JOURNAL OF VIROLOGY, Sept. 1983, p. 568–585
0022-538X/83/090568-18$02.00/0

Vol. 47, No. 3

Genetic and Phenotypic Analysis of Herpes Simplex Virus Type 1 Mutants Conditionally Resistant to Immune Cytolysis

BETTE A. PANCAKE, DORIS P. ASCHMAN, AND PRISCILLA A. SCHAFFER*

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

Received 14 February 1983/Accepted 8 June 1983

Nine temperature-sensitive (*ts*) mutants of herpes simplex virus type 1 selected for their inability to render cells susceptible to immune cytolysis after infection at the nonpermissive temperature have been characterized genetically and phenotypically. The mutations in four mutants were mapped physically by marker rescue and assigned to functional groups by complementation analysis. In an effort to determine the molecular basis for cytolysis resistance, cells infected with each of the nine mutants were monitored for the synthesis of viral glycoproteins in total cell extracts and for the presence of these glycoproteins in plasma membranes. The four mutants whose *ts* mutations were mapped were selected with polypeptide-specific antiserum to glycoproteins gA and gB; however, three of the four mutations mapped to DNA sequences outside the limits of the structural gene specifying these glycoproteins. Combined complementation and phenotypic analysis indicates that the fourth mutation also lies elsewhere. The *ts* mutations in five additional cytolysis-resistant mutants could not be rescued with single cloned DNA fragments representing the entire herpes simplex virus type 1 genome, suggesting that these mutants may possess multiple mutations. Complementation tests with the four mutants whose *ts* lesions had been mapped physically demonstrated that each represents a new viral gene. Examination of mutant-infected cells at the nonpermissive temperature for the presence of viral glycoproteins in total cell extracts and in membranes at the cell surface demonstrated that (i) none of the five major viral glycoproteins was detected in extracts of cells infected with one mutant, suggesting that this mutant is defective in a very early function; (ii) cells infected with six of the nine mutants exhibited greatly reduced levels of all the major viral glycoproteins at the infected cell surface, indicating that these mutants possess defects in the synthesis or processing of viral glycoproteins; and (iii) in cells infected with one mutant, all viral glycoproteins were precipitable at the surface of the infected cell, despite the resistance of these cells to cytolysis. This mutant is most likely mutated in a gene affecting a late stage in glycoprotein processing, leading to altered presentation of glycoproteins at the plasma membrane. The finding that the synthesis of both gB and gC was affected coordinately in cells infected with six of the nine mutants suggests that synthesis of these two glycoproteins, their transport to the cell surface, or their insertion into plasma membranes is coordinately regulated.

Herpes simplex virus type 1 (HSV-1) induces the synthesis of at least four major viral glycoproteins (gB, gC, gD, and gE), all of which have been detected in virion envelopes and on the surfaces of lytically infected cells in culture (3, 45). A number of studies have shown that virus-specific neutralizing antibodies and cytotoxic lymphocytes are induced in experimental animal hosts after infection with HSV and that the viral glycoproteins constitute the major inducers and targets of both humoral and cell-mediated immunity (5, 11, 21, 29).

HSV mutants exhibiting altered synthesis or incorporation of individual glycoproteins into infected cell membranes should prove useful in elucidating the specific role of each viral glycoprotein in the induction of immunity to HSV. Mutants currently available for this purpose include the following: (i) *ts*A1, a DNA-negative mutant defective in the gene for the major viral DNA-binding protein and exhibiting reduced synthesis of certain of the major viral glycoproteins (38, 40); (ii) *ts*B5 and *ts*J12, two late mutants in the structural gene specifying the precursor product and hence, antigenically related glycoproteins gA and gB; these mutants are

unable to induce the synthesis of, or to direct the incorporation of gB into, the plasma membranes of infected cells at the nonpermissive temperature (23, 37); (iii) *ts*10, which exhibits altered transport to, or insertion of, viral glycoproteins into membranes at infected cell surfaces (14); (iv) *cs*50B, a cold-sensitive mutant which induces the synthesis of an antigenically altered form of gE in infected cells (32, 46); and (v) antigenic variants of glycoproteins gB and gC (19). To obtain additional viral mutants conditionally defective in the synthesis or insertion of individual glycoproteins into infected cell membranes, we have isolated a series of mutants selected for their inability to render infected cells susceptible to immune cytolysis mediated by complement and monospecific rabbit antisera to glycoproteins gA, gB, and gC at 39.6°C (25). By virtue of their immune cytolysis-resistant temperature-sensitive phenotype, we term these mutants *icr ts* mutants.

