JOURNAL OF VIROLOGY, Sept. 1985, p. 796–805
0022-538X/85/090796-10$02.00/0

Vol. 55, No. 3

Herpes Simplex Virus Type 1 ICP27 Is an Essential Regulatory Protein

WENDY R. SACKS, CONSTANCE C. GREENE, DORIS P. ASCHMAN, 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 11 March 1985/Accepted 14 May 1985

The five immediate-early genes of herpes simplex virus are expressed during the initial stages of the infectious cycle, and certain immediate-early proteins have been shown to play a regulatory role in subsequent viral gene expression. Until recently, the functional properties of only one immediate-early protein, ICP4, had been examined in any detail, primarily because mutants had been isolated only in the gene for ICP4. We report herein the genetic and phenotypic characterization of four temperature-sensitive mutants of herpes simplex virus type 1 (*ts*Y46, *ts*E5, *ts*E6, and *ts*LG4) that have begun to elucidate the function(s) of a second immediate-early protein, ICP27. The four mutants complemented each other inefficiently or not at all, indicating that they are defective in the same function. Marker rescue tests placed the mutations in *ts*Y46 and *ts*E5 in sequences that encode the transcript for ICP27; the mutations in *ts*E6 and *ts*LG4 lie in or near these sequences. The ability of wild-type ICP27 expressed from a cloned gene to complement *ts*Y46 and *ts*LG4 constitutes additional evidence that these mutants are defective in an ICP27-associated function. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of mutant-infected cell polypeptides showed that certain immediate-early (α) polypeptides were overproduced, whereas significant levels of early (β) and drastically reduced levels of several late (γ) proteins were synthesized at the nonpermissive temperature. Interestingly, the mutants were observed to form a spectrum with regard to their relative abilities to induce the expression of a number of polypeptides, especially those of the delayed-early (βγ) class. Consistent with their ability to induce expression of early polypeptides, all of the mutants induced the synthesis of substantial levels of viral DNA at the nonpermissive temperature. Taken together, the results of these studies demonstrate (i) that ICP27 plays an essential regulatory function in virus replication, (ii) that this function is required after the onset of early gene expression and viral DNA synthesis, and (iii) that the inability of the mutants to induce the synthesis of late proteins is independent of viral DNA synthesis.

Herpes simplex virus type 1 (HSV-1) specifies at least four classes of proteins—immediate early (α), early (β), delayed early (βγ), and late (γ)—based on the times of their maximum rates of synthesis and their requirements for expression (17). The immediate-early proteins are expressed first during the infectious cycle in the absence of prior viral protein synthesis and are thought to play a role in the regulation of subsequent viral gene expression (17, 18). Regulation is thought to be mediated mainly at the level of transcription, and the differences in regulation exhibited by the four classes of genes reflect—at least in part—differences in their promoter-regulatory sequences (13, 18, 32, 34, 35, 43).

To date, five proteins in the immediate-early class of polypeptides have been identified and mapped: ICP0, -4, -22, -27, and -47 (17, 31, 37). Until recently, the functional properties of only one of these proteins, ICP4, had been examined in any detail primarily because temperature-sensitive mutants were available only with mutations in the gene for ICP4. By using these mutants, ICP4 has been shown to play an essential role throughout the viral replicative cycle regulating the expression of early, delayed-early, and late genes (6, 11, 34–36, 42). Whether these regulatory effects are direct or indirect and whether ICP4 acts alone or in concert with other immediate-early polypeptides remain open questions.

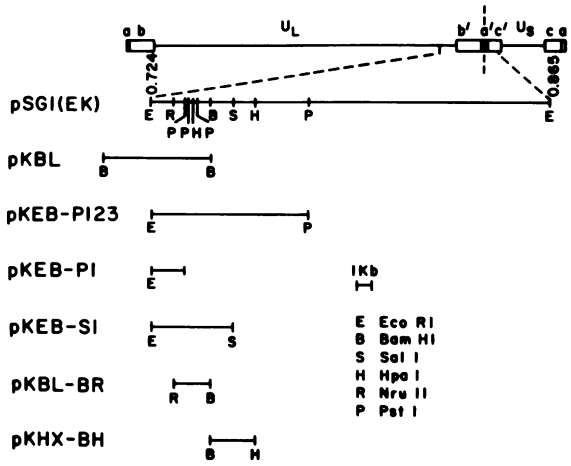
As in the case of ICP4, mutants (and cloned viral genes) have proven useful in efforts to elucidate the functions of other immediate-early genes. Thus, through the characterization of deletion and insertion mutants, ICP22 has been shown to be essential for virus replication in some cell types, but not in others (33; I. W. Halliburton, L. E. Post, and B. Roizman, Abstr. 8th Intl. Herpesvirus Workshop, Oxford, England, 1983, p. 19). Through the use of plasmids able to express various combinations of immediate-early genes after transfection, evidence has begun to accumulate that ICP0 is similar to ICP4 in its ability to stimulate expression from early promoters (10a, 27, 37a). Whether ICP0, -27, and -47 perform essential functions during replication has not yet been established. Clearly, the availability of *ts* mutants in ICP0, -27, and -47 would be useful in elucidating the functions of these proteins and in evaluating the possibility of interplay among immediate-early gene products in mediating viral gene expression.

We describe herein studies of four *ts* mutants of HSV-1 whose mutations reside in the gene encoding ICP27. Genetic and phenotypic characterizations of these mutants demonstrate that ICP27, like ICP4, performs an essential role in the regulation of viral gene expression.

MATERIALS AND METHODS

Cells and Virus. African green monkey kidney (Vero and CV-1) and human embryonic lung (HEL) cells were propagated in Dulbecco modified Eagle minimal essential medium containing 10% fetal calf serum, 0.03% glutamine, and 0.25% $NaHCO_3$. Vero cells were utilized for growth of virus stocks, plaque assays, complementation tests, infectivity assays,

* Corresponding author.



