Alterations in Glycoprotein gB Specified by Mutants and Their Partial Revertants in Herpes Simplex Virus Type 1 and Relationship to Other Mutant Phenotypes

MARY L. HAFLEY AND PATRICIA G. SPEAR*

Department of Microbiology, The University of Chicago, Chicago, Illinois 60637

The *tsB5* mutant of herpes simplex virus type 1 (HSV-1) strain HFEM was shown previously to be temperature sensitive for accumulation of the mature form of glycoprotein gB, for production or activity of a factor required in virus-induced cell fusion, and for production of virions with normal levels of infectivity. In addition, a previous study showed that virions produced by *tsB5* at permissive temperature were more thermolabile than HFEM virions and contained altered gB that did not assume the dimeric conformation characteristic of HFEM. Results presented here demonstrate that, at permissive temperature, *tsB5* differs from HFEM in another respect: plaques formed by *tsB5* are syncytial on Vero cells (but not on HEp-2 cells), whereas plaques formed by HFEM are nonsyncytial on both cell types. In addition, our results indicate that *tsB5* produces an oligomeric form of gB, but that it differs in electrophoretic mobility and stability from the gB dimers of HFEM. The major purpose of this study was to investigate the dependence of the various *tsB5* mutant phenotypes on the temperature sensitivity of gB accumulation and on the alterations in oligomeric conformation of gB produced at permissive temperature. For this work the following HSV-1 strains related to *tsB5* or HFEM were analyzed: (i) phenotypic revertants selected from *tsB5* stocks for nonsyncytial plaque morphology on Vero cells or for ability to form plaques at restrictive temperature (38.5°C); (ii) a plaque morphology variant of HFEM selected for its syncytial phenotype on Vero cells; (iii) temperature-sensitive recombinants previously isolated from a cross between *tsB5* and the non-temperature-sensitive syncytial strain HSV-1(MP); and (iv) a phenotypic revertant selected from one of the recombinant stocks for its ability to form plaques at 39°C. These strains were all compared with *tsB5* and HFEM at three different temperatures in two different cell lines with respect to plaque formation, yield of infectious progeny, virus-induced cell fusion, and accumulation of gB. The results of our analyses on all the strains tested revealed the following correlations between mutant phenotypes and the accumulation and oligomeric conformation of gB. (i) There was a direct and quantitative relationship between the accumulation in infected cells of infectious progeny and of the mature form of gB, providing strong support for the hypothesis that this form of gB is necessary to the production of infectious virions. The oligomeric conformation of gB characteristic of HFEM is apparently not required for virion infectivity; nor was virion thermostability necessarily related to the presence of the HFEM-like oligomeric form of gB. (ii) The previously reported correlation between temperature sensitivity of gB accumulation and virus-induced cell fusion was confirmed for *tsB5* and extended to other virus strains, and coordinate reversion of these traits was also demonstrated, providing support for the hypothesis that gB has a role in virus-induced cell fusion. At 37°C, intermediate between permissive and restrictive temperatures, some of the mutants and partial revertants induced cell fusion despite reduced accumulations of the mature form of gB, suggesting that the amount of mature gB present did not determine the extent of fusion and that other forms of gB as well as other factors should be investigated with regard to the process of cell fusion. (iii) Some of the mutants and partial revertants could form plaques at 38.5°C despite reduced accumulations of gB and infectious progeny, indicating that the cell-to-cell transmission of viral infection may be at least in part independent of these factors.

Herpes simplex virus type 1 (HSV-1) specifies the synthesis of several glycoproteins that are constituents of virions and membranes in the infected cell (7, 14, 23). Although the functions of each of the HSV-1 glycoprotein species have not been fully defined, it is likely that members of this class of membrane-associated proteins are responsible for the infectivity of virions and for the altered interactions of infected cells, including aggregation and fusion. HSV-induced cell fusion appears to be a complicated process governed by viral products capable of inducing membrane fusion and other products whose activities can apparently prevent cell fusion (reviewed in J. Tooze [ed.], *The Molecular Biology of Tumor Viruses*, in press). Paradoxically, the fusion-inducing or syncytial (*syn*) phenotype can be considered a mutant phenotype resulting from the absence or failure of the viral gene products that modulate or inhibit fusion-inducing activity. In addition, host factors may influence the effect of HSV infection on cellular interactions because some *syn* mutants express the *syn* phenotype only in certain cell types and not in others (18).

Previous studies indicated that the glycoprotein designated gB is one of the HSV-1 products required in the virion envelope for infectivity and in the membranes of infected HEp-2 cells for virus-induced cell fusion. These studies of a temperature-sensitive (*ts*) mutant, designated HSV-1(HFEM)*ts*B5 (19), and of *ts* recombinant viruses obtained from a cross between HSV-1(HFEM)*ts*B5 and the non-*ts* syncytial virus strain, HSV-1(MP) (13), revealed that the failure to accumulate the mature form of gB at restrictive temperature correlated with reduced specific infectivity of virions produced and with the inability to induce cell fusion, respectively. In addition, other altered phenotypes have been associated with the *ts*B5 mutation; these alterations include enhanced thermolability of HSV-1(HFEM)*ts*B5 virions produced at permissive temperature and inability to accumulate the detergent-stable dimeric form of gB that appears to be characteristic of wild-type HSV-1 (19, 20).

To investigate the extent to which the various altered phenotypes associated with the *ts*B5 mutation were related to the temperature-sensitive accumulation of gB and to each other, we analyzed several partially reverted strains isolated from HSV-1(HFEM)*ts*B5, a non-*ts* revertant of the recombinant virus HSV-1(*ts*B5MP3) and an independently isolated plaque morphology variant of HFEM that is temperature sensitive for gB accumulation. The ability of each virus strain to produce plaques, to induce cell fusion, and to accumulate both gB and infectious

virus was assessed at three temperatures in two different cell lines, Vero and HEp-2. Previous studies of HSV-1(HFEM)*ts*B5 and the *ts* recombinant viruses had been done exclusively with HEp-2 cells. Because some of the strains derived from HSV-1(HFEM) and HSV-1(HFEM)*ts*B5 induce fusion at permissive temperature in Vero cells, but not in HEp-2 cells, we chose to include Vero cells in this study to assess directly the relationship between the temperature sensitivity of gB accumulation and fusion induced by these viruses. The use of Vero cells was also informative because the mutants were found to be less temperature sensitive in Vero cells than in HEp-2 cells. By analyzing glycoprotein synthesis over a range of temperatures in the two cell types, we were able to monitor the differential, cell-dependent accumulation of gB and the expression of other mutant traits as a function of temperature. In addition, the ability of the virus strains to produce thermostable virions and the wild-type dimeric form of gB at permissive temperature was monitored.