In this paper, we describe the genetic and phenotypic characterization of nine such mutants. The mutations in four of nine *icr ts* mutants map to DNA sequences outside the limits of the structural gene for gA and gB, and the mutants exhibit a variety of phenotypes with respect to the production of mature forms of gA, gB, gC, and gD, both intracellularly and at the infected cell surface. The studies presented herein attest to the usefulness of the selection procedure used for the enrichment of glycoprotein-defective mutants and suggest the potential value of *icr ts* mutants for immunological and biochemical studies of individual HSV-1 glycoproteins.

MATERIALS AND METHODS

Cells and cell culture. African green monkey kidney (Vero) cells were used for preparation of virus stocks, in plaque assays, in immune cytolysis tests (as indicator cells), in complementation tests, and in certain instances, for the production of viral DNA. Viral DNA infectivity and marker rescue tests with selected mutants were also conducted in Vero cells. Human embryonic lung (HEL) fibroblasts served as target cells in immune cytolysis tests and were used to determine mutant DNA phenotypes; they were also used initially in complementation tests. Human epidermoid carcinoma (HEp-2) cells were used routinely for the preparation of infectious viral DNA and for studies of the synthesis and expression of viral glycoproteins. Secondary rabbit kidney (RK) cells were used in marker rescue tests with seven of the nine mutants investigated.

All cells were grown at 37°C in Dulbecco modified Eagle medium (Flow Laboratories, McLean, Va.) supplemented with 10% fetal calf serum for HEL cells or 10% newborn calf serum for all other cell types as described previously (49).

Viruses and virus assays. The selection of KOS mutants resistant to immune cytolysis at the nonpermissive temperature (*icr ts* mutants) has been described by Machtiger et al. (25). The nine *icr ts* mutants included in this study were *icr ts*62, 78, 108, 116 and 149 (selected with polypeptide-specific rabbit antiserum prepared to the antigenically related HSV-1 glycoproteins gA and gB); *icr ts*132 and 184 (selected with antiserum to gC); and *icr ts*112 and 125 (selected with antiserum to glycoproteins gA/gB and gC).

Relevant properties of the series of *ts* mutants used in complementation tests are presented in Table 1. Mutant *ts*1201 was kindly provided by Valerie Preston (Institute of Virology, Glasgow, Scotland); *ts*10 was provided by Rozanne Sandri-Goldin (University of Michigan, Ann Arbor, Mich.). The isolation and properties of these mutants have been described (14, 34).

Virus stocks were prepared in HEL or Vero cells as described previously (39). The permissive temperature for virus growth and assay was 34°C, and for reasons described below, 39.6°C was used as the nonpermissive temperature. All genetic and biochemical tests were conducted in water baths with temperature variations of ±0.1°C. Plaque assays were carried out with a 2% methylcellulose overlay in incubators with temperature variations of ±0.2°C.

Immune cytolysis. The procedure employed for immune cytolysis tests has been described by Machtiger et al. (25). Monospecific rabbit antisera to sodium dodecyl sulfate (SDS)-denatured gA/gB and gC were kindly provided by Richard Courtney (University of Tennessee, Knoxville, Tenn.). The preparation and characterization of these antisera have been reported by Eberle and Courtney (11).

Preparation of infectious viral DNA. Except for *icr ts*62 and 78, all wild-type and mutant viral DNAs were extracted from virions produced in infected HEp-2 cells as described by Weller et al. (49). Repeated attempts to obtain infectious DNA from *icr ts*62 and 78 in HEp-2 and RK cells were unsuccessful, although both viruses could produce infectious progeny in both cell types. However, infectious DNAs of both mutants were readily obtained from virions produced in Vero cells. The infectivities of mutant DNAs were determined in RK or Vero cells as described by Weller et al. (49).