Plasmid	Map Coordinates	Marker Rescue Efficiency ($\times 10^4$) With the Following Mutant DNAs		
		tsY46	tsE5	tsE6
pSG1	0.724-0.865	195	580	7138
pKBL	0.707-0.745	0.036	0.200	0.140
pKEB-P123	0.724-0.780	339	300	127
pKEB-P1	0.724-0.736	<0.042	<0.037	<0.053
pKEB-S1	0.724-0.753	<0.031	<0.027	0.019
pKBL-BR	0.732-0.745	<0.038	0.047	<0.078
pKHX-BH	0.745-0.761	44	3.3	56
No Fragment	--	<0.045	<0.030	0.078

FIG. 1. Marker rescue of *ts* mutations in *ts*Y46, *ts*E5, and *ts*E6. The region of the HSV-1 strain KOS genome between the *Bam*HI site at map coordinate 0.707 and the *Eco*RI site at coordinate 0.865 has been expanded to show the locations of subclones of the *Eco*RI-EK and *Bam*HI-L fragments used in marker rescue experiments with the three mutants. The locations of restriction sites between the *Eco*RI and *Pst*I sites at coordinates 0.724 and 0.780, respectively, are those of Bond and Person (4). The results of marker rescue experiments in which purified mutant DNAs were cotransfected with individual linearized plasmids are shown on the right and are expressed as $[(PFU/ml)_{39°C}/(PFU/ml)_{34°C}] \times 10^4$.

and marker rescue experiments. Infectious viral DNA was isolated from infected HEL cells.

Stocks of the KOS strain of HSV-1 and the *ts* mutants used in this study were prepared and assayed as described previously (41). *ts*Y46, *ts*E5, and *ts*E6 were derived from KOS as described below. *ts*LG4, derived from KOS strain 1.1 (39), was kindly provided by R. Sandri-Goldin (University of California, Irvine). Unless otherwise stated, 34°C was the permissive temperature and 39°C was the nonpermissive temperature for virus growth and assays.

Recombinant DNA plasmids. The genomic locations of viral DNA inserts in the plasmids used in this study are shown in Fig. 1. Plasmid pSG1 containing the *Eco*RI-EK fragment (coordinates 0.724 to 0.865) in pBR325 (15) was provided by R. Sandri-Goldin and propagated in DH-1, a derivative of *Escherichia coli* K-12 strain 1100. The remaining plasmids were provided by Stan Person (The Pennsylvania State University, University Park) and propagated in *E. coli* strain RR1 (4). Plasmids were propagated in the prescribed hosts as described by DeLuca et al. (8).

DNA isolation. Preparative quantities of plasmid DNA were obtained by the method of Birnboim and Doly (3) and further purified by banding in CsCl.

Infected cell DNA was isolated as described by DeLuca et al. (10).

Isolation and mapping of *Sma*I fragments of pKHX-BH. Subfragments of pKHX-BH for marker rescue experiments were generated by digestion of the plasmid with *Sma*I (Fig. 2) and isolated by electroelution into dialysis bags as described by Maniatis et al. (23), except that 17.8 mM Tris–17.8 mM borate–0.04 mM EDTA (pH 8.0) was used as the elution buffer. DNA was then extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1), ethanol precipitated, and suspended in HBS (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 140 mM NaCl, 0.75 mM Na_2HPO_4).

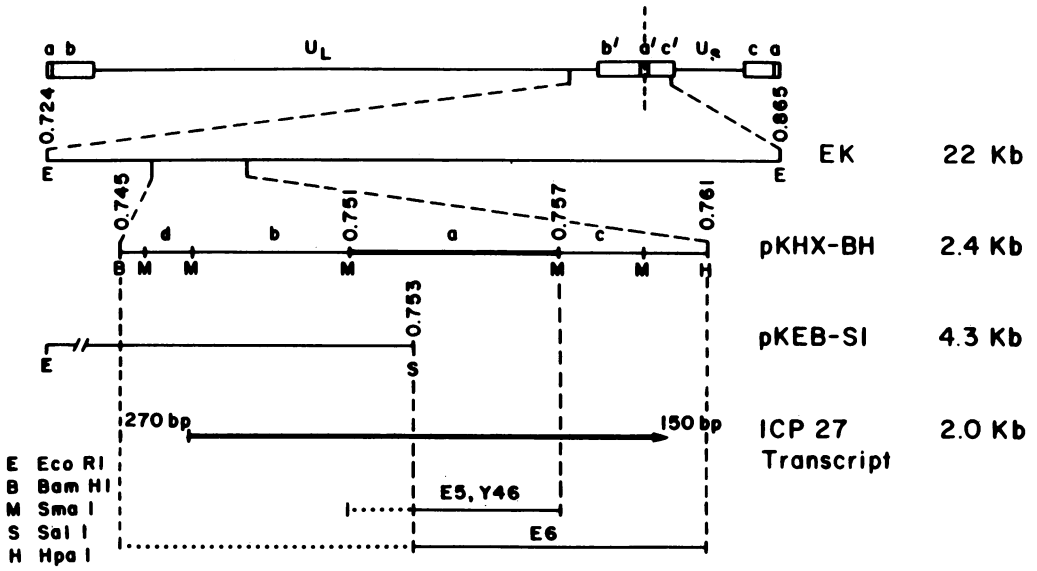
The locations of the *Sma*I sites within the plasmid (Fig. 2) were determined by first cleaving with *Bam*HI, subjecting the linearized plasmid to BAL 31 digestion, removing samples at 5-min intervals, and digesting with *Sma*I. Progressive loss of *Sma*I restriction sites identified their locations with respect to the *Bam*HI site.

Genetic analysis. Marker rescue experiments were carried out as described by Weller et al. (45) with the transfection protocol of Parris et al. (29), except that DNA was precipitated with $CaCl_2$ for 30 min and Vero cells were used.

Complementation analysis was carried out as described previously (40).

Complementation with cloned genes. Complementation of *ts* mutants by proteins expressed transiently from cloned genes was carried out by using a procedure developed by N. DeLuca (personal communication); this procedure is an extension of the monolayer transfection technique of Gorman et al. (16). CV-1 cells were utilized for this procedure because they were found to be more efficiently transfected than Vero cells. Briefly, the cells were plated in 35-mm dishes ($\sim 2 \times 10^5$ cells per dish) and transfected in duplicate after 24 h with 0.3 μg of plasmid DNA and 4.1 μg of salmon sperm DNA in 0.2 ml of HBS. Four hours later, cells were subjected to treatment with 15% glycerol in HBS for 2 min. Approximately 24 h after glycerol treatment, cells were infected at a multiplicity of infection of 2 PFU per cell. After adsorption at 39°C for 60 min, monolayers were washed three times, incubated in growth medium at 39°C, harvested at 18 h postinfection, and processed as for virus stocks (41), and progeny virus was assayed at 34 and 39°C.