Our results demonstrated, first, that the accumulation of infectious virus at various temperatures in both Vero and HEp-2 cells closely correlated with the accumulation of the mature form of gB. This finding supports the hypothesis that gB is required for the production of infectious virions (19). Second, temperature sensitivity of cell fusion was correlated with temperature sensitivity of gB accumulation, as previously reported (13), although there was not necessarily a quantitative relationship between the extent of cell fusion and the amount of the mature form of gB present late in infection. Finally, the capacity of a virus strain to accumulate gB and infectious virus at restrictive temperature was not necessarily associated with the capacity to accumulate the wild-type dimeric form of gB or to produce thermostable virions at permissive temperature. Moreover, some of the phenotypic revertants were capable of forming plaques at 38.5°C despite reduced accumulations of gB and infectious virus at this temperature, indicating that neither may be required in large quantities for the efficient cell-to-cell transmission of HSV infection.

MATERIALS AND METHODS

Cells and media. Monolayer cultures of HEp-2 cells and African green monkey kidney cells (Vero) were grown in the Dulbecco modification of Eagle minimal essential medium containing 10% fetal calf serum. After infection, cells were maintained in medium 199 supplemented with 1% fetal calf serum (199-V). Cultures used for titrations of infectious units were maintained in medium 199 containing 1% fetal calf

serum and 0.1 to 0.3% pooled human gamma globulin (199-0) (18). Cells were purchased from Flow Laboratories, Inc., Rockville, Md.; media and sera were obtained from K. C. Biologicals, Inc., Lexana, Kans.

Viruses. The HSV-1 strains used in this study are listed in Table 1 and described further below and in the Results section. Virus stocks were lysates (mixed 1:1 with sterile skimmed milk) prepared from HEp-2 cells that had been infected at an input multiplicity of 0.01 PFU per cell and maintained at 34°C for 3 to 4 days.

Isolation and plaque purification of R5/tsB5, R2A/tsB5 and R3C/tsB5. Monolayer cultures of Vero cells were infected with serial dilutions of HSV-1(HFEM)tsB5 in 1 ml of medium 199-V. The inoculum was incubated with the cultures at 37.0°C for 2 h and then aspirated and replaced with 5 ml of medium 199-0. Infected cultures were incubated at either 33.5 or 38.5°C until small plaques were visible (~24 h). At that time the medium was removed from dishes with the lowest numbers of visible plaques and replaced by an overlay of 0.5% agarose in medium 199-V. Incubation was continued at the appropriate temperature for 12 to 24 h. A bent, sterile Pasteur pipette was used to aspirate agarose plugs over well-isolated plaques selected according to the criteria given in Table 1 for the isolation of spontaneous variants of tsB5. The plugs were placed in 0.5 ml of sterile skimmed milk and frozen at -70°C until use. Each sample was then sonicated on ice, serially diluted, and plated onto Vero monolayers for the second round of selection and purification from well-isolated plaques, as described above. A seed pool of virus was prepared in HEp-2 cells from the second plaque isolate and titrated, and subsequent stocks were prepared as described above.

Chemicals. D-[1-¹⁴C]glucosamine hydrochloride (45 to 55 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass. Chemicals used for polyacrylamide gel electrophoresis were supplied by Bio-Rad Laboratories, Richmond, Calif.

Preparation and analysis of radiolabeled cell extracts. Monolayer cultures of HEp-2 cells and Vero cells (4×10^6 cells per culture) were infected at an input multiplicity of 5 PFU of the appropriate virus strain per cell. Adsorption was for 2 h at 37°C; at that time the inoculum was replaced with medium 199-V, and the cultures were shifted to the appropriate temperature. At 3 h after infection the overlay medium was replaced with medium 199-V containing D-[1-¹⁴C]glucosamine hydrochloride (0.9 μ Ci/ml). Cells were harvested at 20 h after infection by decanting the medium, rinsing with phosphate-buffered saline, and adding 0.5 ml of sodium dodecyl sulfate (SDS) solubilization mixture (0.5 M Tris-hydrochloride [pH 7.0], 5% 2-mercaptoethanol, 2% SDS, and a trace of bromophenol blue dye) to each culture flask. The flasks were then frozen at -20°C until use, when each sample was thawed and sonicated, and a portion (the quantity of lysate from approximately 2×10^6 cells) was analyzed by electrophoresis on polyacrylamide gels by the technique of Laemmli (11) modified as previously described (6). For some analyses, the heat-treatment of samples before electrophoresis was omitted. The cross-linker used for polymerization of the acrylamide was *N,N'*-diallyltartardiamide. Radioactive proteins were detected by fluorography (2).

Efficiency of plating and virus titrations. Cell monolayers were infected with the appropriate dilutions of virus and, after an adsorption period of 2 h at 37°C, overlaid with medium 199-0 and shifted to the appropriate temperature for incubation. After 48 h the monolayers were rinsed, fixed with methanol, and stained with Giemsa for counting of the plaques.

Virus yields. Cell monolayers (4×10^6 cells per flask) were infected at a multiplicity of 2 PFU per cell. After an adsorption period of 2 h at 37°C, the inoculum was replaced with 3 ml of medium 199-V, and incubation was continued at the appropriate temperature. At 20 h after infection, morphology of the infected monolayers was scored, 3 ml of sterilized skim milk

TABLE 1. HSV-1 strains, mutants, and revertants used in this study

Virus strain [abbreviated designation] ^a	Parental strain(s)	Conditions for isolation of mutants		Source or reference
		Phenotype selected	Cell line	
HSV-1 (HFEM) [HFEM]				Obtained from A. Buchan
HSV-1 (HFEM) <i>syn</i> [HFEM _{syn}]	HFEM	Syncytial plaque at permissive temperature	Vero	Bauke and Spear (1)
HSV-1 (HFEM) <i>tsB5</i> [tsB5]	HFEM	Temperature sensitive for plaque formation	BHK	Obtained from A. Buchan; Manservigi et al. (13); Sarmiento et al. (19)
HSV-1 (R5/ <i>tsB5</i>) [R5/ <i>tsB5</i>]	<i>tsB5</i>	Nonsyncytial plaque at permissive temperature	Vero	This study
HSV-1 (R2A/ <i>tsB5</i>) [R2A/ <i>tsB5</i>]	<i>tsB5</i>	Syncytial plaque at restrictive temperature	Vero	This study
HSV-1 (R3C/ <i>tsB5</i>) [R3C/ <i>tsB5</i>]	<i>tsB5</i>	Nonsyncytial plaque at restrictive temperature	Vero	This study
HSV-1 (MP) [MP]	HSV-1 (NT)	Syncytial plaque	FL	Hoggan and Roizman (8)
HSV-1 (<i>tsB5</i> MP3) [tsB5MP3]	<i>tsB5</i> and MP	Syncytial plaque and temperature sensitive for plaque formation	HEp-2	Manservigi et al. (13)
HSV-1 (R/ <i>tsB5</i> MP3) [R/ <i>tsB5</i> MP3]	<i>tsB5</i> MP3	Syncytial plaque at restrictive temperature	HEp-2	Manservigi et al. (13)

^a Abbreviated designations for the virus strains are those used in the text.

was added, and the flasks were frozen at -70°C until titration. The flasks were then thawed (shaking during the thawing process to remove cells from the plastic), and the contents were sonicated and titrated as described above, using Vero cell monolayers and an incubation temperature of 33.5°C .

