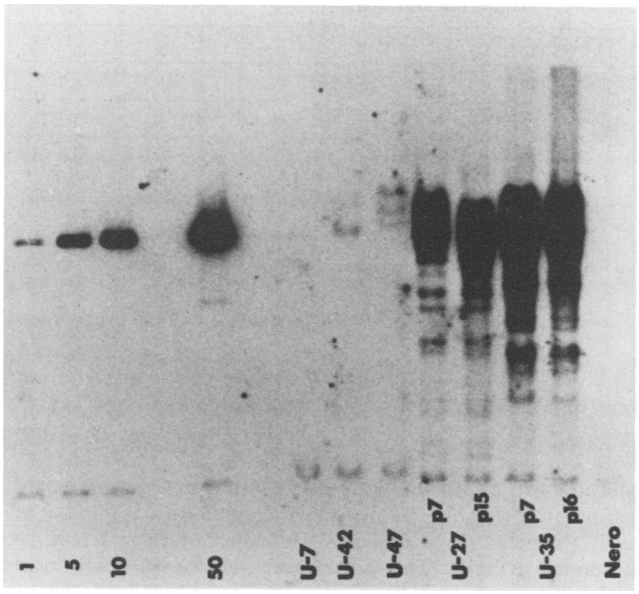

FIG. 3. Southern blot analysis of the presence and copy number of ICP8 gene sequences in selected cell lines. Amounts of pKEF-P4 representing 1, 5, 10, or 50 copies of ICP8 per haploid genome or 10 μg of total cellular DNA was digested with *Eco*RI, electrophoresed in an agarose gel, and transferred to a nitrocellulose filter. The filter was probed with the *Sal*I-*Kpn*I fragment shown in Fig. 1 (line 10) labeled with ^{32}P by nick translation. U-7 and U-47 were tested at passage 7 (p7) U-42 at passage 15, U-27 at passages 7 and 15, and U-35 at passages 7 and 16.

the copy number and cellular DNA associations of the ICP8 gene were stable through eight passages in U-47 cells (not shown).

Expression of ICP8 in complementing cell lines. SDS-PAGE analysis showed that U-47 cells did not constitutively express detectable levels of ICP8 (Fig. 4, lane 2). Infection with HSV-1 KOS at a multiplicity of 0.2 PFU/cell revealed approximately equal amounts of ICP8 in U-47 (lane 6) and Nero cells (lane 5). At higher multiplicities, the amount of ICP8 produced in KOS-infected Nero cells was greater than that produced in infected U-47 cells (lanes 9 and 10, lanes 13 and 14). Thus, although the intensity of ICP8 and other viral polypeptide bands increased as a function of the increased multiplicity of KOS in Nero cells, no multiplicity dependence was seen in KOS-infected U-47 cells. In addition, KOS-infected U-47 cells exhibited more efficient shutoff of host protein synthesis at all multiplicities tested than did KOS-infected Nero cells (Fig. 4; see also Fig. 8).

Characterization of cells which failed to complement ICP8 *ts* mutants and inhibited plaque formation by wild-type HSV-1. Among the cell lines that failed to complement *ts*A24 and *ts*A42 (Table 1), two, designated U-27 and U-35, significantly inhibited plaque formation by wild-type HSV-1 KOS. This phenomenon was further investigated in a series of plaque assays of wild-type virus and of the ICP8 deletion mutant *d*61 (to be described below) on U-27 and U-35 cells at various passage levels (Table 2). This table shows a representative sampling of titers of a single KOS stock and a single *d*61 stock obtained by plating on U-27 and U-35 cells and on control Nero, U-7, and U-47 cells on different occasions. The reduction in titer of the KOS stock observed on U-27 and U-35 cells varied from test to test, but in most cases was in the range of 10- to 40-fold. In addition to the reduced plating efficiency of KOS on U-27 and U-35 cells, plaque sizes were quite heterogeneous. The data in Table 2 also indicate that a functional ICP8 polypeptide must be produced in both U-27 and U-35 cells, since they supported the growth of *d*61 several orders of magnitude more efficiently than did control Nero cells, which do not contain the ICP8 gene.

When the DNAs of U-27 and U-35 cells were subjected to Southern blot analysis, the two cell types were estimated to

