

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

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We report on the properties of a temperature-sensitive mutant produced by transfection of cells with intact DNA and a specific DNA fragment mutagenized with low levels of hydroxylamine. The plating efficiency of the mutant at 39°C relative to that at 33.5°C was 5×10^{-6} . The pattern of polypeptides produced at the nonpermissive temperature was similar to that seen with wild-type virus in infected cells treated with inhibitory concentrations of phosphonoacetic acid in that α and β polypeptides were produced, whereas most γ polypeptides were either reduced or absent. Consistently, the mutant did not make viral DNA, although temperature sensitivity of the viral DNA polymerase could not be demonstrated. Marker rescue studies with herpes simplex virus type 2 (HSV-2) DNA mapped the mutant in the L component within map positions 0.385 and 0.402 in the prototype (P) arrangement of the HSV-1 genome. Analysis of the recombinants permitted the mapping of the genes specifying infected cell polypeptides 36, 35, 37, 19.5, 11, 8, 2, 43, and 44, but only the infected cell polypeptide 8 of HSV-2 was consistently made by all recombinants containing demonstrable HSV-2 sequences. Marker rescue studies with cloned HSV-1 DNA fragments mapped the temperature-sensitive lesion within less than 10^3 base pairs between 0.383 and 0.388 map units. Translation of the RNA hybridizing to cloned HSV-1 DNA, encompassing the smallest region containing the mutation, revealed polypeptide 8 (128,000 molecular weight), which was previously identified as a β polypeptide with high affinity for viral DNA, and a polypeptide (25,000 molecular weight) not previously identified in lysates of labeled cells.

Approximately 50 infected cell polypeptides (ICPs) produced in cells infected with either herpes simplex virus type 1 (human herpesvirus 1, HSV-1) or HSV-2 appear to be virus specific (12, 25). The functions of some of the ICPs have been identified (33), but the functions of the majority of the ICPs are not known. One approach to the identification of the functions of these polypeptides is the production of functional mutants by mutagenesis of the entire genome (4, 5, 32) or of selected regions (6) of the viral DNA. In this paper, we report the production and characterization of a temperature-sensitive mutant produced by mutagenesis of a restriction endonuclease fragment of HSV-1 DNA. The mutation appeared to map within the coding sequences specifying two viral polypeptides

which probably play a role in viral DNA synthesis.

Pertinent to this report are the following. (i) HSV-1 and HSV-2 ICPs form at least three groups, designated α , β , and γ , whose synthesis is coordinately regulated and sequentially ordered in a cascade fashion (13). α polypeptides are made first; functional α polypeptides are required for the synthesis of β polypeptides, which in turn shut off the synthesis of α polypeptides and induce the synthesis of γ polypeptides, i.e., late structural polypeptides. In turn, γ polypeptides shut off the synthesis of β polypeptides. (ii) The synthesis of viral DNA begins at 3 h postinfection and coincides with the onset of synthesis of β polypeptides (29). Indeed, two viral enzymes involved in viral DNA synthesis, the thymidine kinase and DNA polymerase, appear to be β gene products (9, 19, 27). (iii) Several genes specifying α , β , and γ gene products have been mapped (21, 23). Several β genes have been shown to cluster in the region of the genome between 0.30 and 0.42 map units on the

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physical map of the HSV-1 genome (23). To delineate the function of some of these genes, we have selected for mutagenesis a DNA fragment mapping between 0.31 and 0.41 map units.

MATERIALS AND METHODS

Viruses and cells. The isolation and relevant properties of HSV-1(F) (8), HSV-1(m̄P) (11), and HSV-2(G) (8) strains were published elsewhere. Virus stocks were prepared and titrated on Vero or HEp-2 cells as previously described (30). Rabbit skin cells were originally obtained from J. McLaren. Human embryonic lung (HEL) cells were originally obtained from Flow Laboratories, Rockville, Md.

Enzymes and radioisotopes. The restriction endonucleases *Bam*HI and *Kpn*I were obtained from New England Biolabs, Boston, Mass., and Bethesda Research Labs, Bethesda, Md. The purification of the restriction endonucleases *Bg*III, *Eco*RI, *Hsu*I, and *Hpa*I has been described (22). T₄ DNA ligase was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. The radioisotopes [*methyl*-³H]thymidine (59 Ci/mmol), [*methyl*-³H]dTTP, tetrasodium salt (80 Ci/mmol), [¹⁴C]leucine, [¹⁴C]isoleucine, and [¹⁴C]valine (each >250 mCi/mmol), and L-[³⁵S]methionine (>500 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass.

Preparation of viral DNA and restriction endonuclease digestion. Intact viral DNA was purified by NaI density gradient centrifugation (36) of DNA extracted from cytoplasmic nucleocapsids or from infected whole cell lysates. Digestion of viral DNA, preparative agarose gel electrophoresis, and isolation of individual restriction endonuclease-derived fragments were described elsewhere (22). Analytical gel electrophoresis was done on a horizontal apparatus at

2 V/cm. In some experiments, *Bam*HI HSV-1 DNA fragments cloned in pBR322 (24) were used. These were *Bam*HI-G (pRB102), *Bam*HI-Q (pRB103), and *Bam*HI-T (pRB119). The plasmids carrying the cloned HSV-1(F) DNA were extracted from bacteria and centrifuged to equilibrium in cesium chloride-ethidium bromide gradients as described elsewhere (7).

The plasmid pRB401 contained the HSV-1(F) sequences mapping between the *Sal*I cleavage site at 0.372 map units and the *Bam*HI cleavage site at 0.388 map units and enclosed the left portion of the *Sal*I fragment O (ΔO) (Fig. 1). To construct the plasmid, pRB102 was digested with *Sal*I, annealed, ligated in 66 mM Tris (pH 7.6), 6 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM ATP, and 1.0 U of T₄ DNA ligase, and used to transform *Escherichia coli* C600SF8 as described (20). pRB401 was propagated and purified as described elsewhere (24).

Mutagenic treatment of the DNA fragment and isolation of the temperature-sensitive mutant. The *Bg*III fragment I (molecular weight, 10.2 × 10⁶; mapping in the prototype arrangement of HSV DNA between coordinates 0.309 and 0.411) was prepared from digests of HSV-1(m̄P) DNA. This fragment was incubated for 1 h at 70°C in 5 mM hydroxylamine-0.1× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate). The fragment was dialyzed overnight against 1× HBS (8 g of NaCl, 0.37 g of KCl, 0.125 g of Na₂HPO₄ · 2H₂O, 1.0 g of dextrose, and 5.0 g of HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Calbiochem, La Jolla, Calif.] per liter, pH 7.05), and cotransfected with intact HSV-1(m̄P) DNA into rabbit skin cells (18, 31). Virus progeny from the transfection stock were isolated and examined for growth at 33.5 and 39°C in Vero cells. The isolates exhibiting temperature-sensitive phenotype were further purified by

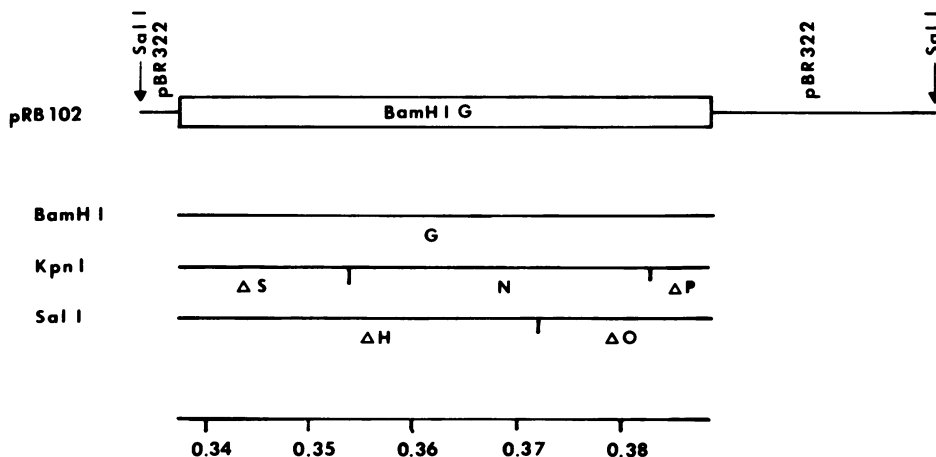


FIG. 1. *Bam*HI, *Kpn*I, and *Sal*I maps of the HSV-1(F) *Bam*HI fragment G contained within the plasmid pRB102. The plasmid DNA is shown in linear form, produced by cleavage at the unique *Sal*I site within the pBR322 sequences. The numbers below the lower line refer to map coordinates of HSV-1(F) DNA in the prototype arrangement of HSV-1 DNA. The symbol Δ indicates that only a portion of the designated *Kpn*I or *Sal*I fragment is contained within the *Bam*HI fragment G. Plasmid pRB401, not shown in this figure, contained the *Sal*I fragment ΔO; it was produced by digestion of pRB102 with *Sal*I, ligation, and transformation of *E. coli* with the products of ligation.

three successive plaque isolation passages. Stocks were prepared at a multiplicity of 0.01 PFU/cell in HEp-2 cell roller-bottle cultures.