Marker rescue. In marker rescue tests with seven of the nine *icr ts* mutants, RK cells were cotransfected with infectious mutant DNA and recombinant plasmid DNA linearized by cleavage with *Eco*RI or *Bgl*II (New England Biolabs, Beverly, Mass.) as described by Weller et al. (49). Vero cells were used for marker rescue with *icr ts*62 and 78. Recombinant plasmids consisting of *Eco*RI fragments spanning the entire KOS genome inserted into plasmid pBR325 were obtained from Rozanne Sandri-Goldin (16), and recombinant plasmids consisting of the KOS *Bgl*II fragments I, D, and G inserted into plasmid pKC7 were obtained from Wai-Choi Leung (University of Alberta, Edmonton, Alberta, Canada). *Xba*I fragment G was excised from a 0.5% agarose gel and was eluted by the glass powder elution procedure of Vogelstein and Gillespie (47) as modified by D. M. Coen, P. T. Gelep, D. P. Aschman, S. K. Weller, and P. A. Schaffer (submitted for publication). Infectious progeny virus from marker rescue tests was assayed in Vero cells at 34 and 39.6°C. The efficiency of plating was calculated as (PFU per milliliter at 39.6°C)/(PFU per milliliter at 34°C).

Isolation of ts^+ revertants and rescuants. To confirm

TABLE 1. *ts* mutants used in complementation tests

Wild-type virus	Mutant (reference)[a]	Complementation group[b]	DNA phenotype[c]
KOS	*ts*A1 (38)	1–1	−
	*ts*A15 (38)	1–1	−
	*ts*A24 (38)	1–1	−
	*ts*A42 (49)	1–1	−
	*ts*C4 (38)	1–3	−
	*ts*C7 (38)	1–3	−
	*ts*D9 (38)	1–4	−
	*ts*E5 (38)	1–5	+
	*ts*E6 (38)	1–5	+
	*ts*F18 (38)	1–6	+
	*ts*G3 (38)	1–7	±
	*ts*J12 (38)	1–9	+
	*ts*K13 (38)	1–10	±
	*ts*M19 (38)	1–10[d]	±
	*ts*N20 (38)	1–13	+
	*ts*O22 (38)	1–14	−
	*ts*T36 (7)	1–27	±
	*ts*V37 (7)	1–29	+
17	Glasgow *ts*F (4)	1–11	+
	Glasgow *ts*A (4)	1–16	+
	Glasgow *ts*1201 (34)	ND[e]	+
HFEM	*ts*B7 (20)	1–20	−
	*ts*LB5 (18)	1–22	−
	*ts*LB7 (18)	ND	+
KOS 1.1	*ts*LG4 (36)	ND	+
	*ts*10 (14)	ND	+
186[f]	*ts*39 (10)	ND	+
	*ts*201 (10)	ND	+

[a] References pertain to original descriptions of mutants.

[b] Complementation group assignments are those reported by Schaffer et al. (39) for groups 1–1 through 1–23 and by Chu et al. (7) for groups 1–24 through 1–29.

[c] DNA phenotypes are either described in references (see above, footnote a) or in Schaffer et al. (39).

[d] Based on physical mapping data and reevaluation of complementation, the complementation group assignment of *ts*M19 has been changed from 1–12 to 1–10 (S. K. Weller, D.P. Aschman, W. R. Sacks, D. Coen, and P. A. Schaffer, submitted for publication).

[e] ND, not determined.

[f] HSV-2.

the genetic basis for the complex phenotypes exhibited by *icr ts* mutants, ts^+ revertants and rescuants (from marker rescue tests) were isolated. Isolates were plaque purified at 39.6°C, and stocks were prepared in Vero cells (38, 39).

Complementation. Complementation tests were performed in water baths at 39.6°C as described by Schaffer et al. (38). Complementation indices were calculated as $[(A + B)/A + B]$ where A and B represent the yields of two mutants grown at 39.6°C and assayed at 34°C. Values of 10 or greater were taken to indicate positive complementation.