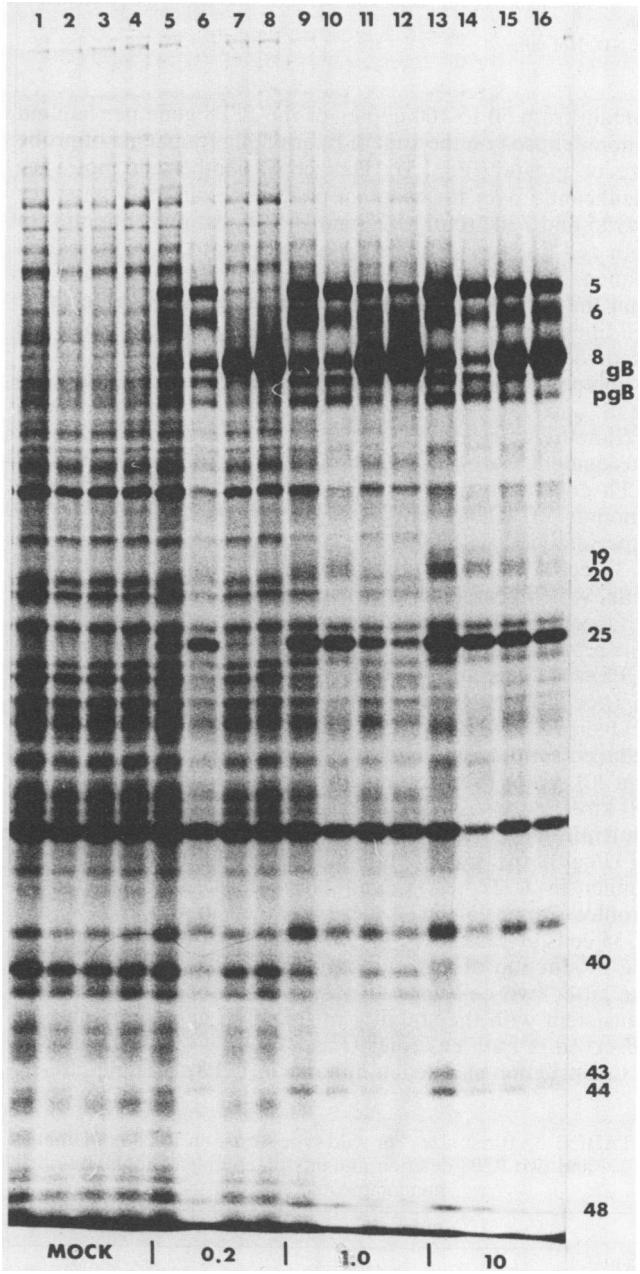

FIG. 4. SDS-PAGE analysis of ^{35}S-labeled polypeptides in cells infected with wild-type HSV-1 KOS at different multiplicities. Cells were infected with 0.2 (lanes 5 to 8), 1.0 (lanes 9 to 12), or 10 (lanes 13 to 16) PFU/cell or mock-infected (lanes 1 to 4). Polypeptides were labeled with [^{35}S]methionine from 5 to 18 h postinfection. Cell lines: Nero (lanes 1, 5, 9, and 13), U-47 (lanes 2, 6, 10, and 14), U-27 (lanes 3, 7, 11, and 15), and U-35 (lanes 4, 8, 12, and 16). gB, Glycoprotein B; pgB, precursor to glycoprotein B.

TABLE 2. Plaque assays of virus stocks at 37°C on different cell lines[a]

Virus	Titer (PFU/ml)[b]						
	Nero	U-7 (p22)	U-47 (p9)	U-27		U-35	
				p8	p21	p9	p21
KOS	5.4×10^7	5.4×10^7	6.3×10^7	4.0×10^6	1.8×10^6	2.3×10^6	1.5×10^6
*d*61	1.0×10^2[c]	ND[d]	2.3×10^7	3.9×10^5	ND	2.4×10^5	ND

[a] One stock of KOS and one stock of *d*61 were assayed at different times on the cell lines listed.
[b] The passage number of the cell lines tested is indicated (p22 = passage 22, etc.).
[c] The appearance of *d*61 plaques on Nero cells was due to the generation of occasional recombinants when the mutant was propagated in ICP8-containing U-47 cells.
[d] ND, Not done.

contain from 50 to 100 copies of the ICP8 gene per haploid genome, based on the sum of hybridizing fragments of probe size or greater (Fig. 3). This copy number did not vary significantly over the range of passages examined (passages 7 to 15 and 7 to 16 for U-27 and U-35 cells, respectively). In both cell lines, a significant proportion of the ICP8-related sequences appeared as bands of greater and lesser mobility than that of pKEF-P4 (the plasmid used to generate these cell lines), indicating that rearrangements and deletions had occurred.

Polypeptide synthesis in U-27 and U-35 cells infected with KOS and labeled from 5 to 18 h postinfection with [^{35}S]methionine was examined by SDS-PAGE (Fig. 4). It was evident that while neither U-27 nor U-35 cells expressed ICP8 constitutively (lanes 3 and 4; see also Fig. 7), large amounts of ICP8 were detected in both cell types after superinfection with KOS. The quantity of ICP8 detected in U-35 cells was noticeably greater than that seen in U-27 cells, which correlates with the greater copy number of the ICP8 gene in U-35 cells indicated in Fig. 3. This observation was reproducible in repeat experiments. Additionally, more ICP8 was detected in these cells at a multiplicity of 1 PFU/cell than at 0.2 or 10 PFU/cell.

Overproduction of ICP8 was accompanied by markedly reduced synthesis of viral polypeptides of the β (gB, ICPs 6 and 40), γ_1 (ICPs 5, 25, and 44), and γ_2 (ICPs 19, 20, 43, and 48) kinetic classes. This effect was most pronounced at low multiplicity (0.2 PFU/cell). At high multiplicity (10 PFU/cell), the polypeptide phenotype of KOS was most similar in U-27, U-35, and Nero cells. The polypeptide profiles shown in Fig. 4 were obtained in U-47, U-27, and U-35 cells at passage 5. Virtually identical results (including the production of large amounts of ICP8) were obtained in the latter two cell lines at passage 20 (not shown). This is consistent with the stability of the ICP8 gene copy number observed in both cell lines (Fig. 3).

Construction of deletion mutants of ICP8. Plasmid pSG18-*Sal*IA (19) contains the portion of the HSV-1 genome between map units 0.386 and 0.418, which includes the 5′ two-thirds of the ICP8 gene (Fig. 1). A 780-base-pair (bp) deletion in the ICP8 coding sequence in pSG18-*Sal*IA was generated by digestion with *Xho*I and religation to yield plasmid pDX (Fig. 1). Analysis of the nucleotide sequence of the ICP8 gene (27) indicates that the reading frame is maintained in pDX. This plasmid also carries a deletion of approximately 55 bp in ori$_L$ sequences. The deletion arose spontaneously as a result of instability of the ori$_L$ palindrome when cloned in bacteria (14, 40). The size of this deletion was estimated by comparison with pKEF-P4, whose 55-bp deletion was determined by DNA sequencing (40) (not shown).

ICP8 deletion mutant *d*61 was constructed by cotransfection of U-47 cells with intact, infectious KOS DNA and pDX (linearized at the *Eco*RI site). Progeny of the cotransfection were plated on U-47 cells, and isolates were tested for growth on U-47 and Vero cells. Of 240 plaque isolates, 1 (*d*61) grew efficiently in U-47 but not in Vero cells (Tables 2 and 3). Mutant *d*61 was plaque purified three times.

Comparison of *d*61 and KOS DNAs by restriction enzyme analysis and Southern blot hybridization revealed that they differed in several respects. First, as expected, the *Sal*I L fragment of *d*61 DNA (map units 0.386 to 0.418; equivalent to pSG18-*Sal*IA in Lee and Knipe [19]) comigrated with the *Sal*I insert of pDX, indicating that the 780-bp deletion in ICP8 in the plasmid had been incorporated into *d*61 DNA (Fig. 5, lanes 2 and 5; Fig. 6, lanes 2 and 5).