Intertypic marker rescue. Intertypic marker rescue was done as previously described (18, 31). Briefly, approximately 0.1 μg of intact HSV-1 mutant DNA was mixed with 1.0 μg of HSV-2(G) DNA which was previously digested with either *Bgl*II, *Eco*RI, or *Hsu*I restriction endonuclease. Rabbit skin cell flask cultures were incubated at 33.5°C for 4 to 5 days, and the progeny was then titrated at 33.5 and 39°C on Vero cells. Recombinants which exhibited a ratio of titers at 39 and 33.5°C that was greater than 0.5 were selected from the transfection stocks and plaque-purified three times at 39°C before preparation of virus stocks.

Intratyptic marker rescue. Intratyptic marker rescues were done by transfection of rabbit skin cells with mixtures of intact mutant DNA and HSV-1(F) DNA fragments as described above. The HSV-1 fragments used for intratyptic rescue were (i) *Bam*HI-G cloned in pBR322 (pRB102) and mapping between coordinates 0.337 and 0.388 in the prototype arrangement, (ii) the *Kpn*I fragment N (map position 0.353 to 0.384) derived by digestion of *Bam*HI fragment G, and (iii) the *Sa*II fragments Δ H and Δ O derived by digestion of pRB102. These fragments contained the left and right portions of the *Bam*HI fragment G, respectively, as well as adjacent pBR322 sequences. The progeny obtained after transfection of rabbit skin cells were titrated at 33.5 and 39°C on Vero cells, and the plating efficiency was determined.

Synthesis of viral DNA in infected cells. Confluent, resting, monolayer cultures of HEL cells were infected and labeled with [*methyl*-³H]thymidine at 20 $\mu\text{Ci/ml}$. In experiments in which the sensitivity of DNA synthesis to phosphonoacetic acid (PAA) was determined, HEL cell monolayers were pretreated with 300 μg of PAA per ml for 4 h before infection, and that level of PAA was maintained throughout the course of infection. DNA was extracted from infected cell monolayers as described (15). The DNA was banded in NaI equilibrium density gradients in a Beckman Ti75 rotor, 40,000 rpm, 48 h, at 20°C. Fractions of equal volume were collected from the gradients, and radioactivity was measured as described (15).

Determination of HSV-1-induced DNA polymerase in infected cells. Replicate HEp-2 cell 32-oz. (ca. 0.95-liter) bottle cultures were infected with parental or mutant virus at 10 PFU/cell. At 12 h post-infection, infected cell extracts were prepared by a modification of the procedure described by Huang (14). Briefly, cells were washed with 0.05 M Tris (pH 7.4)–0.15 M NaCl, scraped from dishes, and suspended in a hypotonic buffer consisting of 0.05 M Tris (pH 7.6), 1 mM MgCl_2 , and 1 mM dithiothreitol. The cells were allowed to swell for 15 min at 4°C and were sonically disrupted at maximum output in three successive 20-s cycles with intermittent cooling. The extracts were cleared of cell debris by centrifugation at 27,000 rpm at 4°C for 1 h in a Beckman SW50.1 rotor equipped with small-volume adaptors. DNA polymerase activity was measured in a reaction mixture consisting of 20 mM Tris (pH 7.8), 5 mM MgCl_2 , 0.2 mM dithiothreitol, 0.25 mg of bovine serum albumin per ml, 0.005 mM TTP, 1 μCi of [³H]TTP, 100 mM

$(\text{NH}_4)_2\text{SO}_4$, 2 μg of the synthetic poly(dA)·oligo-(dT)_{12–18} template primer, and the extract. The reactions were incubated for 1 h at either 33.5 or 39°C, and trichloroacetic acid-precipitable radioactivity was then measured. No polymerase activity was demonstrable in lysates of mock-infected cells under the conditions tested.

In vivo inactivation of HSV-1-induced DNA polymerase activity was done as described (1). Briefly, replicate cultures infected at 10 PFU/cell with parent or mutant virus were shifted at 12 h postinfection from 33.5 to 39°C. At hourly intervals for 4 h, replicate cultures were harvested, and the activity of the viral polymerase was determined as described above.

Isolation of RNA. HEp-2 roller-bottle cultures, which had been pretreated with 300 μg of PAA per ml for 4 h, were infected with HSV-1(F) at a multiplicity of 35 PFU/cell. Cultures were maintained in medium 199-V plus 300 μg of PAA per ml (17). Ten hours postinfection, the cells were harvested, washed with phosphate-buffered saline, and lysed in a buffer consisting of 1% Nonidet P-40, 10 mM Tris (pH 7.6), 50 mM NaCl, and 1 mM EDTA. The cytoplasmic extract was collected and extracted with an equal volume of buffer-saturated phenol. The phenol phase was reextracted with an equal volume of 10 mM Tris (pH 9.0)–50 mM NaCl–1 mM EDTA. The aqueous phases were pooled, extracted twice more with phenol and three times with chloroform-isoamyl alcohol (24:1), and precipitated with ethanol at –20°C.

RNA selected from DNA bound to diazobenzoyloxymethyl-paper. HSV-1(F) DNA fragments, as pBR322 recombinant plasmids, were prepared and covalently bound to diazobenzoyloxymethyl-paper by the method of Stark and Williams (34). Hybridization of the bound DNA to RNA extracted from HSV-1(F)-infected cells was carried out by procedures to be described in detail later (S. Mackem and B. Roizman, manuscript in preparation). Briefly, 0.5 mg of RNA from cells infected in the presence of PAA was hybridized with the filters in a solution containing 50% formamide, 0.8 M NaCl, 20 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid); Calbiochem, LaJolla, Calif.] (pH 6.4), 5 mM EDTA, 0.2% sodium dodecyl sulfate for 7 h at 56°C. After hybridization, the filters were washed three times with 2 \times SSC containing 0.2% sodium dodecyl sulfate and two times with 0.1 \times SSC containing 0.2% sodium dodecyl sulfate at 60°C for 15 min. The specifically selected RNAs were eluted with 99% formamide–20 mM PIPES (pH 6.4) at 70°C. The eluted RNA, in formamide, was diluted twofold with water and precipitated with ethanol at –20°C in the presence of 250 mM sodium acetate and 10 μg of carrier tRNA.

In vitro translation of selected RNA. The selected, eluted RNAs were translated in vitro using a rabbit reticulocyte lysate system (New England Nuclear Corp.) and using [³⁵S]methionine to label polypeptides synthesized. The translated polypeptides were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels and visualized by fluorography as previously described (3).

Protein labeling and gel electrophoresis. Labeling of HSV-infected cells with [¹⁴C]leucine, [¹⁴C]isoleucine, and [¹⁴C]valine, sodium dodecyl sulfate-

polyacrylamide gel electrophoresis of the cell lysates, and autoradiography were performed as previously described (23).

RESULTS

Isolation of the temperature-sensitive mutant. Approximately 300 plaques were isolated from the viral progeny obtained after transfection of the rabbit skin cells with the hydroxylamine-treated *Bgl*II fragment I and intact HSV-1(\bar{m} P) DNA. A single stable mutant, designated HSV-1(\bar{m} P)*ts*HA1 and exhibiting a ratio of plating efficiencies (39/33.5°C) of 5×10^{-6} , was selected for further studies. HSV-1(\bar{m} P)*ts*HA1, like its parent HSV-1(\bar{m} P), has *syn*⁺ plaque morphology (11).