DNA phenotypes. Viral DNA phenotypes of mutants were determined by the procedure of Aron et al. (1). Infected cells were labeled from 4 to 24 h at 34 or 39.6°C with [*methyl*-^{3}H]thymidine (60 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.). CsCl gradient centrifugation was performed with a Beckman Vti 65 rotor at 225,000 × *g* for 12 h. Counts in gradient fractions precipitable by 5% trichloroacetic acid were measured with a Beckman LS7000 liquid scintillation counter.

Metabolic labeling of viral glycoproteins. HEp-2 cells were used for studies of glycoprotein phenotypes because wild-type virus production and isotopic labeling were more efficient in these cells. In comparative studies of immune cytolysis of KOS-infected HEp-2 and HEL cells by hyperimmune anti-HSV-1 and glycoprotein-specific rabbit antisera, cytolysis was equally efficient in both cell types. Moreover, the glycoprotein phenotypes of the wild-type virus were also indistinguishable in the two cell types. For metabolic labeling, HEp-2 cell monolayers in 25-cm^2 flasks (1×10^6 to 3×10^6 cells per flask) were infected at multiplicities of infection ranging from 10 to 30 PFU per cell. After adsorption for 1 h at 37°C, inocula were removed, monolayers were washed twice with phosphate-buffered saline (PBS; 0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.4]), and infected cells were incubated at 34 or 39.6°C for 3 h

TABLE 2. Growth of *icr ts* mutants as a function of temperature[a]

Virus	Virus yield (PFU/ml) at (°C):				Class	No. of mutants in class
	34	39.0	39.6	40		
KOS (wild-type)	1×10^8	6×10^7	5×10^6	4×10^4		
*ts*B2[b]	5×10^7	4×10^2	3×10^1	$<10^1$	Maximally *ts*	
*icr ts*184 *icr ts*62	2×10^7	2×10^2	2×10^1	$<10^1$	Maximally *ts*	2
*icr ts*78 *icr ts*108 *icr ts*116 *icr ts*149 *icr ts*132 *icr ts*112 *icr ts*125	1×10^7	3×10^4	2×10^3	NT[c]	Moderately *ts*	7
*icr ts*117 14 others	2×10^8	8×10^6	4×10^5	2×10^3	Minimally *ts*	15

[a] Vero cells were infected at a multiplicity of infection of 5, and progeny virus was assayed at 34°C after 18 h of incubation at the designated temperature. Data are presented for representative viruses of each temperature class; other members of each class are listed below the class representative.

[b] *ts*B2 in complementation group 1–2 is a DNA$^-$ mutant of HSV-1 strain KOS (39).

[c] NT, Not tested.

more in Dulbecco modified Eagle medium supplemented with 2% newborn calf serum. At 4 h postinfection, 1 to 2 μCi of D-[1-^{14}C]glucosamine (New England Nuclear Corp., Boston, Mass.; 54.2 mCi/mmol) was added to each culture, and flasks were again incubated at 34 or 39.6°C for an additional 20 h. Labeled cells were harvested by scraping into medium and were washed twice in ice-cold PBS. Cell pellets were frozen at −20°C in 200 μl of PBS. Before gel electrophoresis, pellets were thawed and disrupted by sonication for 2 min at 80% maximum efficiency in a Heat Systems sonicator (Heat Systems Ultrasonics, Inc., Plainview, N.Y.). Immediately before electrophoresis, 25 μl of cell lysate was boiled for 2 min with SDS sample buffer (8), and the resulting suspension was clarified by centrifugation in an Eppendorf microfuge.