Second, and unexpectedly, the ori$_L$-containing *Kpn*I V fragment of *d*61 DNA (0.406 to 0.419) was approximately 55 bp shorter than its KOS counterpart (Fig. 5, lanes 6 and 8; Fig. 6, lanes 6 and 8). This indicated that the ori$_L$ deletion present in pDX had also been incorporated into *d*61 DNA. The replication competence of *d*61 in U-47 cells suggested that ori$_L$ may not be essential for replication of HSV-1 in vitro. This interesting possibility has been explored in greater detail and will be considered elsewhere (manuscript in preparation).

Third, and also unexpectedly, *d*61 DNA was found to contain an insertion or duplication of approximately 100 bp in the "b" sequences of the inverted repeats surrounding U$_L$. This insertion or duplication can be seen in Fig. 5, lanes 6 and 8, in which the dimolar *Kpn*I U fragment (0.025 to 0.043 and 0.777 to 0.795 map units) was larger in *d*61 than in KOS; the same was true for the *Sal*I F fragment (0.740 to 0.784) (Fig. 5, lanes 3 and 5), although this is not clearly visible in the gel. These diploid insertions or duplications mapped at or near the tandem reiterated sequence identified by Rixon et al. (28).

Fourth, *d*61 DNA also carried an insertion or duplication of about 300 bp, which mapped in U$_L$ between coordinates

TABLE 3. Burst sizes[a] of wild-type virus, an ICP8 *ts* mutant, and two ICP8 deletion mutants on complementing and noncomplementing cells

Cells	Virus yield (PFU/cell)				
	KOS, 37°C	*ts*A24		*d*21, 37°C	*d*61, 37°C
		34°C	39.4°C		
Vero	250	23	7.8×10^{-3}	5.2×10^{-3}	7.2×10^{-3}
U-47	92	ND[b]	2.5	62	12

[a] 6.0×10^5 cells were infected at a multiplicity of 1 PFU/cell, washed, incubated at the indicated temperature for 18 h, harvested, and assayed for infectious virus.
[b] ND, Not done.

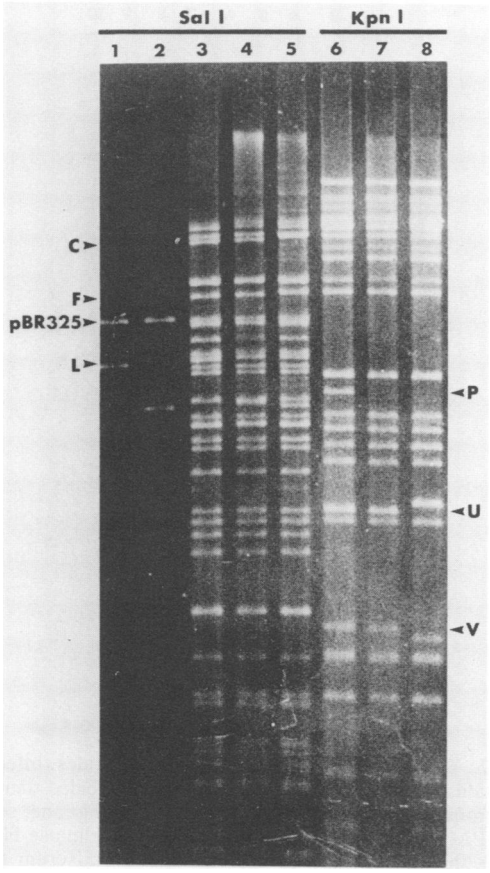

FIG. 5. Agarose gel electrophoresis of plasmid and viral DNA digests. Lanes: 1 to 5, *Sal*I digests; 6 to 8, *Kpn*I digests; 1, pSG18-*Sal*IA; 2, pDX; 3 and 6, KOS; 4 and 7, *d*21; 5 and 8, *d*61. Relevant *Sal*I (left) or *Kpn*I (right) fragments are indicated.

0.060 and 0.063. As a result of this ~300-bp insertion or duplication and the ~100-bp insertion or duplication mentioned above, the *Sal*I C fragment (coordinates 0.036 to 0.095) of *d*61 migrated noticeably more slowly than its KOS counterpart (Fig. 5, lanes 3 and 5). Digestions with several restriction enzymes were used to fine-map the ~100- and ~300-bp alterations in *d*61 DNA (data not shown).

The presence of multiple alterations in the DNA of *d*61 made it unacceptable for genetic and phenotypic analysis, necessitating the construction of a second ICP8 deletion mutant. For this purpose pDX was digested with *Sal*I and *Kpn*I, and the 2,340-bp *Sal*I-*Kpn*I fragment was purified (Fig. 1). This fragment, with map coordinates 0.386-0.406, includes the *Xho*I deletion within the ICP8 gene but excludes ori_L-related sequences. U-47 cells were cotransfected with this *Sal*I-*Kpn*I fragment and infectious KOS DNA, and a deletion mutant, *d*21, was isolated by the same strategy used to identify *d*61. It is notable that homology consisting of 748 bp to the right of the 780-bp deletion in the cotransfected fragment was sufficient to ensure incorporation of the deleted fragment into the viral genome at a frequency similar to that observed when more extensive homology was available (i.e., pDX and the resulting mutant *d*61). Restriction enzyme digestion, agarose gel electrophoresis, and Southern blot analyses revealed that *d*21 had indeed incorporated the 780-bp deletion within ICP8 coding sequences. As shown in Fig. 6, the *Kpn*I P fragment (which contains the engineered *Xho*I deletion in the ICP8 gene) was of the same size in *d*21 and *d*61. Importantly, *d*21 DNA exhibited no detectable change in the mobility of the ori_L-containing *Kpn*I V fragment or any other fragment relative to KOS DNA (Fig. 5 and 6).