Characterization of *ts*HA1: DNA synthesis. Two series of experiments were done. The purpose of the first experiment was to determine whether the *ts* lesion in HSV-1(\bar{m} P)*ts*HA1 has an effect on viral DNA synthesis. The incorporation of [³H]thymidine into viral DNA was measured at both 33.5 and 39°C, the permissive and nonpermissive temperatures, respectively. The results of the NaI equilibrium density centrifugation of the DNA extracted from labeled infected cells are shown in Fig. 2. Both HSV-

1(\bar{m} P) and HSV-1(\bar{m} P)*ts*HA1 synthesized viral DNA at 33.5°C. In contrast to the parent HSV-1(\bar{m} P), the mutant failed to synthesize viral DNA in cells infected and maintained at the nonpermissive temperature. In addition, there was no viral DNA synthesis in either HSV-1(\bar{m} P)- or HSV-1(\bar{m} P)*ts*HA1-infected cells at either temperature in the presence of 300 μ g of PAA per ml (data not shown).

In the second series of experiments, we tested the temperature sensitivity of the DNA polymerase. In the first experiment, replicate cultures were infected with mutant or parental virus, incubated for 12 h at the permissive temperature, and then extracted and inactivated at 39°C for intervals up to 30 min. Analyses of viral DNA polymerase activity failed to show preferential inactivation of the polymerase specified by the mutant. In the second experiment, cells infected with mutant or parental virus were shifted from 33.5 to 39°C at 12 h postinfection. At hourly intervals, up to 16 h postinfection, replicate cultures were harvested and cell extracts were tested for DNA polymerase activity. No differences in DNA polymerase activity between parent- and mutant-infected cells were found. These studies indicated that the mutation was not in the viral DNA polymerase.

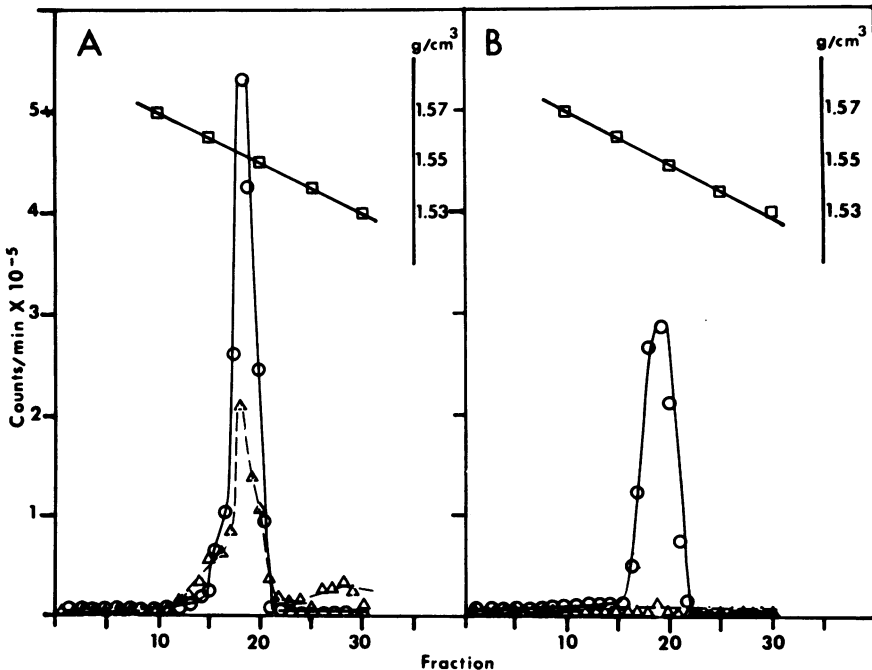


FIG. 2. Distribution in NaI density gradients of [³H]thymidine-labeled DNA from HEL cells infected with parent or mutant virus and incubated at the permissive and nonpermissive temperatures. Note that confluent HEL cells at rest produce little or no host DNA after infection (15). (A) 33.5°C; (B) 39°C. (○—○) HSV-1(\bar{m} P); (△—△) HSV-1(\bar{m} P)*ts*HA1; (□—□) density of NaI solution as determined from its refractive index.

Characterization of *tsHA1*: protein synthesis at the permissive and nonpermissive temperatures. In this series of experiments, replicate HEP-2 cultures were infected with HSV-1($\bar{m}P$) or HSV-1($\bar{m}P$)*tsHA1* and incubated at 33.5 and 39°C. At various times postinfection, the cells were labeled for 2-h intervals with ^{14}C -amino acids. Autoradiographic images of the electrophoretically separated polypeptides are shown in Fig. 3. The most obvious feature of the

results was the reduction in the synthesis of γ polypeptides during the 10- to 12-h labeling interval in cells infected with the mutant virus and incubated at 39°C. Specific γ polypeptides, e.g., ICPs 15, 19, 20, 37, and 43, were not synthesized, whereas others, e.g., ICPs 5, 25, and 32, were synthesized in greatly reduced amounts. The synthesis of γ polypeptide ICP 11, however, was not appreciably affected. In addition, in *tsHA1*-infected cells incubated at 39°C, the β polypep-

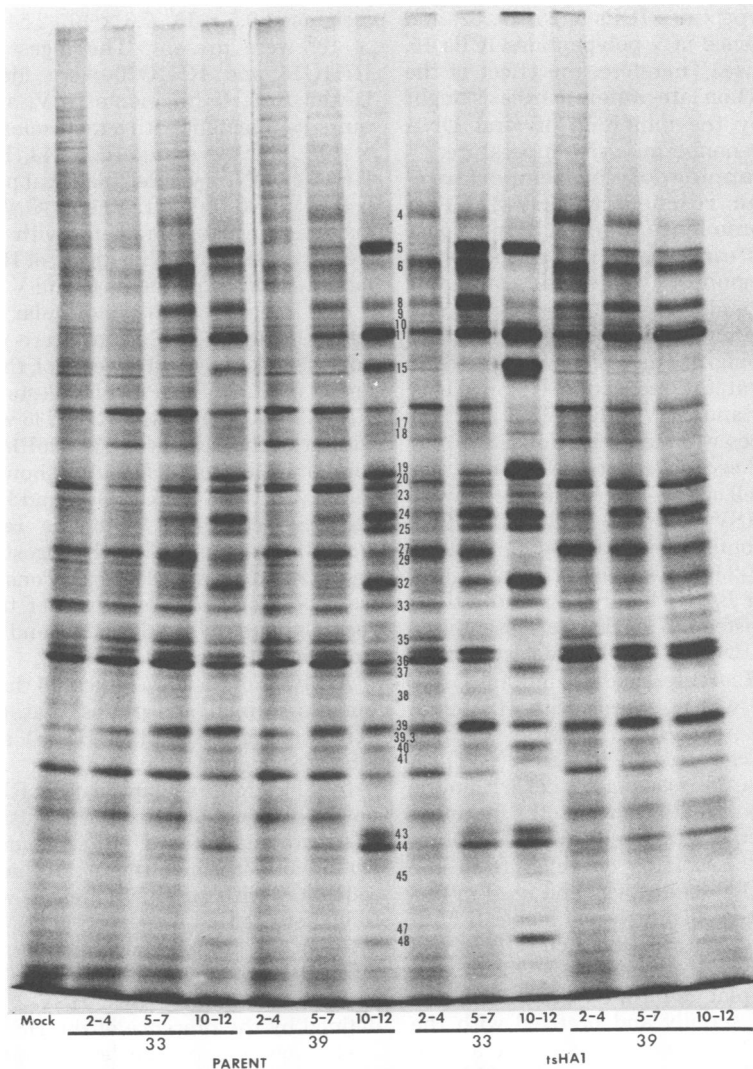


FIG. 3. Autoradiographic images of electrophoretically separated polypeptides labeled at different times in cells infected with parent or mutant virus and maintained at permissive and nonpermissive temperatures. The labeling intervals were 2 to 4, 5 to 7, and 10 to 12 h postinfection. The ICPs were labeled according to Honess and Roizman (12) as modified by Morse et al. (23). Note that in this experiment the infection in cells with the mutant virus at 33.5°C is more advanced than that in cells infected with the parent. In other experiments the patterns at the permissive temperature were identical.

tides were made late in infection in amounts far greater than in cells incubated at the permissive temperature. For example, the β polypeptides ICPs 6, 8, 36, and 39 and the β/γ polypeptide ICP 24 were synthesized in large amounts during the 10- to 12-h labeling interval.

The electrophoretic profile seen at the non-permissive temperature in HA1-infected cells was similar to that seen in cells infected with PAA-sensitive virus in the presence of PAA (Fig. 4). This figure shows that the inhibition of viral DNA synthesis resulted in reduction in the synthesis of γ polypeptides ICPs 5, 25, 29, 32, and 33 and the absence of γ polypeptides ICPs 15, 19, 20, 43, and 44. Therefore, the effect of the mutation in HA1 on late protein synthesis might be mediated by the inhibition of viral DNA synthesis at the nonpermissive temperature.