Analysis of infected cell polypeptides. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of radioactively labeled infected cell lysates was performed by the method of Laemmli (19) as modified by Manservigi et al. (24). Monolayers of HEL cells were infected with the indicated virus at a multiplicity of infection of 5 to 10 PFU per cell. Adsorption for 1 h and subsequent incubations were carried out at the indicated temperatures. At the times indicated, 10 to 20 μCi of [^{35}S]methionine per ml, 20 μCi of ^{32}P per ml, or 50 μCi of [^{3}H]mannose per ml (Amersham Corp., Arlington Heights, Ill.) was added at the times indicated. At 15 to 17 h postinfection, cultures were washed with cold saline and solubilized directly in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample solution. Samples were



Mutant Viral DNA	Marker Rescue Efficiency (x 10^4) With pKHX-BH Sma I Fragment d	b	a	c	Vector-associated Fragments	pKHX-BH linearized with Bam HI	pKEB-S1 linearized with Eco RI	No Fragment
tsY46	<0.006	<0.028	326	<0.019	<0.023	818	<0.017	<0.017
tsE5	<0.012	<0.016	4	<0.017	<0.028	200	ND	<0.013

FIG. 2. Fine mapping of *ts* mutations in *ts*Y46 and *ts*E5. The *Eco*RI fragment EK, which rescued the *ts* mutations in all four mutants, is located between map coordinates 0.724 and 0.865. Beneath it, the *Bam*HI-*Hpa*I fragment of pKHX-BH, which was also shown to rescue the three *ts* mutations in Fig. 1, is expanded to show the locations of the *Sma*I sites within it. Subfragments generated by *Sma*I digestion are designated by lowercase letters on the basis of decreasing size. Subfragment *a* (850 bp), which rescued the mutations in *ts*E5 and *ts*Y46, is shown as a bold line. Next is shown the *Eco*RI-*Sal*I fragment of pKEB-S1, which overlaps subfragment *a* and was shown in Fig. 1 not to rescue the three mutations. The location and orientation of the transcript for ICP27 within pKHX-BH is shown next, above the regions which contain the *ts* mutations in *ts*Y46, *ts*E5, and *ts*E6 on the basis of the marker rescue data presented in this figure and in Fig. 1. Dotted lines to the left of the *Sal*I site at coordinate 0.753 denote regions of uncertainty. The results of marker rescue experiments of *ts*Y46 and *ts*E5 DNAs with the gel-purified *Sma*I subfragments of pKHX-BH or the restricted plasmids indicated are shown at the bottom and are expressed as in Fig. 1. The vector-associated fragments include pBR322 derived sequences and HSV sequences remaining after *Sma*I digestion of pKHX-BH. ND, Not done.

then heated at 97°C for 5 min, loaded onto a 9% *N-N'*-diallyltartardiamide cross-linked polyacrylamide gel, and electrophoresed for 17 h at 100 V. After electrophoresis, gels were fixed in methanol-water-acetic acid (5:5:1), equilibrated with water, treated in some cases with Autofluor (National Diagnostics, Somerville, N.J.), dried, and exposed to XAR-5 X-ray film.

Viral DNA phenotypes. HEL cell monolayers were infected at a multiplicity of infection of 5 PFU per cell, incubated at 39°C, and labeled from 4 to 24 h postinfection with 10 μCi of [^{3}H]thymidine per ml. DNA extracts were prepared and analyzed by CsCl equilibrium centrifugation as described by Aron et al. (2), except that 200 μg of proteinase K per ml was used instead of pronase.

RESULTS

Origin of *ts* mutants. The four *ts* mutants examined in this study were generated by three mutagenic procedures. Mutant *ts*Y46 was generated by 2-amino purine mutagenesis of HSV-1 strain KOS and selected for its ability to render infected cells susceptible to immune cytolysis at the permissive temperature, but not the nonpermissive temperature, with complement and polyclonal antiserum to the viral glycoprotein, gB (21, 28). *ts*E5 and *ts*E6 were generated by bromodeoxyuridine mutagenesis of KOS (40). *ts*LG4 was isolated from strain KOS1.1 as described by Sandri-Goldin et al. (39). The latter three mutants were selected for temperature sensitivity of growth at 39°C.

Genetic analysis. (i) Marker rescue. Earlier studies of HSV-1 × HSV-2 recombinants by Morse et al. (25) were consistent with the possibility that the *ts* mutations in *ts*E5 and *ts*E6 lay between map coordinates 0.12 and 0.22. However, the results obtained by using more precise mapping procedures, i.e., marker rescue tests, indicate that these mutations map to sequences within the *Eco*RI joint fragment, EK, but not to the joint fragment JK, suggesting that they map to sequences at the right end of U_L (coordinates 0.724 to ~0.766) in the prototype orientation of the genome (D. Aschman, unpublished results). Likewise, both *ts*Y46 and *ts*LG4 have been mapped to EK, but not JK (15, 28).

(ii) Complementation. Because the mutations in all four mutants had been shown to lie within the ~6.3-kilobase region at the right-hand terminus of U_L, complementation tests were performed to ascertain the number of independent functions represented by the mutants. Earlier complementation tests (28) had shown that *ts*Y46 did not complement

TABLE 1. Complementation among *ts* mutants with mutations mapping between coordinates 0.724 and 0.766

Mutant	Complementation index[a] with the following mutant:			
	*ts*Y46	*ts*E5	*ts*E6	*ts*J12
*ts*LG4	1.6	5.0	7.2	225
*ts*Y46		2.0	1.3	417
*ts*E5			0.54	282
*ts*E6				218

[a] Complementation indices of less than 2 are considered to be negative; indices of 2 to 10 are considered to be negative or of questionable significance; values greater than 10 are considered positive.

*ts*LG4, which was the only other mutant then known to map to the far right end of U_L. The results shown in Table 1 demonstrate that all four mutants complemented one another poorly or not at all, whereas all four exhibited efficient complementation with the control mutant, *ts*J12, a mutant defective in glycoprotein gB (10, 20, 24). These data suggest that the four mutants contain mutations in the same gene.

(iii) Fine mapping by marker rescue. To further localize the mutations in *ts*Y46, *ts*E5, and *ts*E6 (the KOS1.1-derived mutant *ts*LG4, isolated and mapped by R. Sandri-Goldin, was not included in these tests), plasmids containing different subclones of sequences comprising the right-hand terminus of U_L were used in marker rescue experiments with the mutant viral DNAs (Fig. 1). Efficient rescue of the wild-type phenotype by plasmids pKEB-P123 and pKHX-BH, but not pKEB-S1, places the *ts* mutations in all three mutants between the *Bam*HI (coordinate 0.745) and *Hpa*I (coordinate 0.761) sites and most probably between the *Sal*I and *Hpa*I sites at coordinates 0.753 and 0.761, respectively.