Phenotypic characterization of ICP8 deletion mutants. (i) Growth properties. As anticipated, the burst sizes of both *d*21 and *d*61 mutants in nonpermissive Vero cells were negligible and similar to that of the ICP8 mutant *ts*A24 in the same cells at the nonpermissive temperature (Table 3). Clearly, expression of the resident ICP8 gene in U-47 cells was required for growth of *d*21 and *d*61, although virus replication was less efficient than that of KOS in either Vero or U-47 cells. The nearly threefold-lower burst size of KOS in U-47 cells than in Vero cells may reflect an inhibitory effect of the ICP8 induced following infection, such as that seen in U-27 and U-35 cells. Consistent with these observations, the plaques produced by the wild-type virus in U-47 cells were larger than those produced by either *d*21 or *d*61, with *d*61 producing the smallest plaques.

(ii) ICP8 polypeptide synthesis. The ICP8 polypeptides produced in U-47 and Vero cells, mock-infected or infected with either KOS or *d*61, were examined by Western blot analysis (Fig. 7). As mentioned above, U-47 cells do not appear to express ICP8 constitutively; however, an ICP8 polypeptide of the expected size was detected in U-47 cells infected with *d*61 (lane 7), as was the truncated form of ICP8 specified by *d*61. The larger form of ICP8 presumably constitutes the product of the resident wild-type ICP8 gene.

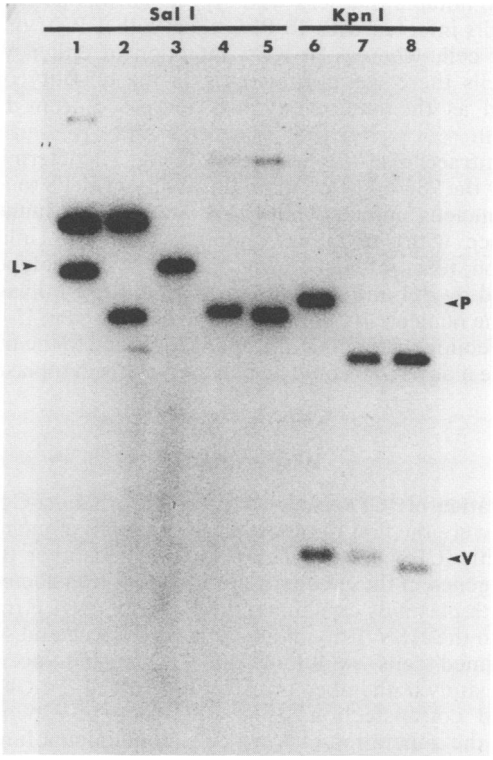

FIG. 6. Southern blot analysis of the gel shown in Fig. 5. The probe used was ^{32}P-labeled pSG18-*Sal*IA (Fig. 1). The *Sal*I L fragment of *d*21 (lane 4) migrated slightly behind the equivalent fragments of pDX and *d*61 (lanes 2 and 5), since only the latter two contain a deletion in ori_L. In the *Kpn*I digests (lanes 6 to 8) the ori_L and ICP8 deletions occur in separate fragments (V and P, respectively).

In Vero cells infected with *d*61, only an ICP8 polypeptide of reduced size was detected (lane 4). The observed size of the truncated polypeptide produced by *d*61 (lanes 7 and 4) agrees well with that predicted from the nucleotide sequence of the ICP8 gene and the size of the deletion in this gene in *d*61 (27). Similar results were obtained with *d*21 (data not shown). As mentioned previously, the *Xho*I deletion introduced into pDX (the plasmid used in the construction of *d*61) should maintain the ICP8 reading frame. Moreover, the loss of 780 bp is equivalent to the deletion of 260 amino acids at the protein level, yielding a polypeptide smaller than wild-type ICP8 by approximately 31,000 daltons. This is in fact the case, as shown in Fig. 7. Additionally, as shown in Fig. 8, the mutant form of ICP8 specified by *d*61, which should have a predicted molecular weight of 97,000, had a slightly greater electrophoretic mobility than ICP15, 103,000 molecular weight (23).

Mutant *d*21 specified an ICP8 polypeptide of the same size as that specified by *d*61 (Fig. 8). The presence of the wild-type form of ICP8 in U-47 cells but not in Vero cells infected with *d*21 or *d*61 was also evident (Fig. 8, lanes 11 to 18). At a multiplicity of 1 PFU/cell, less ICP8 was present in U-47 cells infected with *d*21 or *d*61 than in U-47 cells infected with KOS (lanes 4, 12, and 16). This observation was consistent with the differences in plaque and burst sizes described above. The contribution of the resident wild-type ICP8 gene was required for late gene expression by *d*21 and *d*61, as shown by the fact that polypeptides of the γ_2 kinetic class such as ICPs 15, 19, and 20 were induced by *d*21 and *d*61 only in U-47 and not in Vero cells (Fig. 8, lanes 11 to 18). It is also notable that less wild-type ICP8 was detectable in U-47 cells infected with 10 PFU than with 1 PFU of *d*21 or *d*61 per cell, whereas in KOS infection of either Vero or U-47 cells there was no decrease in the quantity of ICP8 detected as the multiplicity was increased from 1 to 10 PFU/cell.

(iii) Intracellular localization of ICP8. To determine the effect of the 780-bp deletion on the ability of ICP8 to localize to the nucleus, infected cells were analyzed by immunofluorescence with polyclonal antibody to ICP8. Unlike the wild-type form of ICP8, the mutant ICP8 polypeptides specified by *d*21 and *d*61 were present in the cytoplasm and not in the nucleus. By contrast, ICP8 expressed in U-47 cells after infection with either *d*21 or *d*61 localized to the nucleus, and little if any ICP8 could be detected in the cytoplasm (Fig. 9).