Physical mapping of the temperature-sensitive lesion: rescue with HSV-2 (G) restriction endonuclease digests. The purpose of this series of experiments was to locate the approximate region of the genome containing the HA1 lesion and to identify the polypeptides whose genes are located at or near the mutated site. The mapping of the lesion was based on observations that the restriction endonuclease maps of HSV-1 and HSV-2 differ (10) and that HSV-2 sequences will substitute for HSV-1 sequences in marker rescue tests (18, 35).

Rabbit skin cell cultures were transfected with a mixture of HSV-1(mP)*tsHA1* DNA and restriction endonuclease-digested HSV-2(G) DNA. The HSV-2 DNA was digested with either *HsuI*, *EcoRI*, or *BglII*. The results of the titrations of the transfection stocks are presented in Table 1. The HSV-2(G) DNA digested with either *HsuI* or *EcoRI* rescued the mutant DNA. However, there was no rescue with HSV-2(G) DNA digested with *BglII*, possibly because there is a *BglII* cleavage site in HSV-2(G) DNA at map position 0.387 (see Fig. 7), i.e., within the minimal map coordinates in which the mutation has been mapped as described below. The number of stable *ts*⁺ isolates obtained from the *EcoRI* and *HsuI* rescue stocks and used in further experiments is also listed in Table I. Four isolates from the *HsuI* digest rescue stocks and eight from the *EcoRI* digest rescue stocks were analyzed. The plaque-purified rescued clones were numbered serially and prefixed with the designation RH1G, denoting that these are recombinants produced by rescue of the HA1 mutant with HSV-2(G) DNA.

The DNAs of these *ts*⁺ recombinants were digested with the restriction endonucleases *BamHI*, *HpaI*, *BglII*, and *KpnI*. The electrophoretically separated *BamHI* and *KpnI* digests are presented in Fig. 5 and Fig. 6, respectively.

The *BamHI* fragment G was absent in the digests of all the isolates except those of RH1G30 through RH1G36. The digest of RH1G7 DNA did not contain *BamHI* fragments D-H, C', W, R, V, U, Q, or G; i.e., there were no HSV-1 *BamHI* restriction cleavage sites between coordinates 0.28 and 0.45. This recombinant contained a number of new bands with molecular weights of 9.5×10^6 , 7.3×10^6 , 2.68×10^6 , 2.54×10^6 , and 1.6×10^6 . In the digest of RH1G8 DNA, *BamHI* fragments D-H, C', W, R, and V were absent, and new fragments with molecular weights of 9.5×10^6 , 7.3×10^6 , 2.54×10^6 and 1.6×10^6 were present. The digest patterns for RH1G13 and RH1G20 were identical. Each lacked *BamHI* fragments G, V, and R but acquired a fragment with a molecular weight of 7.3×10^6 . The digests of RH1G43, RH1G44, and RH1G47 DNAs yielded identical patterns. Each lacked the *BamHI* fragments G, V, and R but acquired a new fragment with a molecular weight of 8.9×10^6 . The digest of RH1G48 DNA lacked *BamHI* fragments G and V but exhibited a new fragment with a molecular weight of 6.6×10^6 . Similar conclusions were derived from analyses of the *KpnI* digests of these recombinants (Fig. 6). The *KpnI* digests of RH1G30, RH1G31, RH1G35, and RH1G36 were identical to that of the mutant HSV-1(mP)*tsHA1*; therefore, only that of RH1G30 is shown. The *KpnI* digests of RH1G43, RH1G44, and RH1G47 were identical, consistent with the results of the *BamHI* digestions. Also, the digests of RH1G13 and RH1G20 were identical. Consistent results were obtained from analyses of the digests of recombinant DNAs with *BglII* and *HpaI* restriction endonucleases (not shown).

Analyses of the data indicated that the recombinants formed six groups that differ in the extent of replacement of HSV-1 sequences by HSV-2 sequences (Fig. 7).

(i) The group exemplified by RH1G30 could not be differentiated from the mutant virus and therefore represented either revertants or recombinants in which the replacement of HSV-1 sequences with HSV-2 sequences was too small to be detected.

(ii) In the group of recombinants exemplified by RH1G8, the maximum extent of replacement of HSV-1 sequences with HSV-2 sequences is defined by the presence of the HSV-1 *KpnI*-N-P cleavage site on the left boundary and the absence of the HSV-2 *KpnI*-G-D cleavage site on the right boundary. The minimum extent of replacement is defined by the absence of the HSV-1 *BamHI*-G-V cleavage site on the left boundary and the absence of the HSV-1 *HpaI*-B-H cleavage site on the right boundary.

(iii) The recombinants exemplified by RH1G7

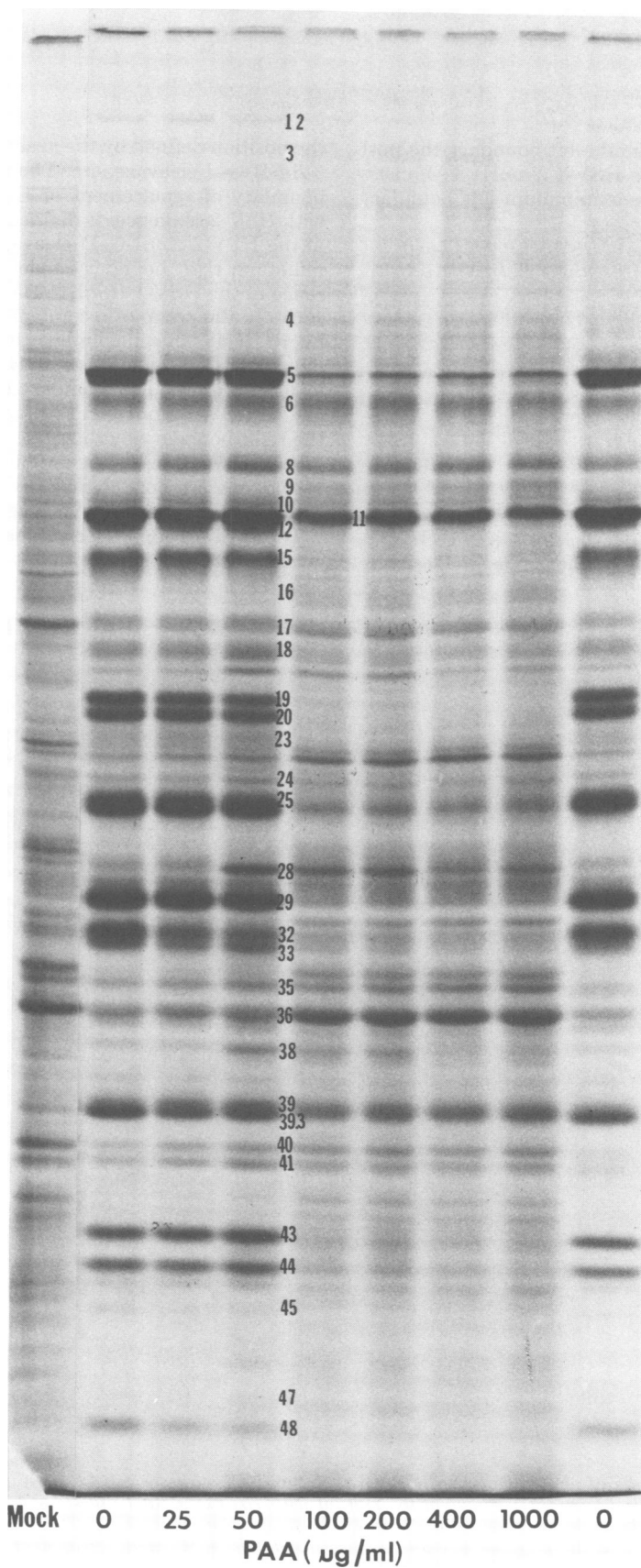


FIG. 4. *Autoradiographic images of electrophoretically separated polypeptides from cells infected with HSV-1(F) and maintained in the presence of the indicated concentrations of PAA from the time of infection until 24 h postinfection. The cells were labeled from 18 to 24 h postinfection.*

have as their maximum left boundary the position defined by the HSV-1 *Bam*HI-T-J' cleavage site and, as their minimum left boundary,

TABLE 1. Rescue of HSV-1(\bar{m} P)*ts*HA1 with restriction endonuclease-digested HSV-2(G) DNA

Endonuclease	Efficiency of plating ^a	No. of stable <i>ts</i> ⁺ isolates ^b
None	$<4.8 \times 10^{-6}$	0
<i>Hsu</i> I	3.0×10^{-3}	2
<i>Hsu</i> I	1.3×10^{-3}	2
<i>Eco</i> RI	4.0×10^{-4}	4
<i>Eco</i> RI	1.1×10^{-3}	4
<i>Bg</i> III	$<5.0 \times 10^{-4}$	0
<i>Bg</i> III	$<2.8 \times 10^{-4}$	0

^a Efficiency of plating of progeny after transfection, expressed as ratio of titers at 39 and 33.5°C.