Plasmid pKHX-BH, containing the smallest fragment that rescued all three mutations (coordinates 0.745 to 0.761), was next cleaved with *Sma*I into five subfragments, the order of which was determined by BAL 31 digestion as described above. These subfragments were tested individually in marker rescue experiments (Fig. 2). Only the 850-base-pair (bp) fragments, *a*, exhibited efficient rescue with *ts*Y46 and *ts*E5, placing the two mutations definitively between coordinates 0.751 and 0.757. However, because there is some overlap between the right end of pKEB-S1, which did not rescue, and fragment *a*, which did, the *ts* mutations in *ts*Y46 and *ts*E5 most probably lie in the 700-bp region between coordinates 0.753 and 0.757 (Fig. 2). Technical difficulties with the transfection of *ts*E6 DNA prevented our obtaining meaningful results with this mutant.

As part of studies which examined the promoter-regulatory regions of immediate-early genes, Mackem and Roizman (22) localized the initiation site of the ICP27 transcript to a position 270 bp to the right of the *Bam*HI site at coordinate 0.745 (Fig. 2). The sequence encoding the 2-kilobase ICP27 transcript, then, comprises the majority of the 2.4-kilobase *Bam*HI-*Hpa*I fragment of plasmid pKHX-BH, which exhibited efficient rescue with mutants *ts*Y46, *ts*E5, and *ts*E6. Moreover, the 850-bp sequence that rescued the mutations in *ts*Y46 and *ts*E5 lies entirely within this region.

(iv) Complementation with cloned genes. As an alternative means of determining the physical location of *ts* mutations, complementation tests were carried out in which the ability of wild-type ICP27 encoded by plasmid pKHX-BH to complement *ts*Y46 and *ts*LG4 at the nonpermissive temperature was assessed. For this purpose, monolayers of CV-1 cells were transfected with the plasmid; after allowing 24 h for the expression of the protein, cells were infected at 39°C with the viruses to be tested, and progeny virus was harvested after 18 h. The yield of progeny virus was determined by plaque assays at 34°C, and titers were compared with those obtained in parallel experiments in which unrelated plasmids were used. The growth of *ts*Y46 and *ts*LG4 was significantly enhanced at the nonpermissive temperature in the presence of the plasmid pKHX-BH compared with pKEB-X2, which contains the gene for ICP4 (9), or with pBR325 (Table 2). In contrast to the results obtained with *ts*Y46 and *ts*LG4, growth of *ts*E5 and *ts*E6 was not enhanced in cells transfected with any of the plasmids utilized in Table 2 or with pKEB-P123 (data not shown), for reasons that are unclear. Growth of *ts*B32, a mutant defective in ICP4 (11), on the other hand, was enhanced in pKEB-X2-transfected cells relative to those transfected with pKHX-BH, demonstrating the specificity of the complementation observed.

Progeny were also assayed at 39°C to determine whether recombinants between mutant genomes and viral DNA sequences in the supercoiled plasmid had contributed to the complementation yield observed. Although no recombinants were detected, *ts*LG4 reverted with relatively high frequency in these tests (data not shown).

To summarize the results of genetic studies, the localization of mutations in four mutants (*ts*Y46, *ts*E5, *ts*E6, and *ts*LG4) to sequences in or near the coding sequences for ICP27 and the failure of the mutants to complement one another indicate that all four are defective in the same gene, that encoding ICP27. Additionally, the fact that the mutants are temperature sensitive for growth demonstrates that ICP27 performs at least one essential function during virus replication.

Phenotypic analysis. (i) Polypeptide synthesis. Because the four *ts* mutations lie in or near sequences that specify ICP27, it was of interest to characterize the effect of these mutations

TABLE 2. Complementation of *ts* mutants by the products of cloned genes

Virus	Expt	Plasmid	Virus yield (PFU/ml)	Fold increase
*ts*Y46	1	pBR325	4×10^2	1.0
		pKEB-X2	4×10^2	1.0
		pKHX-BH	8×10^4	200.0
	2	pBR325	3×10^2	1.0
		pKEB-X2	9×10^1	0.30
		pKHX-BH	8×10^3	27.0
*ts*LG4	1	pBR325	7×10^3	1.0
		pKEB-X2	6×10^3	0.86
		pKHX-BH	4×10^4	5.7
	2	pBR325	1×10^3	1.0
		pKEB-X2	1×10^3	1.0
		pKHX-BH	1×10^4	10.0
*ts*B32	1	pBR325	1×10^3	1.0
		pKEB-X2	5×10^3	5.0
		pKHX-BH	2×10^3	2.0
	2	pBR325	2×10^1	1.0
		pKEB-X2	8×10^2	40.0
		pKHX-BH	<5	<0.25

on polypeptide synthesis at 39°C. To this end, HEL cells were infected with the mutants at 34 and 39°C and labeled from 5 to 17 h postinfection with [^{35}S]methionine. (*ts*E5 reproducibly generated protein profiles indistinguishable from those of *ts*E6; hence these profiles are not shown.)

The polypeptide profiles shown in Fig. 3 demonstrate that the mutants share certain features, but differ significantly in others. With regard to their similarities, the profile of polypeptides synthesized at 34°C resembled that of the wild-type virus at this temperature (data not shown), whereas overproduction of both ICP4 and ICP27 was evident at 39°C. On the other hand, ICP0 which normally migrates between ICP6 and ICP8, did not appear to be overproduced, and the remaining two immediate-early proteins, ICP22 and ICP47, were not detectable under the conditions used. However, unlike the case with the classical mutants in the immediate-early gene specifying ICP4, significant levels of several early proteins (e.g., ICP8 and gB) were synthesized in cells infected with all four mutants, and all four induced the overproduction of ICP6, another early protein. The mutants are also similar in their inability to induce the synthesis of certain true late proteins (e.g., ICP1/2 and ICP48) and to effect the shutoff of host-cell protein synthesis. (Careful inspection of this gel and numerous others not included in this paper reveals that ICP1/2, which migrates close to, but slightly faster than, a mock band, is in fact absent in all of the mutant profiles.) Despite these general similarities, the mutants differed in their abilities to induce the synthesis of a number of polypeptides, especially those of the delayed-early ($\beta\gamma$) class. Thus, a spectrum exists among the mutants with regard to the quantities of these proteins synthesized. *ts*Y46 is clearly the most severely restricted in that it induced drastically reduced levels of delayed-early polypeptides (e.g., ICP5, -25, and -44). *ts*E6 (and *ts*E5, data not shown) is the most permissive in that cells infected with this mutant synthesized apparently wild-type levels of these proteins, whereas *ts*LG4-infected cells synthesized intermediate levels. This variation in expression is also evident in the expression of early polypeptides. ICP8, gB, and pgB were moderately underproduced in *ts*Y46-infected cells, whereas ICP39 and ICP40 were drastically underproduced. Intermediate levels of these latter two polypeptides were induced by *ts*LG4. Likewise, this variation in expression was also noted with regard to certain of the late proteins (e.g., ICP15, -19, and -20).