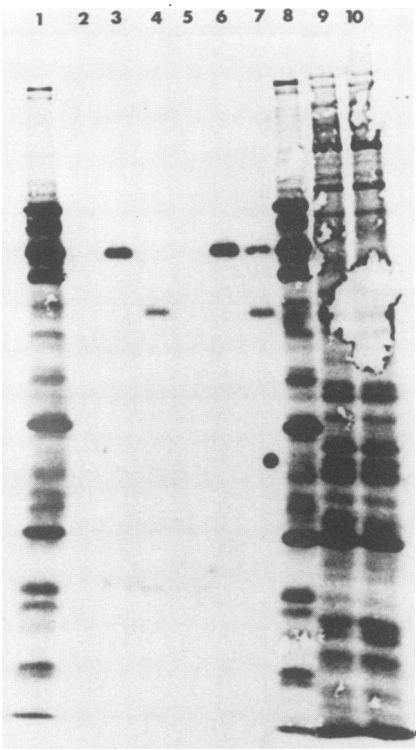

FIG. 7. Western blot analysis of ICP8 polypeptides. Infected-cell polypeptides were not labeled (lanes 2 to 7) or labeled (lanes 1 and 8 to 10) from 5 to 18 h postinfection with [^{35}S]methionine, separated by SDS-PAGE, and electroblotted onto a nitrocellulose filter. The filter was then probed with polyclonal rabbit antiserum to ICP8. Bound antibody was subsequently tagged with ^{125}I-protein A, and the filter was autoradiographed. Cell lines: Nero (lanes 1 to 4 and 8), U-47 (lanes 5 to 7), U-27 (lane 9), or U-35 (lane 10). viruses: KOS (lanes 1, 3, 6, and 8), *d*61 (lanes 4 and 7), or mock infection (lanes 2, 5, 9, and 10). The multiplicity of infection used was 10 PFU/cell, except for 20 PFU/cell in lane 1.

DISCUSSION

Derivation of ICP8-expressing cell lines. Sandri-Goldin et al. (32) were the first to report transformation of mammalian cells with ICP8 and expression of this and other β and γ HSV-1 genes in these cells. These authors transfected Ltk$^-$ cells with plasmids containing the HSV-1 *Eco*RI F fragment linked to the HSV-1 thymidine kinase (*tk*) gene and selected transformed cells exhibiting the TK$^+$ phenotype. In the present study both linked transfections (pKEF-P4-G418) and unlinked cotransfections (pKEF-P4 plus pSV2neo) were used in the generation of Vero cells containing a functional HSV-1 ICP8 gene. The rationale for constructing pKEF-P4-G418 was that covalent linkage of the two genes might increase the probability of isolating cell lines containing the unselected marker (ICP8 gene). On the other hand, because expression of the ICP8 gene in the hybrid plasmid could conceivably be affected by the proximity of the G418 gene and its strong SV40 early promoter, unlinked cotransfections were performed simultaneously. The similarity in the frequency of isolation of cell lines able to complement ICP8 *ts* mutants by the two transfection procedures demonstrated no clear advantage to the linked system.

Complementation of the ICP8 mutant *ts*18 by cells containing stably integrated copies of the HSV-1 ICP8 gene has been reported by Sandri-Goldin et al. (32). These authors assessed complementation by determining virus yields after 48 h of growth at the nonpermissive temperature rather than by plaque assay, as in this study. No constitutive synthesis of ICP8 (or other polypeptides of the β and γ classes) was detected in transformed cells by these investigators, and the stability of the copy number of integrated genes was suggested by widely spaced assays which showed no alteration in the complementing capacity of the transformed cells (32). We report similar findings here.

Cell lines inhibitory to wild-type virus plaque formation and late gene expression. The isolation of the U-27 and U-35 cell lines was of special interest. The amplification of the ICP8 sequences transfected into these cells was spontaneous in the sense that an unselected marker which was not physically linked to the selected marker was amplified. The amplification of the transfected ICP8 sequences was not a rare occurrence in that it was detected in 4% of the cell lines tested (2 of 48). Pülm and Knippers (25) transfected Ltk$^-$ cells with plasmids carrying the HSV-1 *tk* gene and studied