^b Number of isolates plaque-purified and tested.

the position defined by the absence of the HSV-2 *Bgl*II-G-J cleavage site. The maximum right boundary of replacement of HSV-1 sequences with HSV-2 sequences is defined by the absence of the HSV-2 *Kpn*I-G-D cleavage site, and the minimum right boundary is defined by the absence of the HSV-1 *Hpa*I-B-H cleavage site.

(iv) In the group of recombinants exemplified by RH1G48, the maximum extent of replacement of HSV-1 sequences with HSV-2 sequences is defined on the left boundary by the presence of the HSV-1 *Kpn*I-N-P cleavage site and on the right boundary by the presence of the HSV-1 *Bam*HI-V-R site. The minimum left and right boundaries are both defined by the absence of the HSV-1 *Bam*HI-G-V cleavage site.

(v) In the group of recombinants exemplified by RH1G44, the maximum left boundary is de-

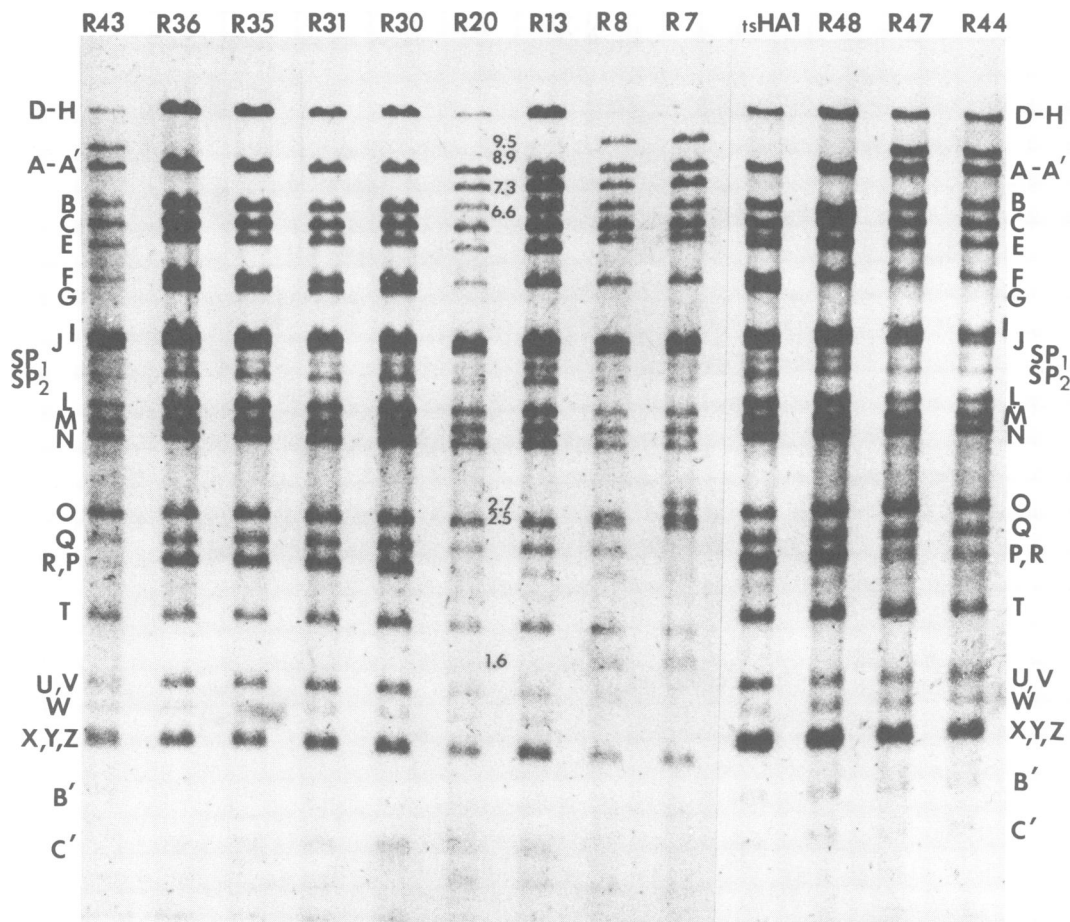


FIG. 5. Photographs of electrophoretically separated *Bam*HI digests of *ts*HA1 DNA and the DNAs of recombinants produced by marker rescue of the mutant with *Hsu*I or *Eco*RI digests of HSV-2 (G) DNA. A reversal image of gels stained with ethidium bromide is shown. Note that in HSV-1(\bar{m} P) DNA, the *Bam*HI fragments D and H and the fragments A and A' are fused. The L-S junction fragment is designated SP according to nomenclature previously established (16).

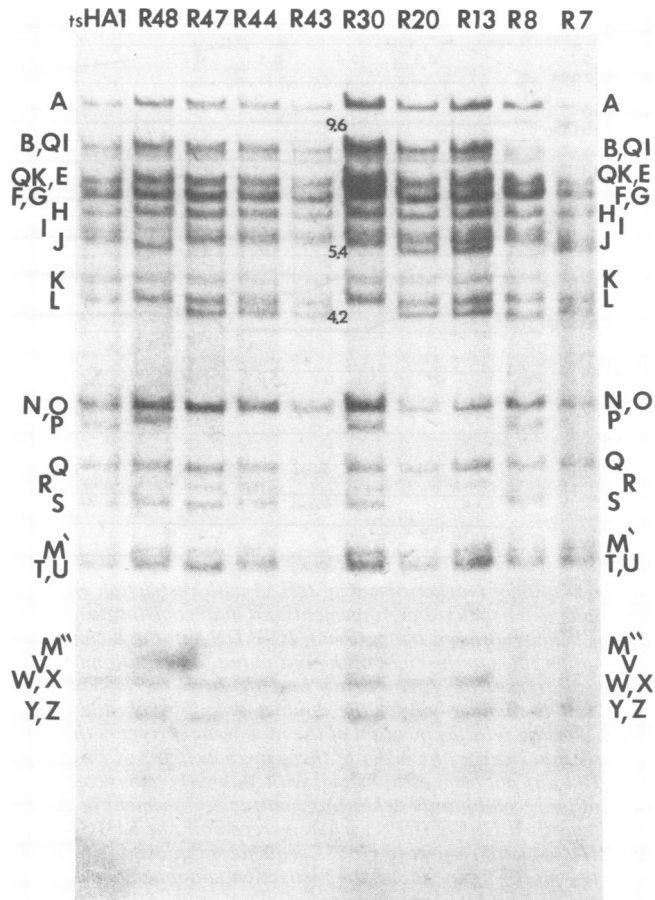


FIG. 6. Photographs of electrophoretically separated *KpnI* digests of *tsHA1* DNA and the DNAs of recombinants produced by marker rescue of the mutant with digests of HSV-2 DNA. A reversal image of gels stained with ethidium bromide is shown. Note that in HSV-1($\bar{m}P$) the *KpnI* fragment M is cleaved to M' and M'' (note direction). The L-S junction fragments are designated QI and QK according to nomenclature previously established (16).

finied by the presence of the HSV-1 *KpnI*-S-N cleavage site, and the minimum is defined by the presence of the HSV-2 *KpnI*-J-M cleavage site. The maximum right border is the HSV-1 *BamHI*-R-W cleavage site, and the minimum is the HSV-1 *KpnI*-P-V cleavage site.

(vi) The maximum left boundary in the group of recombinants exemplified by RH1G13 is the HSV-1 *HpaI*-I-V cleavage site, and the minimum left boundary is the HSV-1 *HpaI*-V-B cleavage site. The maximum right boundary is defined by the HSV-1 *BamHI*-R-W cleavage site, and the minimum right boundary is the HSV-1 *KpnI*-P-V cleavage site.