Heat inactivation of virus. Virus stocks produced at 34°C were diluted 100-fold, and aliquots were distributed into tubes on ice. At time zero all but one aliquot were placed in a 45°C water bath. The contents of the remaining tube were diluted and titrated at 33.5°C as described above. At appropriate time intervals tubes were removed from the 45°C bath and placed on ice for 1 min before dilution and titration.

RESULTS

Virus strains, mutants, and revertants.

Table 1 lists the virus strains used in this study along with criteria for selection of the various mutants and phenotypic revertants, their abbreviated designations, and other pertinent information. HFEM, the strain from which most of the mutants and revertants were derived, is a laboratory strain isolated many years ago from a patient (HF) and passaged first in eggs (E) and mice (M) before being passaged in cell culture (12). We obtained a stock of HFEM [labeled STH₂/BS10/(a)] from Alexander Buchan (University of Birmingham, Birmingham, United Kingdom) and prepared our own working stocks after three cycles of plaque purification by using virus from well-isolated nonsyncytial plaques formed at 39.0°C on Vero cells. In our hands both the original stock of HFEM and our own working stocks contain predominantly virus that forms nonsyncytial plaques but have a higher percentage of syncytial plaque-forming variants than is usually detectable in stocks of nonsyncytial strains. One of these variants, designated HFEM_{syn}, was isolated by Robert Baucke in our laboratory (1) from the virus produced by a large syncytial plaque formed on Vero cells at 37.0°C . The stocks of HFEM_{syn} obtained after three cycles of plaque purification produced syncytial plaques on Vero cells and nonsyncytial plaques on HEp-2 cells.

Mutant *tsB5* was isolated by Alexander Buchan after bromodeoxyuridine mutagenesis of HFEM and was selected for its temperature sensitivity of plaque formation on BHK cells. This mutant is also temperature sensitive for plaque formation on HEp-2 cells (13) and on Vero cells. At permissive temperature *tsB5* resembles HFEM_{syn} in its capacity to induce syncytial plaques on Vero cells and nonsyncytial plaques on HEp-2 cells.

For this study, revertants of *tsB5* were isolated as described in Materials and Methods by the following criteria for their selection (see also

Table 1). Both R2A/*tsB5* and R3C/*tsB5* were isolated from plaques that formed at 38.5°C on Vero cells, the former from a syncytial plaque and the latter from a nonsyncytial plaque. R5/*tsB5*, on the other hand, was selected to resemble parental strain HFEM with respect to its plaque morphology rather than its ability to form plaques at 38.5°C ; this revertant was isolated from a nonsyncytial plaque that formed at 33.5°C on Vero cells. The working stocks of these revertants were prepared after two cycles of plaque purification by the criteria described above and given in Table 1.

The MP strain, selected for its *syn* phenotype on FL cells, was isolated from its parental strain, NT, by Hoggan and Roizman (8). This strain differs from *tsB5* (and also from HFEM_{syn}, as will be seen) in that it is non-*ts* and its *syn* phenotype is expressed in many different cell types (4, 8, 16, 18). Manservigi et al. (13) isolated several recombinant viruses from a cross between MP and *tsB5* to investigate the effect of the *tsB5* mutation on HSV-1-induced cell fusion in HEp-2 cells. These recombinants, including *tsB5*MP3, were selected for their ability to produce syncytial plaques on HEp-2 cells at 34.0°C and for greatly reduced plating efficiency at 39.0°C . The revertant R/*tsB5*MP3 was isolated for this previous study (13) from a syncytial plaque that formed after plating *tsB5*MP3 at 39.0°C on HEp-2 cells.

It should be noted that all the mutants and revertants listed in Table 1, except *tsB5*, were obtained without the use of mutagenic agents.

Efficiency of plating and plaque morphology. The effect of temperature on the plating efficiencies of the virus strains just described was determined by using both Vero and HEp-2 cell monolayers (Table 2). The results obtained at 37.0 and 38.5°C are expressed as the fraction of plaques detected at 33.5°C (columns 4 and 8). The parental strain HFEM plates with slightly reduced efficiency only at 38.5°C on HEp-2 cells and plates efficiently on Vero cells at the three temperatures tested. The isolate designated HFEM_{syn} has a plating efficiency close to that of its HFEM parent on both cell types. In contrast, mutant *tsB5* is highly temperature sensitive for plaque formation on Vero cells as well as on HEp-2 cells at restrictive temperature (38.5°C) but plates efficiently at 37.0°C on both cell types. The spontaneous revertants isolated on Vero cell monolayers from *tsB5*, designated R2A/*tsB5*, R3C/*tsB5*, and R5/*tsB5*, plate with high efficiency on that cell line at the temperatures tested, unlike parental strain *tsB5*. However, two of these virus strains, R3C/*tsB5* and R5/*tsB5*, were found to plate on HEp-2 cells

TABLE 2. *Effect of temperature and host cell on plating efficiency and plaque morphology of HSV-1 strains*