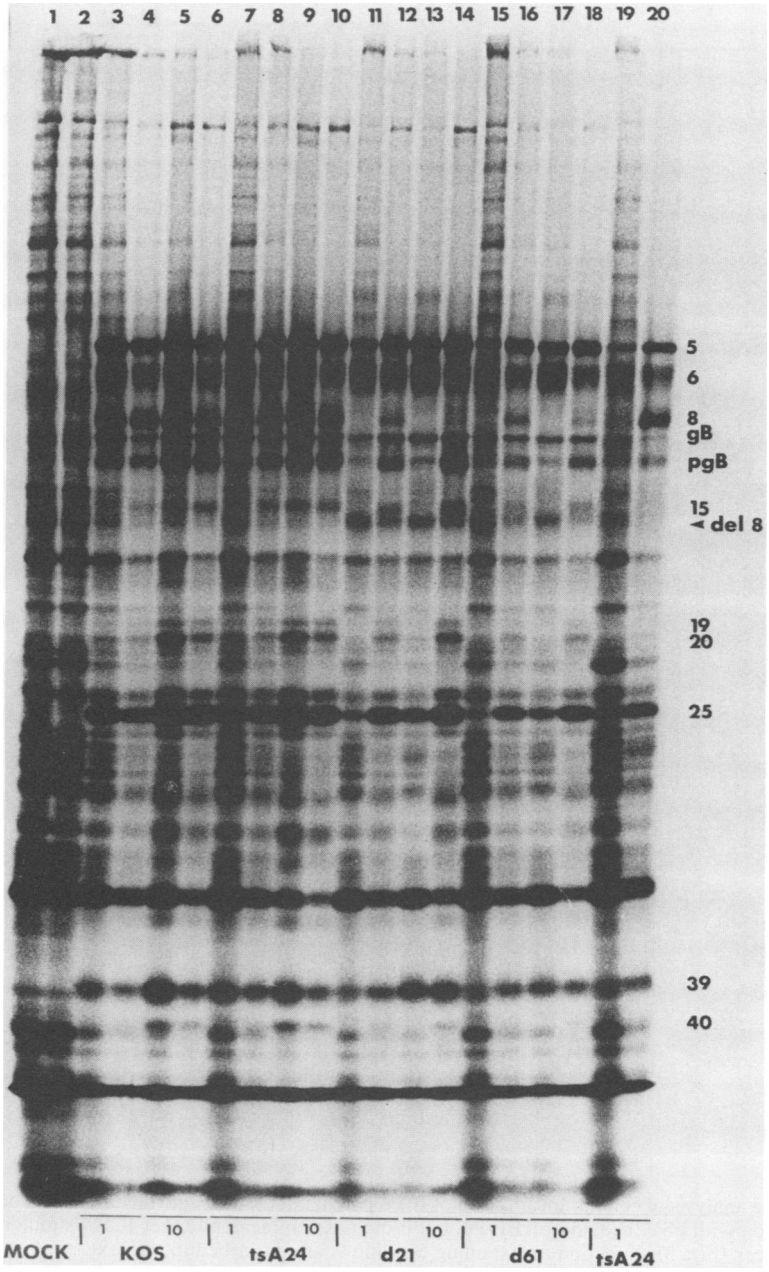

FIG. 8. SDS-PAGE of infected-cell polypeptides: comparison of KOS, *ts*A24, *d*21, and *d*61 in Vero and U-47 cells. Infected-cell polypeptides were labeled with [^{35}S]methionine from 5 to 18 h postinfection. Odd-numbered lanes contain polypeptides from Vero cells; even-numbered lanes contain polypeptides from U-47 (ICP8-expressing) cells. The multiplicity of infection used was 1 PFU/cell (lanes 3, 4, 7, 8, 11, 12, 15, 16, 19, and 20) or 10 PFU/cell (lanes 5, 6, 9, 10, 13, 14, 17, and 18). The temperature of infection was 34°C (39.4°C in lanes 19 and 20). Lanes 1 and 2, mock infection; lanes 3 to 6, KOS; lanes 7 to 10, *ts*A24; lanes 11 to 14, *d*21; lanes 15 to 18, *d*61; lanes 19 and 20, *ts*A24. The ICPs are numbered on the right. The smaller ICP8 polypeptide specified by *d*21 and *d*61 is indicated by an arrow (del 8).

the number and structure of integrated gene copies in 65 resulting cell lines. One of these lines exhibited wide variation in the size of integrated *tk* sequences and was estimated to contain 85 copies of *tk* per cell (25). These findings closely resemble our findings for U-27 and U-35 cell lines, except that in the present case an unselected marker was amplified.

To our knowledge, inhibition of HSV replication in cells carrying multiple copies of an HSV gene has not been reported previously. U-27 and U-35 cells offer a unique opportunity to examine the regulatory role of ICP8 in the HSV-1 growth cycle. The data presented here constitute independent evidence for the participation of ICP8 in the negative regulation of β, γ_1, and γ_2 viral genes postulated by others (10–12), since viral polypeptides of these kinetic classes were markedly underproduced concomitantly with overproduction of ICP8.

It is notable that the inhibitory effect of the resident ICP8 gene in U-27 and U-35 cells was reduced at a high multiplicity of infection and that less ICP8 was actually made in these cells at this multiplicity than at a multiplicity of 1 PFU/cell.

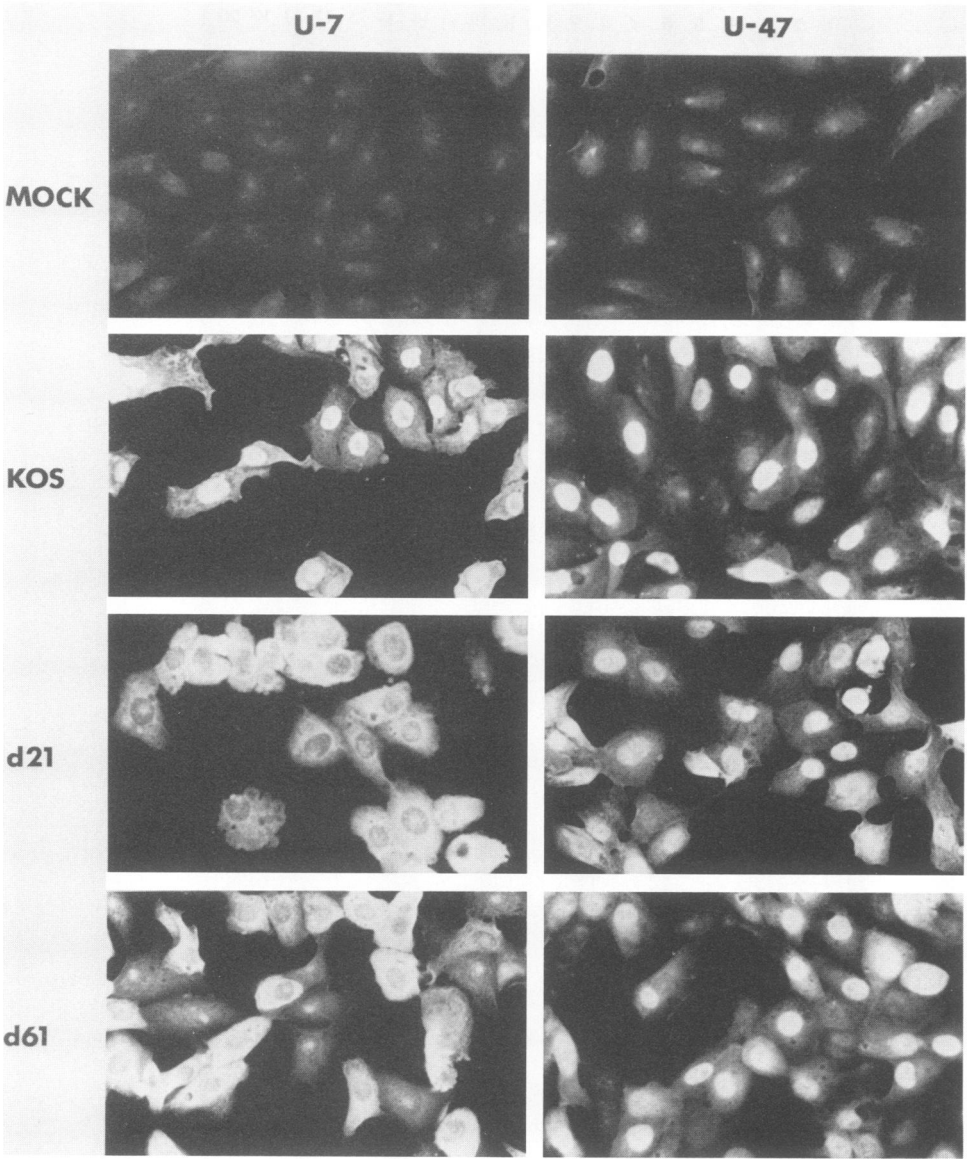

FIG. 9. Immunofluorescence analysis of cellular localization of ICP8 polypeptides. The cell line used as a control, U-7, was G418 resistant and obtained after cotransfection with pSV2neo and pKEF-P4, but it did not complement either ICP8 *ts* mutant (*ts*A24 or *ts*A42), nor did it contain any ICP8 gene sequences (Fig. 3). Infection and staining conditions are described in the text.