Mapping polypeptide genes specified by HSV-2 sequences in the recombinant genomes within map units 0.28 to 0.48. Mapping of the polypeptide genes was based on known differences in electrophoretic mobility of

HSV-1- and HSV-2-specific polypeptides in sodium dodecyl sulfate-polyacrylamide gels (12, 23). In these experiments, HEp-2 cultures infected with HSV-1($\bar{m}P$), HSV-1($\bar{m}P$)*tsHA1*, HSV-2(G), and representative *tsHA1* × HSV-2 (G) recombinants were pulse-labeled with ^{14}C -amino acids at various times postinfection. Figure 8 shows the autoradiographic images of the electrophoretically separated ICPs. A summary of the map positions of the ICP templates deduced from these analyses is included in Fig. 7.

The electrophoretic separations shown in Fig. 8 indicate that the recombinants expressed the HSV-2 ICPs 2, 8, 11, 35, 36, 43, and 44, which have been previously identified and mapped (23), and a new polypeptide ICP 19.5, which was not previously detected. Specifically, the band designated as ICP 19 was maximally labeled late in infection and was previously thought to con-

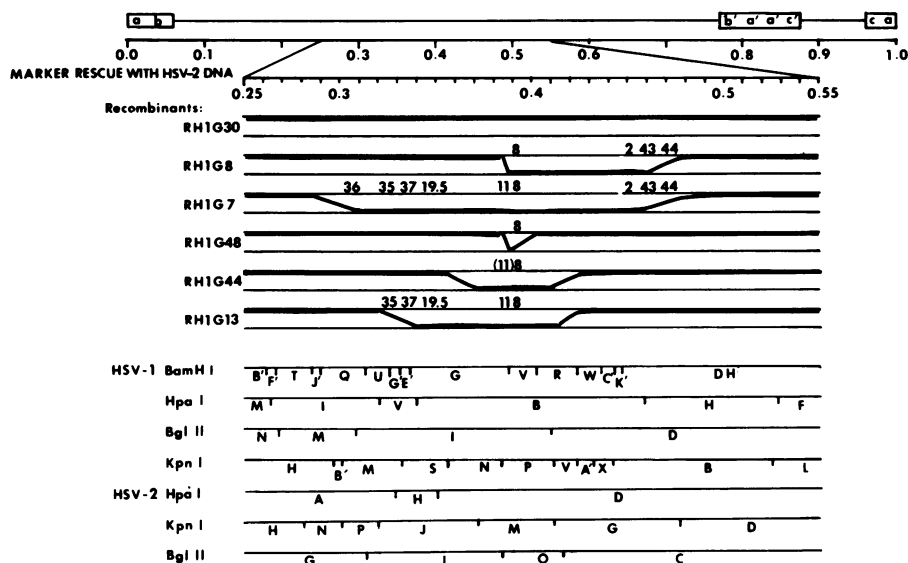


FIG. 7. Summary of the results of marker rescue of *tsHA1* with restriction endonuclease digests of HSV-2 DNA. The top two lines represent sequence arrangement and map coordinates of HSV DNA in the prototype arrangement. All the lines below represent the region of HSV DNA in the L component between 0.25 and 0.55 map units. The top and bottom lines to the right of the individual designations of the recombinants represent HSV-1 and HSV-2 sequences, respectively. The heavy line identifies the HSV-1 or HSV-2 sequences present in the recombinant DNAs. The numbers above the doublet lines identify the HSV-2 ICPs specified by the individual recombinants, and the relative position of the numbers represents the approximate location of the genes specifying ICPs 2, 43, and 44 has not been established. Although only one recombinant is listed for each line, each is representative of other recombinants exhibiting similar replacement of HSV-1 sequences with HSV-2 DNA. Note that RH1G44 specified HSV-2 ICP 11 whereas recombinant RH1G43, with identical borders and indistinguishable from RH1G44 with respect to HSV-1 sequences replaced with HSV-2 DNA, specified HSV-1 ICP 11. The bottom portion of the figure shows the restriction endonuclease maps of the region of the HSV-1 and HSV-2 DNAs between 0.25 and 0.55 map units.

tain a single γ polypeptide. In recombinants RH1G7, RH1G13, and RH1G20, the intensity of this band was greatly diminished, but a new band appeared. The γ polypeptide ICP 19 made late in infection was previously mapped between 0.66 and 0.7 map units (23), and translation products of RNA selected with DNA fragments from that region did indeed contain a polypeptide with the electrophoretic mobility of ICP 19 (data not shown). We suspect, therefore, that the polypeptide from lysates of cells harvested early in infection and comigrating with ICP 19 is different; therefore, we have designated it as ICP 19.5.

The genes specifying HSV-2 ICPs 19.5, 35, and 37 must lie to the right of map position 0.32 and to the left of map position 0.37. These polypeptides were not specified by RH1G8 or RH1G44; hence, their genes are located to the left of map position 0.37. The left border of these genes is defined by sequences present in RH1G13 at map position 0.32. The gene specifying ICP 36 must be located to the left of ICP

35 inasmuch as ICP 36 was not specified by RH1G8 or RH1G13. The genes specifying ICPs 2, 43, and 44 are located to the right of map position 0.415, defined by the minimal border of HSV-2 sequences in RH1G13, and left of map position 0.48, defined by the right maximum border of HSV-2 sequences in RH1G7 and RH1G8. Studies by D. M. Knipe, W. Batterson, and B. Roizman (submitted for publication) have localized the left border of ICPs 2, 43, and 44 at map position 0.45.

The mapping of the gene specifying ICP 11 is more complex. RH1G7 and RH1G13 both specified HSV-2 ICP 11. Whereas RH1G44 specified an HSV-2 ICP 11, the recombinants RH1G43 and RH1G47 specified an HSV-1 ICP 11. Although no differences in restriction endonuclease patterns were observed, RH1G43 and RH1G47 could have a smaller replacement of HSV-1 sequences with HSV-2 sequences at their borders. Previous studies have also mapped HSV ICP 11 in this region (23). Because RH1G8 did not specify an HSV-2 ICP 11, the data sug-

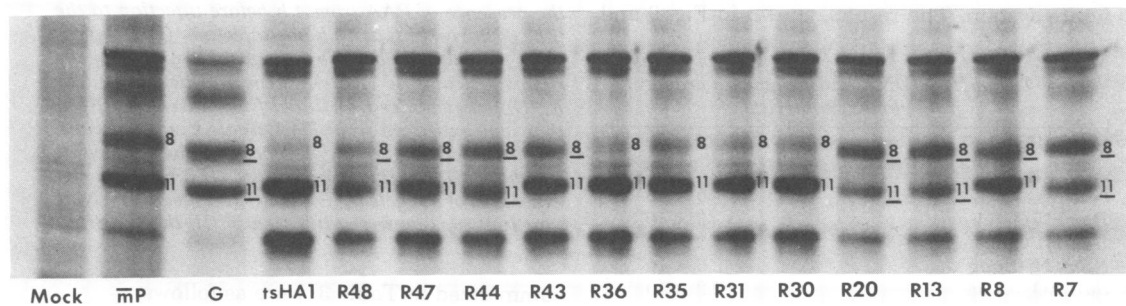
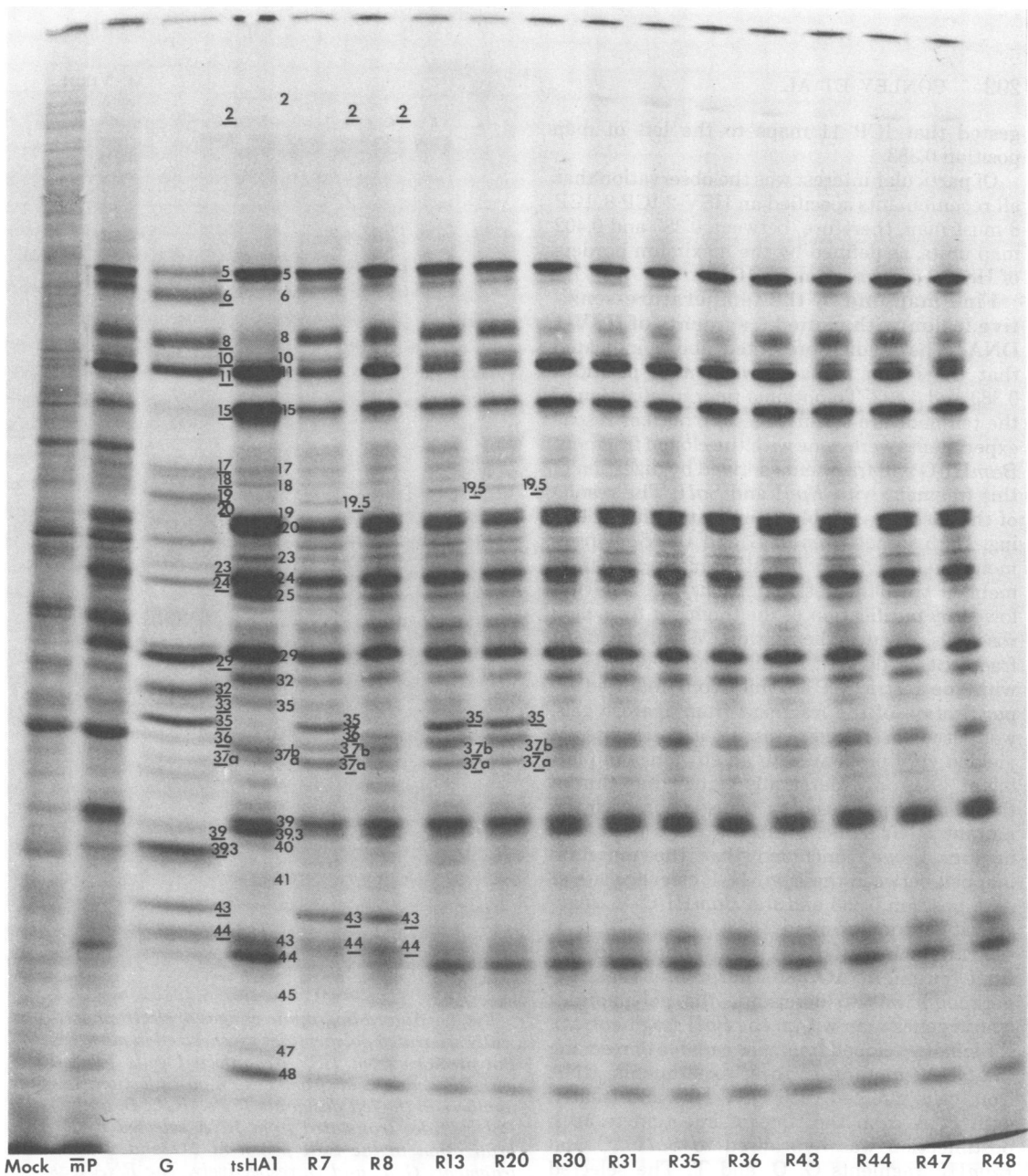