Virus strain	Temp (°C)	HEp-2 cells				Vero cells				PFU/ml HEp-2/ Vero
		Plating efficiency		Plaque morphol- ogy	Fusion at high MOI ^a	Plating efficiency		Plaque morphol- ogy	Fusion at high MOI ^a	
		PFU/ml	Fraction of 33.5°C value			PFU/ml	Fraction of 33.5°C value			
HFEM	33.5	1.5 × 10 ⁹		Non- <i>syn</i>	—	2.2 × 10 ⁹		Non- <i>syn</i>	+4	0.68
	37.0	1.7 × 10 ⁹	1.11	Non- <i>syn</i>	—	1.8 × 10 ⁹	0.82	Non- <i>syn</i>	+4	
	38.5	7.3 × 10 ⁸	0.49	Non- <i>syn</i>	—	1.6 × 10 ⁹	0.73	Non- <i>syn</i>	+3	
HFEM _{syn}	33.5	2.8 × 10 ⁸		Non- <i>syn</i>	+3	6.3 × 10 ⁸		<i>syn</i>	+4	0.44
	37.0	3.4 × 10 ⁸	1.21	Non- <i>syn</i>	+2	5.3 × 10 ⁸	0.84	Mixed	+4	
	38.5	1.3 × 10 ⁸	0.46	Non- <i>syn</i>	+1	5.1 × 10 ⁸	0.81	Non- <i>syn</i>	+4	
<i>tsB5</i>	33.5	7.0 × 10 ⁷		Non- <i>syn</i>	+1	2.4 × 10 ⁹		<i>syn</i>	+4	0.03
	37.0	5.0 × 10 ⁷	0.71	Non- <i>syn</i>	+1	1.8 × 10 ⁹	0.75	Non- <i>syn</i>	+4	
	38.5	<10 ⁴	(<0.001)	—	—	<10 ⁴	(<0.001)	—	—	
R2A/ <i>tsB5</i>	33.5	1.6 × 10 ⁸		Non- <i>syn</i>	+1	8.0 × 10 ⁸		<i>syn</i>	+4	0.20
	37.0	2.0 × 10 ⁸	1.25	Non- <i>syn</i>	+2	6.3 × 10 ⁸	0.78	Mixed	+4	
	38.5	1.0 × 10 ⁸	0.63	Non- <i>syn</i>	+1	8.0 × 10 ⁸	1.00	Mixed	+4	
R3C/ <i>tsB5</i>	33.5	1.6 × 10 ⁸		Non- <i>syn</i>	—	5.6 × 10 ⁸		Mixed	+4	0.29
	37.0	1.0 × 10 ⁸	0.63	Non- <i>syn</i>	—	7.0 × 10 ⁸	1.25	Mixed	+4	
	38.5	<10 ⁴	(<0.001)	—	—	2.0 × 10 ⁸	0.36	Non- <i>syn</i>	—	
R5/ <i>tsB5</i>	33.5	7.0 × 10 ⁷		Non- <i>syn</i>	—	4.0 × 10 ⁸		Mixed	+4	0.18
	37.0	6.0 × 10 ⁷	0.86	Non- <i>syn</i>	—	7.4 × 10 ⁸	1.85	Mixed	+4	
	38.5	<10 ⁴	(<0.001)	—	—	4.6 × 10 ⁸	1.15	Non- <i>syn</i>	—	
<i>tsB5</i> MP3	33.5	4.3 × 10 ⁸		<i>syn</i>	+4	2.8 × 10 ⁸		<i>syn</i>	+4	1.54
	37.0	4.2 × 10 ⁸	0.97	<i>syn</i>	+3	2.6 × 10 ⁸	0.93	<i>syn</i>	+4	
	38.5	2.9 × 10 ⁴	(<0.001)	—	(±)	5.0 × 10 ⁷	0.18	<i>syn</i>	+3	
R/ <i>tsB5</i> MP3	33.5	4.0 × 10 ⁸		<i>syn</i>	+4	5.4 × 10 ⁸		<i>syn</i>	+4	0.74
	37.0	4.1 × 10 ⁸	1.03	<i>syn</i>	+4	5.7 × 10 ⁸	1.06	<i>syn</i>	+4	
	38.5	1.2 × 10 ⁸	0.30	<i>syn</i>	+4	4.2 × 10 ⁸	0.78	<i>syn</i>	+4	
MP	33.5	5.1 × 10 ⁷		<i>syn</i>	+3	8.9 × 10 ⁷		<i>syn</i>	+4	0.57
	37.0	8.3 × 10 ⁷	1.63	<i>syn</i>	+4	1.1 × 10 ⁸	1.24	<i>syn</i>	+4	
	38.5	6.5 × 10 ⁷	1.28	<i>syn</i>	+4	7.7 × 10 ⁷	0.87	<i>syn</i>	+4	

^a MOI, Multiplicity of infection. Extent of cellular fusion in synchronously infected cultures (2 PFU per cell) at 20 h after infection. The minus (—) symbol denotes absence of fusion, the plus-minus (±) symbol denotes occasional small syncytial foci, and the other symbols denote approximate extent of fusion (+1, 25%; +2, 50%; +3, 75%; +4, 100% of cells in syncytia).

with greatly reduced efficiency at 38.5°C, unlike R2A/*tsB5*, which forms plaques efficiently at all three temperatures on HEp-2 cells. It is interesting that revertants R5/*tsB5* and R3C/*tsB5* exhibit similar plating efficiencies on both Vero and HEp-2 cells at all three temperatures tested, despite the fact that they were selected at permissive and restrictive temperatures, respectively, for similar plaque morphologies on Vero cells. The recombinant virus, *tsB5*MP3, resembles *tsB5* in its temperature sensitivity for plaque formation on HEp-2 cells but is much less temperature sensitive than *tsB5* on Vero cells. The revertant of this recombinant, R/*tsB5*MP3, and the MP virus strain plate with high efficiency on both HEp-2 and Vero cells at the temperatures tested.

The results presented in Table 2 (column 11) also show that most of the HSV-1 strains used

in this study plate with somewhat lower efficiency on HEp-2 cells than on Vero cells at permissive temperature. We find this to be true for most HSV strains tested in our laboratory (unpublished data). When aliquots of inoculum are plated on both cell types, the number of plaques detected on HEp-2 cell monolayers is usually between 10 and 50% of the number detected on Vero cells. However, two of the virus strains used in this study exhibit relative plating efficiencies outside this range. The mutant *tsB5* has a greatly reduced plating efficiency on HEp-2 cells relative to Vero cells, whereas the opposite is true for the recombinant *tsB5*MP3, which plates more efficiently on HEp-2 cells than on Vero cells. At the present time, we cannot explain the unusually low and high cell-dependent plating efficiencies of *tsB5* and *tsB5*MP3, respectively, and can conclude only that this aber-

rant behavior is not simply related to the *ts* mutation shared by the two virus strains.

As mentioned in the preceding section, virus strains related to HFEM usually form nonsyncytial plaques on HEp-2 cells and exhibit variability in syncytial plaque formation on Vero cells. Even the revertants isolated from nonsyncytial plaques (R3C/*ts*B5 and R5/*ts*B5) form mixtures of syncytial and nonsyncytial plaques on Vero cells. Despite this variability, Table 2 (column 9) reveals a trend in that the *syn* phenotype of plaques appears to be temperature sensitive in Vero cells for the HFEM mutant strains analyzed in this study. Similar results have been reported for a different variant of HFEM (18). Although the strains derived from HFEM usually form nonsyncytial plaques on HEp-2 cell monolayers, some isolates are capable of fusing both HEp-2 and Vero cell monolayers in synchronously infected cultures (2 PFU per cell) (Table 2, columns 6 and 10). In general,

the strains that fuse HEp-2 cells are the same strains that produce predominantly syncytial plaques on Vero cells at permissive temperature. The factors which govern this temperature-, cell-, and multiplicity-dependent variability of *syn* phenotype in the HFEM strain remain to be elucidated and will be discussed further below. It should be noted that in the group of viruses under investigation here, only viruses related to the MP strain of HSV-1 are consistently capable of forming syncytial plaques on both HEp-2 and Vero cells.