Surface labeling of viral glycoproteins. HEp-2 cell monolayers in 25-cm^2 flasks (1×10^6 to 3×10^6 cells per flask) were infected at a multiplicity of infection of 10 to 30 PFU per cell and were incubated at 34 or 39.6°C for 20 h. Infected cells were harvested by scraping into the medium and were washed twice with ice-cold PBS. Cells were counted, and cell suspensions were adjusted to contain 10^6 cells in 1.0 ml of PBS. Cells were pelleted, resuspended in 1.0 ml of PBS, and transferred to glass tubes (10 by 75 mm) coated with Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Pierce Chemical Co., Rockford, Ill.) as described by Markwell and Fox (26). Each tube contained the precipitate from 100 μg of Iodogen evaporated from 1.0 ml of chloroform. Tubes were rinsed with PBS before the addition of cells. One-half mCi of ^{125}I (sodium iodide, 17 Ci/mg; New England Nuclear Corp.) was added to each cell suspension, and iodination was permitted to proceed for 15 min at room temperature in an iodination hood. The iodinated cell suspensions were transferred to new tubes containing 10 ml of Dulbecco modified Eagle medium, pH 7.4, and cells were washed twice in Dulbecco modified Eagle medium and once in PBS. Cells were counted in the presence of trypan blue before freezing to assess their viability and to ensure that each sample placed on gels contained the same number of cells. Cell pellets were stored at −20°C. A control suspension of KOS-infected cells was also treated with ^{125}I-labeled sodium iodide under similar test conditions, except that no Iodogen was used. Under these conditions, nonspecific iodination was not detected.

Preparation of hyperimmune rabbit antiserum to HSV-1. Rabbits were immunized with HSV-1 (strain KOS). Inoculum virus was grown in RK cells supplemented with 5% rabbit serum as described by Esparza et al. (12). On days 0 and 7, rabbits were inoculated both subcutaneously and intramuscularly with 1.0 ml of UV-inactivated (noninfectious) virus containing approximately 6×10^6 particles mixed 1:1 with complete Freund adjuvant by each route of inoculation. On days 14 and 21, rabbits were inoculated subcutaneously and intramuscularly with 5×10^6 PFU of infectious virus. Animals received 10^9 PFU of infectious virus 60 days later and were bled after 1 week. Serum antibody titers ranged from 1:1,000 to 1:2,000 as determined by plaque reduction neutralization tests. These sera were then used for immune precipitation as described below.

Immune precipitation. Cell pellets were thawed and incubated for 30 min at 5°C with lysis buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40 in PBS) as described by Para et al. (31). Lysates were centrifuged at 100,000 × *g* for 30 min, and 50-μl samples of supernatant fluids were incubated with 4 μl of preimmune rabbit serum for 60 min at 5°C. A 200-μl

portion of a 10% suspension of *Staphylococcus aureus* (Ig Sorb, Enzyme Center, Boston, Mass.) prewashed and suspended in lysis buffer was then added; samples were mixed and incubated for 30 min at 5°C. Nonspecific material adsorbing to preimmune rabbit serum or to *S. aureus* was removed by pelleting in an Eppendorf microfuge. Supernatant fluids, transferred to new tubes, were incubated at 5°C for 1 h with 25 μl of hyperimmune rabbit antiserum or monoclonal antibodies and for 30 min with 200 μl of 10% *S. aureus* in lysis buffer. Pelleted material was then washed three times in lysis buffer and twice in wash buffer (50 mM Tris-hydrochloride, pH 7.5, containing 100 mM NaCl) (31). Precipitates were stored at −20°C before analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

To identify HSV-1 glycoprotein-specific bands in SDS gels, lysates of KOS-infected HEp-2 cells labeled with either [^{14}C]glucosamine or ^{125}I were immune precipitated with monoclonal antibodies to HSV-1 glycoproteins gA/gB (monoclonal antibody 3-S), gC (monoclonal antibody 19-S), or gD (monoclonal antibody 1-S) by procedures described above with hyperimmune rabbit antisera. The monoclonal antibodies used were obtained from Martin Zweig (Frederick Cancer Research Center, Frederick, Md.). The isolation and properties of these antibodies have been described by Showalter et al. (41).