The same effect was observed in U-47 cells. In a similar vein, Leiden et al. observed that more tk was expressed in transformed Ltk$^+$ cells infected with a *tk*$^-$ mutant of HSV-1 at a multiplicity of 2 PFU/cell than at 5 or 10 PFU/cell (20). Sandri-Goldin et al. (32) also reported that the induction of glycoprotein gB and ICP8 from resident genes in cells transfected with the *Eco*RI F fragment of HSV-1 decreased as the multiplicity of infection increased. Similarly, the optimum multiplicity of infection of induction of glycoprotein gC from a gene integrated into mouse cells was 2 PFU/cell, and no gC could be detected at a multiplicity of 10 PFU/cell (1). Sandri-Goldin et al. (32) suggested that these effects could be due to the virus-induced early host protein shutoff function (15), which might decrease expression of integrated viral genes as it would for bona fide cellular genes.

On the other hand, it is notable that at low multiplicity, unexpectedly large amounts of ICP8 polypeptide were expressed in U-27 and U-35 cells (even considering the high gene copy number), since ICP8 is known to repress its own synthesis in the context of the viral genome (12). This may reflect the need for an additional viral protein(s) for the shutoff of ICP8. This protein(s) would not be present in sufficient amounts to mediate suppression during infection at low multiplicity.

In addition to their utility for studies of the regulation of HSV-1 gene expression, the U-27 and U-35 cell lines should prove to be excellent sources of wild-type ICP8 protein for use in antiserum production or other studies.

ICP8 deletion mutants. The primary reason for initiating the present study was the desire to examine HSV-1 gene expression in the absence of functional ICP8 without the complicating factors of leak and reversion. DeLuca et al.

have shown that a *ts* form of ICP4 can lower the efficiency of complementation by ICP4 expressed from a wild-type ICP4 gene (7). This interference was not observed in two ICP4 deletion mutants isolated and studied by those authors. Because a similar situation could conceivably occur with respect to ICP8, we constructed the deletion mutants described here and compared them with the ICP8 *ts* mutant *ts*A24. Under nonpermissive conditions, the phenotypes were equally restricted for both types of mutant. Additionally, complementation of *ts*A24, *d*21, and *d*61 by resident ICP8 as seen in the polypeptide phenotype was much more efficient at 1 PFU/cell than at 10 PFU/cell. However, unlike the situation observed with ICP4 (7), there was no significant difference in the polypeptide phenotypes of *ts*A24, *d*21, or *d*61 at either multiplicity of infection. This suggests that the ICP8 polypeptide encoded by *ts*A24 has no significant inhibitory effect on complementation by the wild-type ICP8 made in U-47 cells. An alternative explanation is that if such an effect exists, it cannot be detected because at high multiplicity—when an inhibitory effect would be most readily observed—expression of resident wild-type ICP8 is turned down and therefore complementation is already too inefficent for further impairment to be detected. This hypothesis is supported by the fact for all three ICP8 mutants, the polypeptide phenotypes at a multiplicity of 10 PFU/cell were similar in complementing (U-47) and in noncomplementing (Vero) cells (Fig. 8).

The defective ICP8 polypeptides specified by *d*21 and *d*61 were excluded from the cellular nucleus, as were the *ts* forms of ICP8 expressed by *ts*A24 and most other ICP8 *ts* mutants (39). For this reason it is unlikely that *d*21 and *d*61, like *ts*A24 at 39°C, induce the synthesis of viral DNA. By contrast to these mutants, *ts*HA1 (4) produces an ICP8 molecule that can associate with the cell nucleus (19), although abnormally (26). This is consistent with the fact that there is only a small overlap between the engineered deletion in *d*21 and *d*61 (which eliminates amino acids 328 through 587 on the ICP8 molecule [27]) and the mutation site in *ts*HA1 (between amino acids 563 and 685; D. Knipe, personal communication). *ts*HA1 is also DNA negative at the nonpermissive temperature, indicating that this phenotype is a consequence of alterations in ICP8 not associated with nuclear localization. Whether the 780-bp deletion in *d*21 and *d*61 includes the nuclear localization signal or whether failure to reach the nucleus reflects a folding problem in the truncated form of the protein remains to be determined. Interestingly, virtually no cytoplasmic ICP8 could be detected in U-47 cells infected with either deletion mutant, suggesting that the wild-type form of the polypeptide may have facilitated the nuclear localization of the defective form of the protein.

The deletion mutants generated in this study should prove useful for studies of the in vivo biology of HSV-1, the influence of the deleted domain on ICP8 DNA-binding capacity (19, 30), and interactions of ICP8 with other viral proteins (3, 38).

ACKNOWLEDGMENTS

We thank N. DeLuca for valuable discussions, R. Byrn, N. DeLuca, M. Polvino-Bodnar, and W. Sacks for assistance in many of the experiments, D. Coen for critical reading of the manuscript, and M. Datz for manuscript preparation.

This investigation was supported by Public Health Service grant CA-21082 from the National Cancer Institute. P.K.O. was supported by Postdoctoral Fellowship DRG-840 from the Damon Runyon-Walter Winchell Cancer Fund.