FIG. 8. Autoradiographic images of electrophoretically separated polypeptides from labeled mock-infected cells and from cells labeled from 10 to 12 h postinfection with HSV-1($\bar{m}P$), HSV-2(G), *tsHA1*, and recombinants produced by marker rescue of *tsHA1* with digests of HSV-2(G) DNA. The bottom insert shows an expanded portion of another gel which better illustrates the difference in the electrophoretic mobilities of HSV-1 and HSV-2 ICPs 8 and 11.

gested that ICP 11 maps to the left of map position 0.383.

Of particular interest was the observation that all recombinants specified an HSV-2 ICP 8. ICP 8 must map, therefore, between 0.383 and 0.402 map units, as defined by the maximum borders of HSV-2 sequences in RH1G48.

Fine mapping of the temperature-sensitive lesion with cloned fragments of HSV-1 DNA. The studies presented above indicated that the lesion maps between map positions 0.383 and 0.402. To obtain a finer localization of the temperature-sensitive lesion, marker rescue experiments were done with the cloned fragment *Bam*HI-G and fragments derived by digestion of this fragment with *Kpn*I and *Sal*I. The results of these studies (Table 2) indicated the following. Both intact *Bam*HI-G and the *Sal*I fragment Δ O, derived by digestion of *Bam*HI fragment G with the restriction enzyme *Sal*I followed by purification of the fragment from agarose gels, rescued the mutant. When the *Kpn*I fragment N, derived by digestion of *Bam*HI-G with the restriction enzyme *Kpn*I followed by purification of the fragment from agarose gels, was used in marker rescue experiments, it yielded viral progeny with an efficiency of plating at 39°C only marginally higher than that of the progeny produced by transfection with the mutant DNA alone. From the maps presented in Fig. 1, we concluded that the mutation mapped between the *Kpn*I-N-P cleavage site at map position 0.383 and the *Bam*HI-G-V cleavage site at map position 0.388.

Translation of RNA homologous to the *Sal*I fragment Δ O. The purpose of these experiments was to determine the polypeptides whose genes map within the *Sal*I fragment Δ O, the smallest cloned fragment capable of rescuing the *ts*HA1 mutant. In these experiments, RNA from cells infected with HSV-1(F) and treated with PAA from the time of infection to 10 h postinfection was hybridized to *Sal*I Δ O and *Bam*HI fragments G, Q, and T. The selected RNA was then translated in the reticulocyte lysate system. The results, shown in Fig. 9 and

TABLE 2. Rescue of HSV-1(\bar{m} P)*ts*HA1 with cloned HSV-1 *Bam*HI-G DNA and fragments prepared from cloned *Bam*HI-G

HSV-1(F) DNA Fragment from pRB102	Efficiency of plating ^a
None	5×10^{-6}
<i>Bam</i> HI-G, 0.1 μ g	3×10^{-3}
<i>Bam</i> HI-G, 0.4 μ g	3.2×10^{-2}
<i>Kpn</i> I-N, 0.1 μ g	2.6×10^{-5}
<i>Sal</i> I Δ O, 0.1 μ g	2.2×10^{-3}

^a Efficiency of plating of progeny after transfection, expressed as ratio of titers at 39 and 33.5°C.

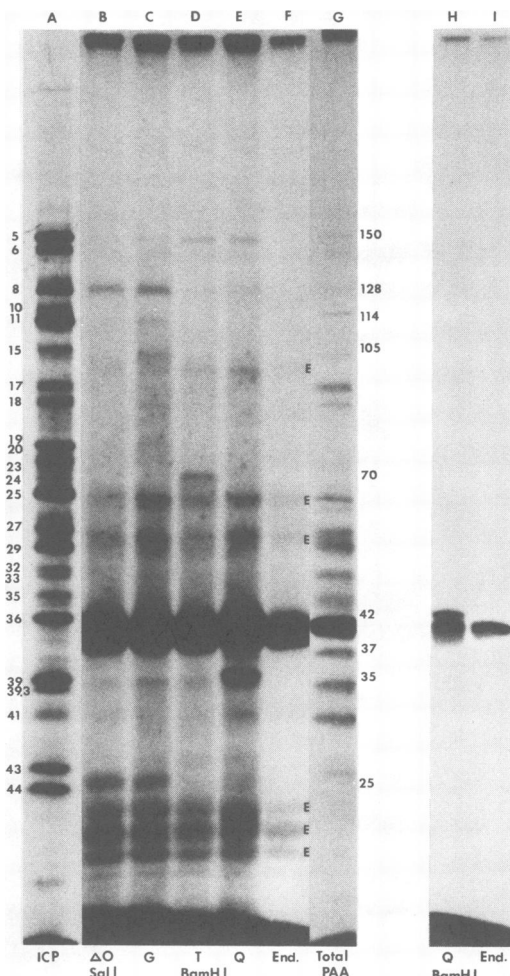


FIG. 9. Autoradiographic images of electrophoretically separated polypeptides synthesized *in vitro*. (A) Polypeptides from HSV-1(F)-infected cells labeled with ¹⁴C-amino acids from 5 to 7 h postinfection. The numbers to the left designate ICPs. (B, C, D, and E) Polypeptides translated from RNA selected by hybridization to the *Sal*I fragment Δ O and *Bam*HI fragments G, T, and Q, respectively. The RNA was extracted from HSV-1(F)-infected cells maintained in the presence of PAA from 4 h before infection to the time of harvesting at 10 h postinfection. (F) Polypeptides translated from the endogenous mRNA of the rabbit reticulocyte lysate system and designated by E. (G) polypeptides translated from unselected RNA that was extracted from PAA-treated infected cells. The numbers to the right represent the molecular weights ($\times 10^3$) of the polypeptides. (H and I) Shorter exposures of the gels in (E) and (F), respectively. Note that ICP 36 is more readily seen in (H) than in (E).

summarized in Table 3, were as follows.