Effect of temperature on yield of progeny virus. The ability of the HSV-1 strains to accumulate infectious progeny virus was determined by titrating the PFU produced by cells infected at a multiplicity of 2 PFU per cell and harvested at 20 h after infection. The results are shown in Fig. 1 as a function of temperature. The parental virus strains, HFEM and MP, and two of the revertant virus strains, designated

FIG. 1. Yield of infectious virus from HEp-2 and Vero cell cultures infected at a multiplicity of 2 PFU per cell, incubated at 33.5, 37.0, or 38.5°C, and harvested at 20 h after infection as described in the text. The results at 37.0 and 38.5°C are expressed as a percentage of PFU produced at 33.5°C. In HEp-2 cells the 33.5°C values (PFU per culture) are as follows: HFEM (2.9×10^8); HFEM_{syn} (2.8×10^8); *ts*B5 (6.0×10^7); R2A/*ts*B5 (2.0×10^8); R3C/*ts*B5 (4.2×10^7); R5/*ts*B5 (2.5×10^8); *ts*B5MP3 (2.5×10^7); R/*ts*B5MP3 (4.6×10^7); and MP (1.6×10^7). In Vero cells the 33.5°C values (PFU per culture) are as follows: HFEM (7.2×10^8); HFEM_{syn} (7.2×10^8); *ts*B5 (4.4×10^8); R2A/*ts*B5 (8.4×10^7); R3C/*ts*B5 (2.4×10^8); R5/*ts*B5 (6.0×10^8); *ts*B5MP3 (3.5×10^7); R/*ts*B5MP3 (6.6×10^7); and MP (1.7×10^7). Quantitation of PFU was done on Vero cell monolayers.

R2A/*tsB5* and R/*tsB5MP3*, are not temperature sensitive for the accumulation of infectious progeny on either HEp-2 cells or Vero cells under the experimental conditions used in this study. The strain designated HFEM_{syn} is temperature sensitive for the accumulation of infectious virus only at 38.5°C in HEp-2 cells but is not temperature sensitive on Vero cells for the accumulation of progeny. In contrast, mutant *tsB5* produces low yields of infectious virus at 37.0°C and greatly reduced yields at 38.5°C in both cell types but is somewhat less temperature sensitive in Vero cells at the more restrictive temperature. Recombinant virus *tsB5MP3* resembles *tsB5* in that it is temperature sensitive for the accumulation of progeny at both 37.0 and 38.5°C in HEp-2 cells, but it differs from *tsB5* in that the yield of virus is reduced only at 38.5°C in Vero cells. Revertant viruses R3C/*tsB5* and R5/*tsB5* are temperature sensitive for yield of infectious virus at 38.5°C in both HEp-2 and Vero cells.

Comparison of the results shown in Fig. 1 and Table 2 indicates that the inability of a virus strain to accumulate high yields of infectious virus at an elevated temperature does not necessarily result in low plating efficiency at the same temperature. Revertants R3C/*tsB5* and R5/*tsB5* and recombinant *tsB5MP3* are capable of forming plaques on Vero cells at a high (38.5°C) temperature but are unable to accumulate high yields of virus under the same conditions. Low yields of infectious virus were also found in HEp-2 cells infected with *tsB5* and *tsB5MP3* at 37.0°C and with HFEM_{syn} at 38.5°C despite the ability of the mutants to plate efficiently on HEp-2 cells at these temperatures. In addition, it appears that the yield of virus as well as the efficiency of plating is somewhat dependent on the host cell used, particularly for recombinant *tsB5MP3*.

As noted earlier, there are apparent differences in the ability of a virus to induce fusion in foci of infected cells (plaques) and after synchronous infection of cells (2 PFU per cell). Although this phenomenon is not understood, it is interesting to note that those viruses (i.e., HFEM_{syn} and *tsB5*) which fuse HEp-2 cells after synchronous infection at permissive temperature, but are temperature sensitive for accumulation of infectious virus, also appear to be temperature sensitive for the *syn* phenotype. This suggests that the temperature sensitivity of infectious virus accumulation and of syncytium formation may be related in these strains and that HSV-induced cell fusion and virion infectivity may depend on an overlapping set of viral products, as previously suggested (13, 19).

Thermostability of virions produced at 34°C. Previous work (19) revealed that *tsB5*

virions made at permissive temperature were more thermolabile than HFEM virions, based on the relative rates of loss of infectivity at 45°C. Figure 2 presents data on the thermolability of virions produced in HEp-2 cells at permissive temperature by several of the strains used in this study. Virions of the HFEM strain are consistently more thermostable than those of mutant *tsB5*. Variant strain HFEM_{syn} reveals an intermediate stability (A), whereas the non-*ts* revertant R2A/*tsB5* retains the thermolability of virions characteristic of *tsB5* (B). The revertant strains R3C/*tsB5* and R5/*tsB5* also resemble *tsB5* with respect to the thermolability of virions (data not shown). In contrast, the revertant of the recombinant, R/*tsB5MP3*, produces more stable virions than its *ts* recombinant parent, *tsB5MP3*, and exhibits a thermostability close to that of the MP parent (C).

The results obtained with R2A/*tsB5* reveal that a strain which produces thermolabile virions (Fig. 2B) does not necessarily yield low titers of infectious virus at restrictive temperature (Fig. 1B). Moreover, we found that temperatures higher than 39°C over an extended period of time were required to demonstrate differences in lability among the various HSV-1 strains. Together these observations suggest that differences among the strains in the effects of temperature on the yield of PFU (Fig. 1) cannot be accounted for solely by differences in heat inactivation of infectivity at restrictive temperature.

Accumulation of glycoproteins as a function of temperature and cell type. To determine the effects of temperature on glycoprotein synthesis and accumulation by all the HSV-1 strains, we analyzed the [¹⁴C]glucosamine-labeled proteins present in infected HEp-2 and Vero cells maintained at different temperatures for 20 h. The fluorograms in Fig. 3 through 5 show the electrophoretic fractionation of SDS-solubilized glycoproteins from lysates of infected cells (2 × 10⁶ cells per sample) maintained at 33.5°C (a), 37.0°C (b), or 38.5°C (c) after adsorption and during the interval of [¹⁴C]glucosamine incorporation (3 to 20 h after infection).

Five HSV-1 glycoproteins, designated gA, gB, gC, gD, and gE, have previously been identified (1, 22), and it has been shown that multiple glycosylated forms of these proteins can accumulate in infected cells (1, 3, 9, 22). For the purpose of these analyses it should be noted that MP, *tsB5MP3*, and R/*tsB5MP3* fail to produce gC, a deficiency that appears not to interfere with viral replication (6, 10, 13, 18). In addition, the gB produced by MP is not well resolved in the electropherograms of Fig. 5. Evidence that MP produces gB at all temperatures tested is presented in the next section.

FIG. 2. Loss of infectivity of virus due to incubation at 45°C as described in the text. The virus preparations used were stocks prepared at 34°C in HEp-2 cells, and quantitation of PFU was done on Vero cell monolayers. The results obtained for preparations heated to 45°C for 30 and 60 min are expressed as the percentage of the following unheated control values (PFU/ml): panel A, HFEM (2.2×10^9), HFEMsyn (1.7×10^9), tsB5 (1.2×10^9); panel B, HFEM (1.6×10^9), R2A/tsB5 (2.2×10^9), tsB5 (3.1×10^9); and panel C, HFEM (4.4×10^9), MP (1.0×10^9), R/tsB5MP3 (1.3×10^9), tsB5MP3 (1.3×10^9), tsB5 (2.0×10^9).