It should be noted that the 43K band absent from gel profiles of cells infected with *ts*E6 (and *ts*E5; Fig. 4) most likely represents ICP36, viral thymidine kinase, since it was

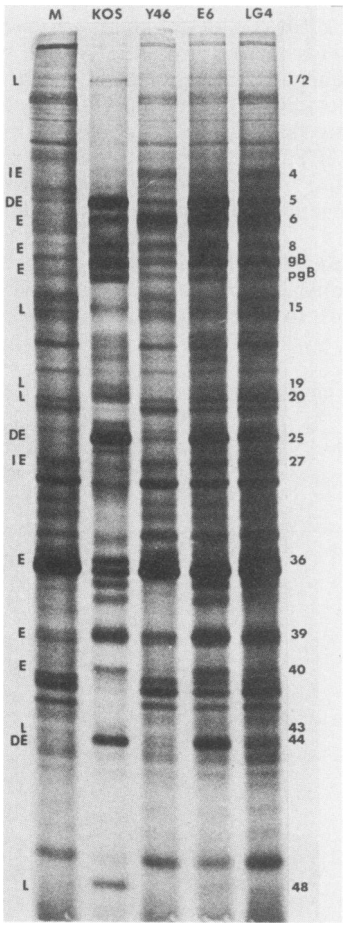


FIG. 3. Polypeptide phenotypes of *ts*E6, *ts*LG4, and *ts*Y46 at 39°C. HEL cells were infected with 10 PFU per cell at 34 and 39°C, labeled with [^{35}S]methionine from 5 to 17 h postinfection, and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the text. Only the 39°C profiles are shown here. Abbreviations: M, mock-infected cells; IE, immediate-early; E, early; DE, delayed-early; L, late polypeptides. The ICP numbers of several polypeptides are shown on the right, as are the locations of gB and pgB.

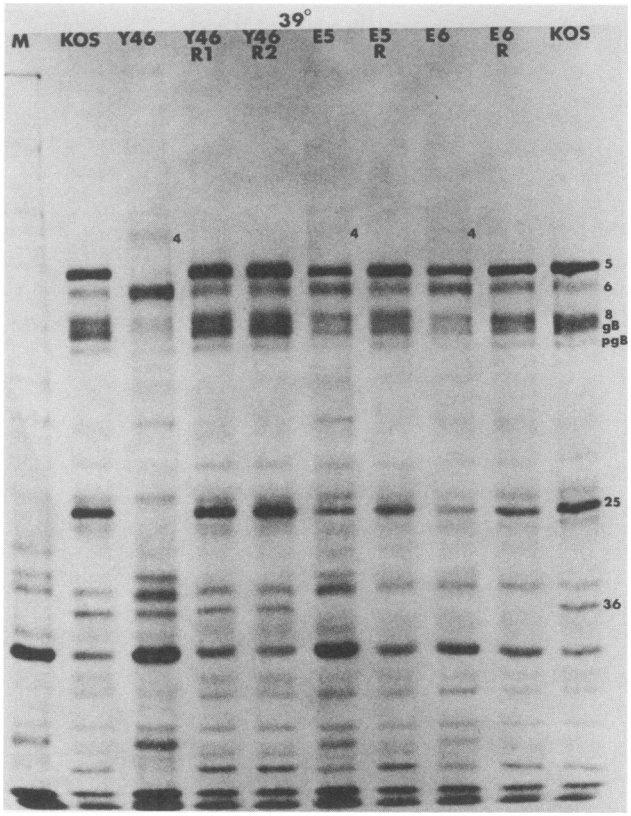


FIG. 4. Polypeptide phenotypes of *ts*Y46, *ts*E5, *ts*E6, and *ts*$^+$ recombinants of these mutants at 39°C. Recombinants were generated in marker rescue experiments with pKHX-BH. Experimental details are identical to those of Fig. 3, except that a multiplicity of infection of 5 PFU per cell was used.

TABLE 3. Growth of ICP27 mutants as a function of temperature[a]

Virus	Virus yield (PFU/ml)	
	34°C	39°C
KOS	9×10^7	2×10^6
*ts*E6	3×10^7	2×10^2
*ts*LG4	3×10^7	3×10^4
*ts*Y46	4×10^7	5×10^3

[a] HEL cells infected in parallel to those used in the analysis of polypeptide phenotypes shown in Fig. 3 were harvested at 17 h postinfection, and progeny virus was assayed at 34°C.

also missing from gel profiles at the permissive temperature and from profiles of a thymidine kinase deletion mutant (provided by D. Coen; N. DeLuca, unpublished results). Additionally, thymidine kinase activity was previously shown to be lacking in cells infected with these mutants at 34 and 39°C (2; D. Coen, personal communication). *ts*E5 and *ts*E6, therefore, contain secondary mutations in this nonessential gene, which is common among mutants induced by bromodeoxyuridine mutagenesis.

One possible explanation for the variation in the early and delayed-early polypeptide profiles of the four mutants is that the more permissive mutants (i.e., *ts*LG4, *ts*E5, and *ts*E6) are "leakier" than *ts*Y46 at the nonpermissive temperature. If this were the case, one would expect to obtain higher titers of these mutants relative to *ts*Y46 at the nonpermissive temperature in one-step growth experiments. At 34°C all three mutants produced titers similar to that of KOS, whereas at 39°C the *ts*E6-infected cultures that gave rise to the profile shown in Fig. 3 yielded the lowest titer of the three mutants, in spite of its extensive permissivity for polypeptide synthesis (Table 3). *ts*LG4 yielded the highest titer at this temperature, and *ts*Y46 yielded an intermediate titer. Permissivity of mutant polypeptide synthesis therefore bears no relation to the quantity of infectious virus produced. Furthermore, tests of polypeptide synthesis in KOS-, *ts*Y46-, *ts*E5-, and *ts*E6-infected cells at 39 and 39.6°C generated profiles that were indistinguishable (data not shown). These data demonstrate that the phenotypic differences in polypeptide synthesis observed do not result in differing degrees of "leak" by the mutants, but rather reflect real differences in the ability of the mutant forms of ICP27 to induce the expression of various members of the later classes of polypeptides.