(i) Two polypeptides with molecular weights of 128,000 and 25,000 were translated from RNA

TABLE 3. *In vitro* translated polypeptides using RNA selected with cloned HSV-1 DNA

Fragment	Map location	Mol wt ($\times 10^3$) of polypeptides translated from selected RNA	Previous identification
<i>Sa</i> I Δ O	0.372-0.388	128	ICP 8
		25	NPI ^a
<i>Bam</i> HI-G	0.337-0.388	128	ICP 8
		114	ICP 11
		105	ICP 15
		25	NPI
<i>Bam</i> HI-Q	0.290-0.312	150	ICP 5
		42	ICP 36
		37	ICP 37
		35	ICP 39
<i>Bam</i> HI-T	0.267-0.286	150	ICP 5
		70	ICP 24

^a NPI, Not previously identified.

selected by hybridization to *Sa*I Δ O. The polypeptide with a molecular weight of 128,000 appeared to comigrate with ICP 8, which was expected to map in that region. A polypeptide with a molecular weight of 25,000 has not been previously mapped in that region. A polypeptide with that approximate molecular weight appeared in the translation of total PAA RNA but not in lysates of cells late in infection. At this time it is unclear whether this is a scarce polypeptide not previously detected or a premature translation termination product of ICP 8. It should be noted that endogenous RNA appeared to be translated more efficiently in the presence of added RNA than in its absence, as evidenced by the intensity of the endogenous polypeptide bands.

(ii) The specificity of the selection was evident from the observation that the same two polypeptides were translated from the RNA selected with the *Bam*HI fragment G and from the observation that these polypeptides were not present in the translation products of the RNA selected with *Bam*HI fragments Q and T. *Bam*HI-G translated two additional polypeptides with molecular weights of 114,000 and 105,000. The polypeptide with a molecular weight of 114,000 corresponded to ICP 11, whereas the polypeptide with a molecular weight of 105,000, probably a gpA or gpB precursor, corresponded to ICP 15.

(iii) The *Bam*HI fragment Q-selected RNA translated polypeptides with molecular weights of 35,000, 37,000, 42,000, and 150,000. The polypeptide with a molecular weight of 35,000 cor-

responded to ICP 39, which was previously mapped to the region. The polypeptide with a molecular weight of 37,000 corresponded to ICP 37, which was mapped to the region by the intertypic recombinants presented in this study. Studies to be presented elsewhere indicate that ICP 37 forms two bands *a* and *b*, corresponding to a precursor and product. The polypeptide with a molecular weight of 42,000 corresponded to ICP 36, the HSV thymidine kinase. Both ICP 36 and the thymidine kinase have been previously mapped within the *Bam*HI fragment Q. The polypeptide with a molecular weight of 150,000 corresponded to ICP 5, based on both its electrophoretic mobility and map location. Previous studies have shown that a large portion of the gene specifying ICP 5 maps between 0.23 and 0.29 map units, and indeed the RNA selected by the *Bam*HI fragment T translated ICP 5. The RNA selected by this fragment also translated a polypeptide with an approximate molecular weight of 70,000 which may correspond to one of the polypeptides which map in the region. ICP 24, molecular weight 68,000, was previously mapped in that region.

DISCUSSION

In this paper we report on the characterization of a mutant isolated by mutagenesis of a specific fragment of the HSV DNA.

Production of the temperature-sensitive mutant. The HSV-1(\bar{m} P)*ts*HA1 mutant was produced by hydroxylamine mutagenesis of an HSV-1 fragment. Unlike the mutants previously produced by Chu et al. (6), the concentration of hydroxylamine used in this study was 100 times lower to reduce the probability of secondary mutations within the mutagenized fragment. Consistently, we isolated only one mutant from 300 plaques screened from the progeny of the transfection of intact DNA and the mutagenized fragment.

Mapping of the *ts* mutation. The mapping of the temperature-sensitive mutation was done to verify that the mutation was within the mutagenized fragment and to localize the mutation within the narrowest possible limits permitted by the procedures employed in this study. Marker rescue with HSV-2 DNA fragments indicated that the mutation was within the HSV-1 *Bgl*II fragment I and localized the mutation between 0.385 and 0.402 map units. Additional marker rescue with cloned HSV-1 DNA fragments localized the mutation between the *Kpn*I-N-P cleavage site and the *Bam*HI-G-V cleavage site, i.e., within a stretch of DNA of less than 1,000 base pairs.

Phenotype of the *ts*HA1 mutant. The effi-

ciency of plating of this mutant at 39°C compared to 33.5°C was 5×10^{-6} , comparable to that of the most stable mutants produced by general mutagenesis of the viral genome. Three properties of this mutant are of particular interest. First, the mutant was DNA⁻; however, the lesion did not appear to be within the DNA polymerase. Second, at the nonpermissive temperature, cells infected with *ts*HA1 produced α and β polypeptides in amounts at least as high as those produced in cells infected with wild-type virus; however, some γ polypeptides (e.g., ICPs 5, 25, and 32) were reduced, and others (e.g., ICPs 15, 19, 20, 37, and 43) could not be detected. The failure to express γ polypeptides in normal concentrations suggested that the mutation was in a β function necessary for the expression of late γ polypeptides. Because the same polypeptide patterns were produced with wild-type virus in infected cells treated with concentrations of PAA sufficient to inhibit viral DNA synthesis, the data suggested that the failure of the mutant to produce normal amounts of γ polypeptides might be related to the absence of viral DNA synthesis at the nonpermissive temperature. Lastly, several experiments, including those shown in Fig. 3, indicated that the electrophoretic pattern at the permissive temperature could not be differentiated from that of cells infected with wild-type virus. This observation was consistent with the expectation that mutants produced by site- or fragment-specific mutagenesis should not contain secondary, nonlethal mutations in genes residing elsewhere in the genome which could affect the pattern of synthesis of polypeptides.

Identification of the gene product containing the temperature-sensitive lesion. Analyses of recombinants produced by marker rescue with HSV-2 DNA digests showed that ICP 8 was the only HSV-2 polypeptide specified by all the recombinants containing detectable HSV-2 DNA sequences. Inasmuch as additional polypeptides specified by the HSV-2 sequences in the recombinant genomes may have electrophoretic mobilities identical to their HSV-1 counterparts, we then proceeded to translate RNA selected by hybridization to the smallest available HSV-1 DNA fragment carrying the sequences which rescued the mutant. The data show that two polypeptides ICP 8 and a previously undetected polypeptide with a molecular weight of 25,000 mapped within the region of the genome rescuing the mutation.

Several points in connection with these results should be noted. (i) The RNA selected for these studies was prepared in PAA-treated cells. Because the shutoff of β polypeptide synthesis is

delayed both in PAA-treated cells infected with wild-type virus and in cells infected with the *ts*HA1 mutant virus at the nonpermissive temperature, we could expect a larger than normal accumulation of transcripts from β genes, including the gene bearing the *ts* mutant. Therefore, if mRNA's specifying other β polypeptides map in that region, they should have been detected. It should be noted parenthetically that other fragments of HSV DNA, used as controls, selected RNAs that specified other polypeptides which in untreated infected cells accumulate in both abundant and scarce amounts.

(ii) The 25,000-molecular-weight polypeptide was not previously detected. It is conceivable that a polypeptide with that molecular weight could comigrate with ICP 43. Although it has been known that HSV-1 ICP 8 is a β polypeptide with strong binding affinity for viral DNA (2, 28, 37), it has not been previously known that its function is required for viral DNA synthesis. The molecular weight of ICP 8 (128,000) is less than the reported molecular weight of the HSV DNA polymerase (26). It should be noted parenthetically that two complementation groups involving *ts* mutations in the viral DNA polymerase have been reported (5). Representative mutants from each group have been mapped by marker rescue but not by the RNA selection and translation technique used in this study. Although the polypeptide carrying the *ts* DNA polymerase lesion has not been identified, these mutants appear to map to the right of the *ts*HA1 mutation (5). These observations appear to reinforce our conclusion that the mutation in *ts*HA1 was not in the viral DNA polymerase.

(iii) Analyses of the recombinants produced by marker rescue with HSV-2 DNA digests have more precisely localized the map locations of HSV genes previously mapped. These are genes for ICPs 36, 35, 2, 43, and 44. In addition, these studies localized the genes specifying two polypeptides, ICP 37 and ICP 19.5 with molecular weights of 40,000 and 84,000, respectively, which have not been previously mapped. The ICP 19.5 is of particular interest because the band containing ICP 19 was considered to contain a single polypeptide. ICP 19.5, produced in more abundant amounts 10 to 12 h postinfection, had not been previously detected. Our data also suggested that ICP 11 maps in close vicinity to, and probably to the left of the ICP 8 gene.

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