FIG. 3. Electrophoretic fractionation of SDS-solubilized, [^{14}C]glucosamine-labeled glycopolypeptides synthesized in HEp-2 or Vero cells infected at 5 PFU per cell with HFEM, HFEMsyn, or tsB5 and incubated at 33.5°C (a), 37.0°C (b), or 38.5°C (c). Samples were prepared as described in the text. The open circle (○) denotes reduced amounts of the glycoprotein species designated gB, and the closed circle (●) denotes absence of detectable gB. The glycoprotein profiles shown for infected HEp-2 cells are not directly comparable to those shown for infected Vero cells because the samples were analyzed on different gel slabs.

FIG. 4. Electrophoretic fractionation of SDS-solubilized, [¹⁴C]glucosamine-labeled glycopolypeptides synthesized in HEp-2 or Vero cells infected at 5 PFU per cell with the revertant strains designated R2A/tsB5, R3C/tsB5, and R5/tsB5 and incubated at 33.5°C (a), 37.0°C (b), or 38.5°C (c). The asterisk (*) denotes restoration of gB accumulation at restrictive temperature; other symbols are used as described in the legend to Fig. 3.

FIG. 5. Electrophoretic fractionation of SDS-solubilized, [¹⁴C]glucosamine-labeled glycopolypeptides synthesized in HEp-2 or Vero cells infected at 5 PFU per cell with MP, tsB5MP3, or R/tsB5MP3 and incubated at 33.5°C (a), 37.0°C (b), or 38.5°C (c). Use of the symbols is as described in the legends to Fig. 3 and 4.

The results shown in Fig. 3 through 5 demonstrate that three of the HSV-1 strains in addition to tsB5 and tsB5MP3 are temperature sensitive for accumulation of the mature form of gB, including two of the revertants, R3C/tsB5

and R5/tsB5, and the mutant HFEMsyn. The degree of temperature sensitivity observed depends on the virus strain and host cell. Comparisons of Fig. 3 and 4 reveal that the revertants R3C/tsB5 and R5/tsB5 fail to accumulate gB

only at 38.5°C in both HEp-2 cells and Vero cells, whereas *tsB5* exhibits this deficiency at both 37.0°C and 38.5°C. The recombinant *tsB5MP3* produces detectable gB at both 33.5°C and 37.0°C in Vero cells but only at 33.5°C in HEp-2 cells. Finally, HFEM_{syn} exhibits some temperature sensitivity of gB accumulation only in HEp-2 cells at 38.5°C.

Comparison of the results shown in Fig. 3 through 5 with those presented in Fig. 1 reveals a striking correlation between the capacity of each virus to accumulate gB under any given condition and its capacity to accumulate infectious progeny. In particular, the revertants capable of producing gB at 38.5°C (R2A/*tsB5* and R/*tsB5MP3*) also produce near-normal quantities of infectious virus at this temperature, whereas revertants R3C/*tsB5* and R5/*tsB5* are deficient both in gB accumulation and in infectious virus accumulation at 38.5°C. Moreover, where cell-dependent deficiencies in infectious virus accumulation were observed (for HFEM_{syn} at 38.5°C and *tsB5MP3* at 37.0°C), cell-dependent deficiencies in gB accumulation were also evident.

The relationship between gB accumulation and HSV-induced cell fusion is more complicated and depends upon whether plaque morphology or behavior of synchronously infected cells is scored. For example, *tsB5* appears to accumulate significantly less gB at 37.0°C than at 33.5°C in Vero cells and also produces non-syncytial plaques at 37.0°C and syncytial plaques at 33.5°C. On the other hand, this mutant appears to induce cell fusion equally well at both temperatures after the synchronous infection (2 PFU per cell) of Vero cells. We cannot fully explain this multiplicity-dependent effect on virally induced cell fusion and can conclude only that the strains which are capable of inducing fusion at permissive temperature and which are temperature sensitive for gB accumulation also exhibit some temperature sensitivity for cell fusion.

Experiments are in progress to determine whether precursors to gB are produced by these mutants and revertants at restrictive temperature and, if so, whether these precursors accumulate, perhaps in some aberrant form. It may be noted from Fig. 3 through 5 that reductions in the amount of gB accumulating as a function of increased incubation temperature are usually accompanied by increases in the quantity of labeled glycopolypeptides comigrating with gA. The results discussed in the next section indicate that "gA" and gB share properties in common and raise the possibility that they could be derived from the same precursor, although this remains to be definitely established.

Incubation temperature appears to affect the accumulation of other glycoproteins in addition to gB. Most of the alterations observed, however, are not consistently associated with any mutant trait and are possibly due to temperature-dependent differences in processing of glycoprotein precursors, which could result in the preferential accumulation of different glycosylated forms at different temperatures. Although the electrophoretic analyses of [¹⁴C]glucosamine-labeled proteins only are shown in this report, we also analyzed [³⁵S]methionine-labeled proteins produced by each of the virus strains. No consistent temperature-dependent effects on viral protein synthesis were observed, except for those evident from the autoradiograms shown in Fig. 3 through 5. Those results are consistent with previously reported findings (13, 19) and were not included here because they reveal no additional information.

Oligomeric conformation of gB. Previous studies (19, 20) have shown that gB can be extracted from wild-type virions (including those of HFEM) in the form of SDS-stable heat-dissociable oligomers of characteristic electrophoretic mobility on acrylamide gels. Evidence was presented that these oligomers are homodimers, and preliminary analyses of extracts prepared from *tsB5* virions (produced at permissive temperature) failed to reveal the presence of gB dimers (19). To determine whether gB dimers could be produced by the strains analyzed in this study, SDS extracts were prepared from purified virions and from unfractionated infected cells, and both heated and unheated samples were analyzed by electrophoresis. Although only a fraction of gB retains its oligomeric conformation in the unheated samples analyzed under these conditions, these oligomers can be detected in overexposed autoradiograms of the acrylamide gels, as are shown in Fig. 6 and 7.

Several new findings emerged from these experiments. First, cells infected with HFEM or MP produced two heat-dissociable glycosylated oligomers; the more slowly migrating form is probably identical to the gB dimer of virions (Fig. 6 and 7). Polypeptides in the faster migrating form have the electrophoretic mobility of gA when dissociated (data not shown), and preliminary studies (unpublished) indicate that the faster form may be a precursor to the slower form. Second, *tsB5* also produces a pair of heat-dissociable glycosylated oligomers at permissive temperature; these oligomers appear to be less stable than those of HFEM (based on the quantities detected), they have a different electrophoretic mobility, and neither is detected at restrictive temperature. The polypeptides forming these oligomers have the electrophoretic mo-

bilities of gB and gA after dissociation (data not shown). Finally, the oligomers produced by recombinant *tsB5MP3* and all the revertant strains had electrophoretic mobilities indistinguishable from those of *tsB5* and, in addition, the temperature sensitivity of their accumulation depended upon the extent to which each strain was temperature sensitive for gB accumulation.