To confirm that the *ts* mutations in *ts*Y46, *ts*E5, and *ts*E6 were, in fact, responsible for the protein profiles observed, infected cell lysates of ts^+ recombinants generated in marker rescue experiments with pKHX-BH (Fig. 1) were similarly analyzed. (Since marker rescue was not carried out by us on *ts*LG4, no ts^+ recombinants of this virus were available for this experiment.) The ts^+ recombinants induced wild-type protein profiles—except for the absence of ICP36 in profiles of ts^+E5R- and ts^+E6R-infected cells (Fig. 4), an observation which is consistent with the existence of secondary thymidine kinase mutations in their respective *ts* parents.

(ii) Late protein synthesis. To document more specifically the restricted ability of the mutants to synthesize late viral polypeptides, infected cells were labeled from 12 to 15 h postinfection with [^{35}S]methionine (Fig. 5). Under these conditions, cells infected with all three mutants underproduced most true late (γ) proteins (e.g., ICP1/2, -15, and -19) at 39°C. Although not visible in this profile, higher percentage gels of the same samples revealed that ICP48 was also greatly underproduced, consistent with the profile shown in Fig. 3. Additionally, it is notable under late labeling conditions that the mutants exhibited varying abilities to express the late protein ICP20 as noted above for the long label.

An alternative approach designed to compare late versus early protein expression involved [^{3}H]mannose labeling to characterize viral glycoprotein synthesis. Because glycoproteins gB and gC are members of the early and late classes of viral polypeptides, respectively (14, 30), we examined their synthesis in mutant and wild-type virus-infected cells at 39°C. Cells infected with *ts*Y46, *ts*E5, and *ts*E6 all synthesized greatly reduced quantities of gC and significant levels of the precursor to gB, pgB (Fig. 6), again implicating a block of these mutants at some point after the early phase of protein synthesis. As expected in cells infected with ts^+ recombinants of all three mutants, wild-type levels of gC were synthesized. All three mutants induced the synthesis of wild-type levels of pgB, gB, and gC at 34°C (data not shown).

(iii) Viral DNA synthesis. After their initial isolation and characterization, *ts*E5 and *ts*E6 were shown to induce the synthesis of significant levels of viral DNA at 39°C (40). This is not surprising, given the abilities of these mutants to induce the synthesis of wild-type levels of most members of the early class of polypeptides, which include those involved

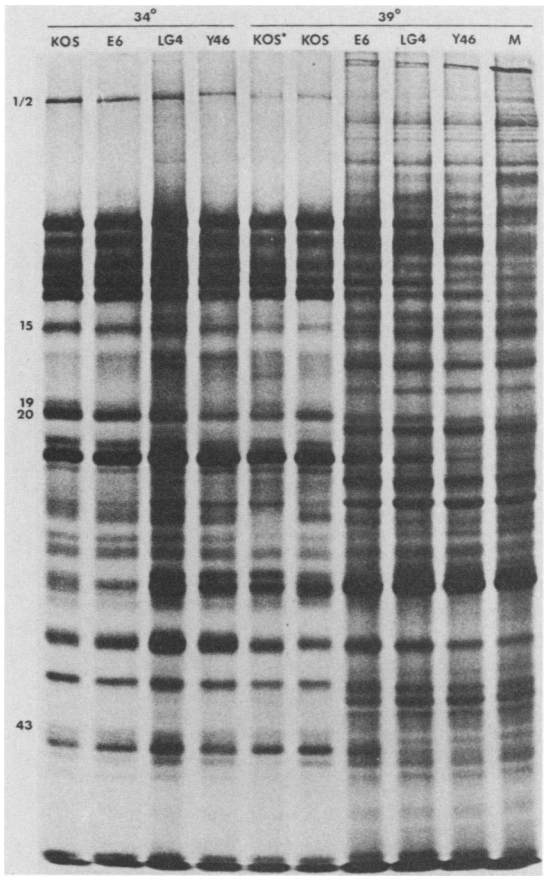


FIG. 5. Polypeptide phenotypes of *ts*E6, *ts*LG4, and *ts*Y46 late in infection at 34 and 39°C. Experimental details are identical to those of Fig. 3, except that labeling was carried out from 12 to 15 h postinfection. The ICP numbers of several late polypeptides are shown on the left. KOS* indicates the polypeptide profile of cells infected with KOS at 39°C and labeled from 5 to 17 h postinfection.

in viral DNA synthesis (2). We therefore sought to confirm this finding and extend it to *ts*Y46. The CsCl gradient profiles of [^{3}H]thymidine-labeled, infected cell extracts from one experiment (Fig. 7) demonstrate that *ts*Y46, like *ts*E5 and *ts*E6, induce the synthesis of viral DNA at 39°C; *ts*Y46 induced 100% of wild-type levels of viral DNA, whereas *ts*E5 induced 46% and *ts*E6 induced 57%. A second determination gave essentially the same results, with some variation in the absolute levels of viral DNA synthesized. *ts*LG4 has also been shown to induce 89% of wild-type levels of viral DNA at 39°C (R. Sandri-Goldin, unpublished results). These findings indicate that the restriction in late polypeptide synthesis observed in cells infected with these mutants is not due to a block in viral DNA synthesis.