LITERATURE CITED

1. **Arsenakis, M., L. F. Tomasi, V. Speziali, B. Roizman, and G. Campadelli-Fiume.** 1986. Expression and regulation of glycoprotein C gene of herpes simplex virus 1 resident in a clonal L-cell line. J. Virol. **58**:367–376.
2. **Burnette, W. N.** 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with an antibody and radioiodinated protein A. Anal. Biochem. **112**: 195–203.
3. **Chiou, H. C., S. K. Weller, and D. M. Coen.** 1985. Mutations in the herpes simplex virus major DNA-binding protein gene leading to altered sensitivity to DNA polymerase inhibitors. Virology **145**:213–226.
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. **DeLuca, N., D. Bzik, V. C. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein gB (VP7). Virology **122**:411–423.
6. **DeLuca, N. A., M. A. Courtney, and P. A. Schaffer.** 1984. Temperature-sensitive mutants in herpes simplex virus type 1 ICP4 permissive for early gene expression. J. Virol. **52**:767–776.
7. **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.
8. **DeLuca, N. A., and P. A. Schaffer.** 1985. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol. Cell. Biol. **5**:1997–2008.
9. **Fenwick, M. L., M. J. Walker, and J. M. Petkevich.** 1978. On the association of virus proteins with the nucleus of cells infected with herpes simplex virus. J. Gen. Virol. **39**:519–529.
10. **Godowski, P. J., and D. M. Knipe.** 1983. Mutations in the major DNA-binding protein gene of herpes simplex virus type 1 result in increased levels of viral gene expression. J. Virol. **47**:478–486.
11. **Godowski, P. J., and D. M. Knipe.** 1985. Identification of a herpes simplex virus function that represses late gene expression prior to DNA replication. J. Virol. **55**:357–365.
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. **Gray, C. P., and H. C. Kaerner.** 1984. Sequence of the putative origin of replication in the U_L region of herpes simplex virus type 1 ANG DNA. J. Gen. Virol. **65**:2109–2119.
15. **Hill, T. M., R. R. Sinden, and J. R. Sadler.** 1983. Herpes simplex virus types 1 and 2 induce shutoff of host protein synthesis by different mechanisms in Friend erythroleukemia cells. J. Virol. **45**:241–250.
16. **Holland, L. E., R. M. Sandri-Goldin, A. L. Goldin, J. C. Glorioso, and M. Levine.** 1984. Transcriptional and genetic analysis of the herpes simplex virus type 1 genome: coordinates 0.29 to 0.45. J. Virol. **49**:957–959.
17. **Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder.** 1984. Improved technique utilizing nonfact dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Tech. **1**:3–8.
18. **Knipe, D. M., and A. E. Spang.** 1982. Definition of a series of stages in the association of two herpes viral proteins with the cell nucleus. J. Virol. **43**:314–324.
19. **Lee, C. K., and D. M. Knipe.** 1983. Thermolabile in vivo DNA-binding protein gene of herpes simplex virus type 1 results in increased levels of viral gene expression. J. Virol. **46**:

909–919.
20. **Leiden, J. M., R. Buttyan, and P. G. Spear.** 1976. Herpes simplex virus gene expression in transformed cells. I. Regulation of the viral thymidine kinase gene in transformed L cells by products of superinfecting virus. J. Virol. **20**:413–424.
21. **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.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 382–389. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
23. **Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer.** 1978. Anatomy of herpes simplex virus (HSV) DNA. XI. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 × HSV-2 recombinants. J. Virol. **26**:389–410.
24. **Powell, K. L., E. Littler, and D. J. M. Purifoy.** 1981. Nonstructural proteins of herpes simplex virus. II. Major virus-specific DNA-binding protein. J. Virol. **39**:894–902.
25. **Pülm, W., and R. Knippers.** 1985. Transfection of mouse fibroblast cells with a promoterless herpes simplex virus thymidine kinase gene: number of integrated gene copies and structure of single and amplified gene sequences. Mol. Cell. Biol. **5**:295–304.
26. **Quinlan, M. P., L. B. Chen, and D. M. Knipe.** 1984. The intranuclear location of a herpesviral DNA-binding protein is determined by the status of viral DNA replication. Cell **36**: 857–868.
27. **Quinn, J. P., and D. J. McGeoch.** 1985. DNA sequence of the region in the genome of herpes simplex virus type 1 containing the genes for DNA polymerase and DNA-binding protein. Nucleic Acids Res. **13**:8143–8163.
28. **Rixon, F. J., M. E. Campbell, and J. B. Clements.** 1984. A tandemly reiterated DNA sequence in the long repeat region of herpes simplex virus type 1 found in close proximity to immediate-early mRNA 1. J. Virol. **52**:715–718.
29. **Ruyechan, W. T.** 1983. The major herpes simplex virus DNA-binding protein holds single-stranded DNA in an extended configuration. J. Virol. **46**:661–666.
30. **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.
31. **Sacks, W. R., C. C. Greene, D. P. Aschman, and P. A. Schaffer.** 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J. Virol. **55**:796–805.
32. **Sandri-Goldin, R. M., A. L. Goldin, L. E. Holland, J. C. Glorioso, and M. Levine.** 1983. Expression of herpes simplex virus β and γ genes integrated in mammalian cells and their induction by an α gene product, Mol. Cell. Biol. **3**:2028–2044.
33. **Schaffer, P. A., V. C. Carter, and M. C. Timbury.** 1978. Collaborative complementation study of temperature-sensitive mutants of herpes simplex virus types 1 and 2. J. Virol. **27**: 490–504.
34. **Smith, H. O.** 1980. Recovery of DNA from gels. Methods Enzymol. **65**:371–380.
35. **Southern, K. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
36. **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.
37. **Spear, P. G., and B. Roizman.** 1980. Herpes simplex viruses, p. 615–745. *In* J. Tooze (ed.), DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor. N.Y.
38. **Vaughan, P. J., L. M. Banks, D. J. M. Purifoy, and K. L. Powell.** 1984. Interactions between herpes simplex virus DNA-binding proteins. J. Gen. Virol. **65**:2033–2041.
39. **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.
40. **Weller, S. K., A. Spadaro, J. E. Schaffer, A. W. Murray, A. M. Maxam, and P. A. Schaffer.** 1985. Cloning, sequencing and functional analysis of ori_L, an HSV-1 origin of DNA synthesis. Mol. Cell. Biol. **5**:930–942.