We also analyzed two of the other *ts* recombinant viruses isolated by Manservigi et al. (13) (*tsB5MP5* and *tsB5MP7*) and found the oligomers produced to be indistinguishable from those of *tsB5*, whereas *HFEMsyn* produced oligomers indistinguishable from those of *HFEM* (data not shown). These results indicate that two of the mutant phenotypes associated with *tsB5*, temperature sensitivity of gB accumulation and aberrant form of gB oligomers, can be readily dissociated, either because two mutations are responsible for the dissociable phenotypes or because a second-site mutation is responsible for the reversion with respect to temperature sensitivity.

DISCUSSION

The results of previous studies demonstrated that the *tsB5* mutant of HSV-1(*HFEM*) differs in several respects from the wild-type parental strain. The mutant was shown to be temperature sensitive for plaque formation, for the accumulation of infectious virions, and for the accumulation of the glycoprotein species designated gB (13, 19). In addition, it was found that the gB produced by *tsB5* at permissive temperature failed to form the dimer characteristic of wild-type gB and that the virions produced by the mutant at permissive temperature were more thermolabile than wild-type *HFEM* virions (19, 20).

It was suggested previously that the failure of the mutant *tsB5* to replicate at nonpermissive temperature was directly related to the absence of gB accumulation and that this absence could be a consequence of a single lesion that alters the processing and thermal stability of gB, perhaps a mutation in the gB polypeptide itself (19). The studies described in this report were undertaken to investigate some of these possibilities, specifically to determine which of the mutant traits described for *tsB5*, or associated with the *tsB5* mutation, could be related to the temperature sensitivity of accumulation of the glycoprotein species designated gB. The arguments for focusing on alterations in the accumulation of gB in investigating the molecular basis for the altered phenotypes associated with the *tsB5* mutation may be summarized as fol-

lows. First, temperature-sensitive accumulation of gB and the failure to produce a dimer of gB with the electrophoretic mobility characteristic of wild-type gB dimers were the only aberrations in viral protein synthesis reproducibly detected in previous studies with *tsB5* (19), with the recombinants derived from *tsB5* and MP (13) and, in this study, with the partial revertants (except for the excessive accumulation of gA at restrictive temperature). Second, an independently isolated variant of *HFEM*, *HFEMsyn*, was found to be somewhat temperature sensitive for gB accumulation (M. Sarmiento and P. G. Spear, unpublished data) and also exhibited some of the altered phenotypes associated with the *tsB5* mutation. Third, an independently isolated *ts* mutant of another virus strain HSV-1(KOS), designated J12, belongs to the same complementation group as *tsB5* (21), has been mapped to the same region of the genome as *tsB5* (D. S. Parris, R. A. F. Dixon, and P. A. Schaffer, Virology, in press) and also exhibits temperature-dependent alterations in the synthesis or processing of gB (S. Little and P. A. Schaffer, personal communication). Finally, reversions of *tsB5* with respect to certain of the altered traits are invariably accompanied by decreased temperature sensitivity in the accumulation of gB, both for the revertants isolated for this study and for the revertant R/*tsB5MP3* isolated by Manservigi et al. (13).

It has not yet been determined whether the *tsB5* mutation is in the structural gene for the gB polypeptide or in some other gene whose product affects the processing or stability of gB. Mapping studies which place the *tsB5* mutation in the region of the HSV-1 genome encoding gB (18) are consistent with either possibility because intertypic differences in electrophoretic mobility of gB were used to map this glycoprotein, and these differences could be due to type-specific alterations either in the gB polypeptide or in its processing. In addition, it is not yet known whether an aberrant form of gB accumulates at restrictive temperature in *tsB5*-infected cells. Preliminary results from immunoprecipitation studies (M. L. Haffey and P. G. Spear, unpublished data) with an antiserum prepared against dimers of gB extracted from wild-type virions (M. Sarmiento, M. L. Haffey, and P. G. Spear, unpublished data) indicate that a polypeptide related to gB is made at restrictive temperature in *tsB5*-infected cells and that the polypeptide incorporates both [³⁵S]methionine and [¹⁴C]glucosamine and comigrates with the gA glycoprotein species. Accumulation of this material may account for increased quantities of gA detected at restrictive temperatures in cells

infected with some of the strains analyzed in this study (Fig. 3 through 5).

These results and those obtained by R. Eberle and R. Courtney (personal communication) indicate that gA and gB are antigenically related; our preliminary analyses of the oligomeric glycoproteins detected in extracts of infected cells (Fig. 6 and 7) indicate that gA as well as gB can form oligomers, but only under conditions permissive for gB accumulation. The structural and functional relationships between these electrophoretically distinct species remain to be defined. It should be emphasized that the form of gB identified in Fig. 3 through 6 is apparently the most mature form of a polypeptide which may undergo several stages of processing and glycosylation (22), and it is only this form whose absence has been correlated with some of the mutant traits of *tsB5* investigated here and in previous studies (13, 19).

Accumulation of gB and infectious virion production. A previous report has shown that *tsB5* produces comparable numbers of virions at restrictive (39.0°C) and permissive (34.0°C) temperatures but that the virions formed at 39.0°C lack gB and have a low specific infectivity (19), indicating that the presence of gB in virions was necessary for their infectivity. The results presented here provide support for the postulated role of gB in virion infectivity. We have found that, in two different cell types, any reductions in the quantities of gB detected at restrictive temperatures are consistently accompanied by reductions in the numbers of accumulated infectious progeny, not only for *tsB5*, but also for each of four other related virus strains. In addition, the revertant R2A/*tsB5* is capable of accumulating high yields of infectious units at permissive or restrictive temperature, although the virions produced at permissive temperature appear to be as thermolabile as those of *tsB5*. Taken together, these results suggest that the number of infectious units accumulating at different temperatures in cells infected with the virus strains used in this study cannot be determined solely by the relative thermolability of virions produced but probably reflects the number of infectious virions produced (not necessarily the total number of virions produced).

Accumulation of gB and the extent of cell fusion. In a previous study (13), recombinant viruses derived from *tsB5* and MP were selected for the ability to form syncytial plaques at permissive temperature (34.0°C) on HEp-2 cells and for greatly reduced plating efficiency at restrictive temperature (39.0°C). These recombinant viruses were found to be temperature sensitive for gB accumulation and for the induction of cell fusion in synchronously infected cultures

of HEp-2 cells (1 to 10 PFU per cell). It was suggested that the temperature sensitivity of gB accumulation in these recombinants was responsible for the temperature sensitivity of virally induced cell fusion and that *tsB5* itself failed to fuse HEp-2 cells at permissive temperature because it expressed a fusion-inhibiting factor not made by the recombinant viruses or MP. The existence of this *tsB5* fusion-inhibiting factor was inferred in part from the finding that HEp-2 cells coinfecting with *tsB5* and MP fused to a much lesser extent than did cells infected with MP alone (13).

Our results are consistent with those of Manservigi et al. (13) in that the quantities of gB detectable in HEp-2 cells infected with recombinant viruses *tsB5*MP3 and R/*tsB5*MP3 correlate with the extent of cell fusion. The relationship of gB accumulation to the extent of cell fusion in HEp-2 cells infected with the other virus strains was difficult to assess because of multiplicity-dependent differences in expression of the *syn* phenotype and also presumably because of expression of the putative fusion-inhibiting activity under certain conditions.