DISCUSSION

To date, information concerning the role of ICP27 in productive infection has been limited due to the unavailability of mutants in this gene. The gene encoding ICP27, a protein with an apparent molecular weight of approximately 63,000, was localized initially by the analysis of polypeptides induced by intertypic recombinants (26, 37) and by mapping viral transcripts produced in the presence of cycloheximide (1, 5, 44)—an assay that functionally defines the immediate-early class of transcripts (17). Detailed analysis of the 2-kilobase ICP27 transcript (22) has localized its coding sequences and control region to the *Bam*HI-*Hpa*I fragment present in plasmid pKHX-BH. Thus, transcription begins

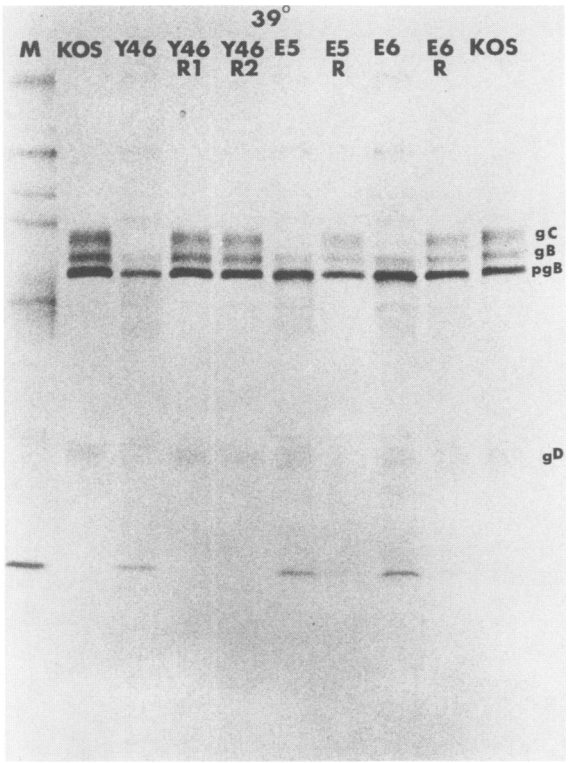


FIG. 6. Glycoprotein phenotypes of *ts*Y46, *ts*E5, *ts*E6, and *ts*$^+$ recombinants of these mutants at 39°C. HEL cells were infected with 5 PFU per cell at 39°C, labeled with [^{3}H]mannose from 5 to 17 h postinfection, and processed as described in the text. Abbreviations are as in Fig. 3. The locations of viral glycoprotein bands are shown at the right.

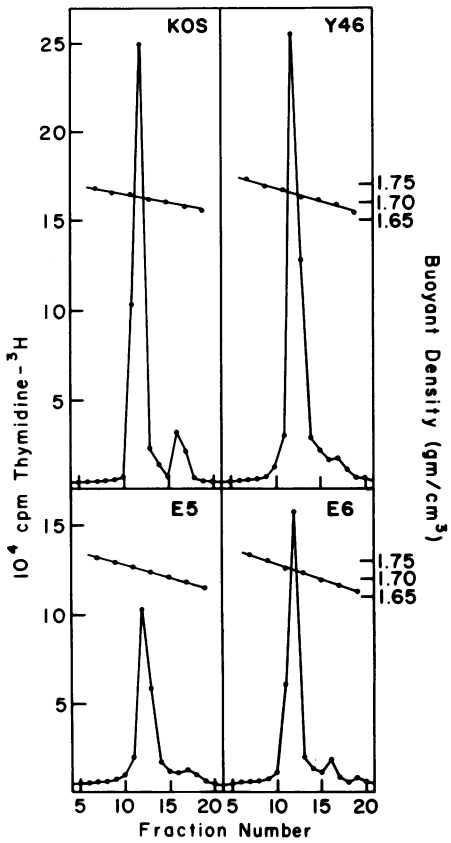


FIG. 7. Viral DNA phenotypes of *ts*Y46, *ts*E5, and *ts*E6 at 39°C. CsCl gradient profiles of infected cell lysates were obtained as described in the text. Viral DNA peaked at a density of 1.725 g/cm^3, and cellular DNA peaked at 1.685 g/cm^3. In this experiment, *ts*Y46 induced 100% of wild-type DNA levels, *ts*E5 induced 46%, and *ts*E6 induced 57%.

270 bp to the right of the *Bam*HI site at coordinate 0.745 and terminates 150 bp to the left of the *Hpa*I site at coordinate 0.761 (Fig. 2).

In this report we describe the preliminary genetic and biochemical characterization of four independently derived temperature-sensitive mutants of ICP27.

Genetic analysis. Support for the contention that the four mutants under consideration are mutated in the gene for ICP27 comes from three observations. (i) All four mutants fail to complement one another, indicating that their respective *ts* mutations affect the same function. (ii) Marker rescue experiments localized the mutations in *ts*Y46, *ts*E5, *ts*E6 to the 2.4-kilobase region between the *Bam*HI and *Hpa*I sites of pKHX-BH and those in *ts*Y46 and *ts*E5 further to the 850-bp region between the *Sma*I sites at coordinates 0.753 and 0.757. These mutations thus map entirely within the ICP27 coding sequence. (iii) Complementation studies with plasmid pKHX-BH demonstrate that it encodes an activity that can complement two of the mutants in *trans*. Since this plasmid contains a total of only 420 bp in excess of the ICP27 transcript, it is highly unlikely that another gene product is responsible for this effect in that any truncated transcripts that might originate from or terminate in this fragment would not be expected to contribute an active gene product to the complementation observed. Preliminary transcriptional mapping studies of this region of the genome indicate that although a second abundant transcript maps to the right of

the gene for ICP27, no compelling evidence exists to suggest overlap between the ICP27 transcript and other transcripts (E. Wagner, personal communication). The four temperature-sensitive mutations, therefore, appear to lie in the gene encoding ICP27, establishing it as essential for lytic HSV growth. In addition, *ts*Y46 and *ts*LG4 may now be assigned to the same complementation group to which *ts*E5 and *ts*E6 are assigned, the complementation group 1-5.

Phenotypic studies. As mentioned above, the only temperature-sensitive mutants to date that have been localized to an immediate-early gene have been those with mutations in the gene for ICP4. That this gene plays a central regulatory role in the course of productive infection was demonstrated in part by the characterization of a class of mutants that overproduce the immediate-early polypeptides and drastically underproduce both early and late proteins at the nonpermissive temperature (6, 11, 35, 36). Recently a second class of mutants in the gene for ICP4 has been described (9). Mutants in this class are characterized by the synthesis of significant levels of the early polypeptides and viral DNA while continuing to overproduce the immediate-early polypeptides.