The new results presented here pertain to the effect of the mutants and newly isolated revertants on the behavior of Vero cells, in comparison with HEp-2 cells, at a broader range of temperatures than was previously reported. All the HSV-1 strains analyzed exhibit a *syn* phenotype in synchronously infected Vero cells maintained at permissive temperature, and all except HFEM form syncytial plaques in Vero cells at permissive temperature. We as yet have no explanation for the finding that HFEM and some of the derivative strains can induce the fusion of synchronously infected HEp-2 cells or Vero cells, whereas plaques on the same cell types are nonsyncytial. Because constituents from the envelopes of input virions have been detected in the surfaces of infected cells (15), the possibility exists that these constituents, and the temperature at which they were made, could influence whether or not fusion of the infected cells occurred. It is also possible that the presence of gamma globulin in the overlay medium used for plaque assays could affect the expression of the *syn* phenotype.

With respect to the possible role of gB in HSV-induced cell fusion, a correlation exists in the sense that those virus strains which are temperature sensitive for cell fusion consistently failed to accumulate gB at 38.5°C, and revertants were invariably less temperature sensitive with respect to both traits. Although virus strains which are temperature sensitive for gB accumulation also exhibit some temperature sensitivity for the induction of fusion, in both

foci of infected cells and synchronously infected cells, the quantities of mature gB that can be detected at 20 h after infection do not necessarily correlate with the apparent extent of cell fusion. In particular, synchronously infected cultures of either cell type can exhibit rather extensive fusion at 37.0°C despite little detectable accumulation of gB. This finding suggests that the mature form of gB itself may not be required or is required only transiently or in small amounts for fusion to occur. It should be noted that if fusion of HEp-2 and Vero cells occurred, it was usually extensive several hours before we harvested infected cells to analyze the accumulation of glycoproteins (20 h after infection).

One other finding of interest emerged from these analyses. The two independently derived mutants, HFEM_{syn} and *tsB5*, which are both temperature sensitive for gB accumulation also differ from the parental strain HFEM in that they induced syncytial plaques on Vero cells at 33.5°C and induced the fusion of synchronously infected HEp-2 cells. Moreover, two of the partial revertants of *tsB5*, R3C/*tsB5* and R5/*tsB5*, which were less temperature sensitive for accumulation of gB more closely resembled HFEM than *tsB5* in their inability to fuse HEp-2 cells and their less *syn* plaque phenotype on Vero cells. These observations raise the possibility that a mutation which alters the synthesis or processing of gB may also influence expression of the *syn* phenotype at permissive temperature, consistent with the previously published proposal (13) that *syn* mutations could result from modifications of fusion-inducing factors as well as elimination of fusion-inhibiting activity.

The results presented here emphasize the complexity of the factors that govern HSV-induced cell fusion. Although our data point to a role for gB in this process, it is clearly not possible to predict the extent of cell fusion solely from the quantities of accumulated gB. The production and activity, not only of gB but also of precursors and possible aberrant forms of gB as well as other viral and cellular products, have to be taken into account to fully understand the role of this glycoprotein in HSV-induced cell fusion.

Plaque formation, accumulation of gB and infectious virus, and HSV-induced cell fusion. *ts* mutants are generally selected for their ability to form plaques at permissive temperatures but not at restrictive temperatures. Most *ts* mutants are also found to be temperature sensitive for the production of infectious progeny in synchronously infected cultures, suggesting that the ability to form plaques usually correlates with the ability to produce infectious progeny.

The previous findings that both syncytial and nonsyncytial HSV strains could form plaques in the presence of antiviral antibodies indicated that plaque formation does not depend on the release of progeny virus to the extracellular fluid (5, 8). Our results suggest that plaque formation does not even depend on the amount of infectious progeny produced by infected cells because revertants R3C/*tsB5* and R5/*tsB5* plated on Vero cells almost as efficiently at 38.5°C as at 33.5°C and yet were nearly as deficient as parental mutant *tsB5* in the accumulation of infectious progeny at 38.5°C. This disparity between plaque formation and accumulation of infectious progeny was observed at 38.5°C only in Vero cells with these revertants, and in HEp-2 cells with the variant HFEM_{syn}. Thus, although host factors influence the capacity of the revertants to form plaques at restrictive temperature, HEp-2 cells as well as Vero cells can support the cell-to-cell transmission of HSV infection under conditions of reduced yields of infectious progeny. Precedence for these findings can be found in the report by Stoker (24), which showed that the number of individual HSV-infected cells capable of forming infectious centers was significantly higher than the number producing detectable infectious progeny.

Accumulation of gB, the dimeric form of gB, and the thermostability of virions. A previous study (19) has shown that mutant *tsB5*, in addition to being temperature sensitive for the accumulation of gB, also fails to accumulate the wild-type dimeric form of gB at permissive temperature. The results presented here indicate that *tsB5* produces an aberrant oligomeric form of gB, with an electrophoretic mobility distinct from that of wild-type gB dimers. The question arises whether this altered conformation of gB in *tsB5* is responsible for the temperature sensitivity of accumulation of this glycoprotein. We have found that, although these mutant traits are linked in three recombinant viruses, the accumulation of gB can be temperature sensitive without alterations in the electrophoretic mobility of the oligomeric form of gB (in HFEM_{syn}), and reversion of the *ts* phenotype with respect to gB accumulation can occur in some virus strains without restoring the dimeric conformation of gB characteristic of wild-type virus. Therefore, temperature sensitivity of gB accumulation is not a necessary consequence of the failure to produce the wild-type dimeric form of gB, although in *tsB5* a single mutation could be responsible for both mutant traits. In addition, our analyses of the revertant virus strains have demonstrated that the failure to form wild-type dimers of gB does not necessarily dictate thermostability of virions and that tem-

perature-stable accumulation of gB does not insure virion thermostability.

The finding that the four revertants analyzed in this study all differed in one or more respects from the wild-type strains indicates either that all the phenotypic reversions resulted from second-site mutations or that *tsB5* differs from HFEM at more than one locus. We cannot distinguish between these two possibilities at the present time. However, it is clear from our analyses of these revertants and mutants that temperature sensitivity in the accumulation of infectious progeny can be accounted for by temperature sensitivity in the accumulation of gB, supporting the hypothesis that gB is an essential constituent of infectious virions (19). In addition, our results confirm and extend the previously reported correlation between temperature-dependent defects in gB processing or accumulation and temperature sensitivity of virus-induced cell fusion. This report also reveals that virus-induced cell fusion and plaque formation are processes which do not directly depend on each other, on the accumulation of infectious progeny, or on the quantities of mature gB present late in infection. The findings that all the revertants resemble *tsB5* with respect to the altered conformation of oligomeric gB and that the altered conformation is apparently not necessarily related to the temperature sensitivity of gB accumulation suggest that the mutation which is responsible for the altered conformation could be distinct from the *ts* mutation of *tsB5*.

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