Characterization of viral polypeptide synthesis in cells infected at the nonpermissive temperature with the mutants in the present study revealed the following. (i) Cells infected with all four mutants in ICP27 overproduce at least two immediate-early gene products. Thus, increased levels of both ICP4 and ICP27 were apparent (although the same was not true of ICP0). (ii) No detectable differences were observed in the electrophoretic mobilities of the mutant forms of ICP27. Since ICP27 has been shown to be phosphorylated (46), we also examined viral phosphoprotein synthesis in mutant-infected cells at the nonpermissive temperature. In infected cultures labeled from 5 to 17 h postinfection with ^{32}P, no differences were apparent among the mutant forms or between the mutant and wild-type forms of the phosphoprotein (data not shown). (iii) Expression of several true late genes (i.e., ICP1/2, -48, and gC) was greatly reduced in cells infected with all four mutants in ICP27. (iv) Cells infected with all four mutants at 39°C exhibited greatly impaired host shutoff. (v) Since ts^+ recombinants of these mutants gave rise to wild-type polypeptide phenotypes, the alterations in viral polypeptide synthesis observed are, by definition, the outcome of mutations that map within the rescuing fragment from which recombinants were derived.

Functional role of ICP27. In view of the polypeptide and viral DNA phenotypes of ICP27 mutants, three points can be made. First, the alterations in the expression of the three classes of viral polypeptides characteristic of these mutants suggests a regulatory role for ICP27. Second, despite the presence of presumably functional ICP4, at least two immediate-early polypeptides were overproduced. Functional ICP4, therefore, is apparently necessary, but not sufficient, to mediate the negative regulation of immediate-early polypeptide synthesis seen early in infection. In light of recent data implicating a late function in negative regulation of immediate-early genes—a function operative after the onset of viral DNA synthesis (9)—and due to the apparent absence of several late gene products in cells infected with these mutants, this component of immediate-early gene regulation may be missing. Third, and in a similar vein, the inefficient shutoff of host protein synthesis in cells infected with each of the mutants is most likely due to the absence of a late gene product dependent for its expression on functional ICP27.

Further characteristics that are common to all four mutants are their ability to induce the synthesis of significant levels of early polypeptides (e.g., ICP6, -8, and gB) and their corresponding ability to induce significant levels of viral DNA synthesis at the nonpermissive temperature. Parenthetically, thymidine kinase (ICP36), should be included in this list of early proteins because cells infected with both *ts*Y46 and *ts*LG4 synthesize wild-type levels of this enzyme. The inability of *ts*E5- and *ts*E6-infected cells to do so is independent of their mutations in ICP27. Functional ICP27, therefore, is apparently not required for the transition from immediate-early to early gene expression or for viral DNA synthesis. Support for this contention comes from a number of laboratories utilizing plasmids containing various combinations of immediate-early genes in transient expression-induction systems to show that ICP27, unlike ICP4 and ICP0, has no stimulatory effect on early gene expression (10a, 12, 27).

Phenotypic variation among mutants in ICP27: functional implications. It is with regard to the expression of a number of polypeptides, especially those of the delayed-early class, that the similarities between the four mutants cease. Thus, a kind of graded continuum exists; *ts*Y46 appears to be highly restricted in the expression of these genes, *ts*E5 and *ts*E6 are the least restrictive, and *ts*LG4 occupies an intermediate position. These graded differences in viral gene expression are rather unusual for mutants in the same gene, yet the genetic data are clear. The differences cannot be explained by leakiness of the mutants, since their polypeptide phenotypes have no bearing on the restriction in their abilities to produce virus at the nonpermissive temperature, and elevating this temperature to 39.6°C had no effect on the polypeptide profiles. Therefore, we favor the idea that the different phenotypes reflect differing abilities of the mutant polypeptides to facilitate the expression of later genes. Several explanations for the phenomenon of graded expression of later polypeptides are possible; the most obvious would ascribe the observed differences to varying affinities of the mutant forms of ICP27 for other immediate-early proteins, RNA polymerase, or the various promoter-regulatory regions of delayed-early genes themselves. Our future efforts to characterize these mutants will focus on this question.

Several additional comments should be made with regard to the polypeptide phenotypes of the mutants. Cells infected with all four mutants displayed drastically reduced levels of several late polypeptides (i.e., ICP1/2, -15, -19, -48 and gC) in the face of significant levels of early gene expression and viral DNA synthesis. Early gene expression and DNA synthesis are therefore not sufficient for the expression of late genes, which apparently requires functional ICP27—either directly or indirectly. In this regard, it should be noted that Ruyechan and co-workers previously reported a locus, Cr, that maps near the right-hand terminus of U_L and that affects the expression of gC (38). In light of our results, it seems likely that this locus is the gene for ICP27. A similar requirement for ICP4 for late gene expression has also been noted in the studies of DeLuca and co-workers (9). Here, members of the "late" class of ICP4 mutants induce drastically increased levels of immediate-early proteins, wild-type levels of early proteins, substantial levels of delayed-early proteins and viral DNA, and greatly reduced levels of late proteins. In view of these findings, it is possible that late viral gene expression requires both functional ICP4 and ICP27.

With regard to the identity of late viral gene functions affected by mutations in ICP27, Dalziel and Marsden (7) recently reported that two late viral proteins of 21 and 22K bind specifically to the *a* sequences of the HSV chromo-

some. The *a* sequences are thought to contain a cleavage signal required for encapsidation of viral DNA. It is possible that these proteins may be involved in the correct cleavage or encapsidation (or both) of concatameric viral DNA. Interestingly, cells infected with *ts*E5 and *ts*E6 have been shown to contain "endless viral DNA," i.e., the mutants appear unable to direct proper cleavage and encapsidation of concatameric viral DNA (S. Bachenheimer, personal communication). It was therefore intriguing to note that all four of our mutants dramatically underproduce ICP48, a late 21K polypeptide.

Taken together, the data presented in this paper indicate that the mutations in *ts*Y46, *ts*E5, *ts*E6, and *ts*LG4 appear to lie in the gene for ICP27. Phenotypic studies of these mutants indicate that ICP27 (i) plays an essential role in viral growth, (ii) is required either directly or indirectly for negative regulation of (at least two) immediate-early genes, (iii) is not required for the expression of a number of early proteins or DNA synthesis, and (iv) is required to facilitate expression of later proteins, independent of viral DNA synthesis.

ACKNOWLEDGMENTS

We thank Rozanne Sandri-Goldin for kindly providing *ts*LG4 and communicating unpublished data concerning this mutant, Stanley Person and Neal DeLuca for generously providing plasmids, Neal DeLuca and Don Coen for technical advice and helpful comments on the manuscript, and Madelon Datz for manuscript preparation.

This investigation was supported by Public Health Service grants CA20260 and CA21082 from the National Cancer Institute. W.R.S. was supported by Public Health Service training grant CA09031 from the National Cancer Institute.

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