Gel electrophoresis. SDS-PAGE was performed as described by Courtney et al. (8) and Little and Schaffer (24). Reagents used for SDS-PAGE were purchased from BioRad Laboratories (Richmond, Calif.). An 8.6% resolving gel cross-linked with *N,N'*-diallyltartardiamide as described by Little and Schaffer (24) was employed with a 4% stacking gel cross-linked with *N,N'*-methylenebisacrylamide, pH 6.8. Resolving gels were 10 cm long. Electrophoresis was conducted at constant current (25 mA per gel) for 3 to 3.5 h. After electrophoresis, gels were fixed, stained with Coomassie brilliant blue R250, destained, fluorographed with En^{3}Hance (New England Nuclear Corp.), dried, and exposed to Kodak SB-5 X-ray film. Gels were stored at −70°C during exposure. X-ray films were scanned with a model 910 densitometer (E-C Apparatus Corp., St. Petersburg, Fla.).

RESULTS

(i) Biological properties of *ts* mutants. Growth of *icr ts* mutants as a function of temperature. Of the 24 cytolysis-resistant mutants which exhibited temperature-sensitive properties (25), only 9 were sufficiently *ts* (i.e., exhibited [efficiency of plating at 39.6°C]/[efficiency of plating at 34°C] of $\geq 10^{-3}$ [38; Table 2]) to be suitable for further genetic and biochemical analysis. Of these, only two (*icr ts*184 and 62) behaved like the majority of previously described *ts* mutants of strain KOS at 39.0 and 39.6°C (38 and, for example, Table 2, *ts*B2). Fifteen other mutants were insufficiently *ts* even at 39.6°C to be used for meaningful biochemical and genetic analysis (e.g., Table 2, *icr ts*117). Because the yield of wild-type virus was greatly reduced at temperatures above 39.6°C and because nine mutants proved suitable for analysis at 39.6°C, this temperature was used in all genetic and biochemical studies as the nonpermissive temperature.

Confirmatory immune cytolysis tests. Before proceeding with genetic and biochemical analysis of these nine *ts* mutants, we reexamined their resistance to immune cytolysis at 39.6°C. For this purpose, cells were infected with each mutant and the wild-type virus at 34 and 39.6°C for 18 h, and infected cells were subjected to immune cytolysis with the same antiserum employed in the selection of a given mutant (Fig. 1).

At 39.6°C, 80% or more of cells infected with the five mutants originally selected with monospecific rabbit antiserum to gA/gB survived treatment with this antiserum and complement. In contrast, only 10% of cells infected with wild-type virus survived this treatment (Fig. 1). In similar tests of mutants selected with antiserum to gC, 65 and 80% of cells infected with *icr ts*132 and 184, respectively, survived at 39.6°C, whereas cells infected with KOS at this temperature exhibited only 15% survival. Approximately 75% of cells infected with *icr ts*112 and 82% of those infected with *icr ts*125 survived treatment with anti-gA/gBgC serum, whereas only 22% of wild-type virus-infected cells survived cytolysis after treatment with this antiserum.

At 34°C, cells infected with eight of the nine mutants were considerably more sensitive to cytolysis than at 39.6°C. Only cells infected with *icr ts*108 exhibited marked resistance to cytolysis at 34°C. Cells infected with this mutant were equally resistant at both temperatures. Thus, except for *icr ts*108, cells infected with *icr ts* mutants exhibited temperature-sensitive resistance to immune cytolysis.

(ii) Genetic analysis of *icr ts* mutants. Physical mapping by marker rescue. Before attempting to localize *ts* mutations by using individual cloned fragments, we tried to demonstrate that mutations could, in fact, be rescued by mixtures of KOS DNA cleaved by either *Eco*RI or *Bgl*II. Successful rescue would signify that individual *Eco*RI and *Bgl*II fragments would be suitable for more detailed mapping studies. Mutations in seven of the nine *icr ts* mutants were rescued by total cleaved KOS DNA (Table 3).

***icr ts*149.** In two marker rescue experiments with infectious *icr ts*149 DNA and individual cloned *Eco*RI DNA fragments representing the entire KOS genome, only tests with *Eco*RI fragment O produced significant numbers of ts^+ plaques of wild-type plaque size at the nonpermissive temperature. The results of one such test are shown in Fig. 2. The low levels of ts^+ virus produced in cotransfections with *Eco*RI fragments I and H proved to be *ts*; no such leaky virus was produced in a second test. Thus, the *ts* mutation in *icr ts*149 lies within the limits of *Eco*RI fragment O (0.448 to 0.457).

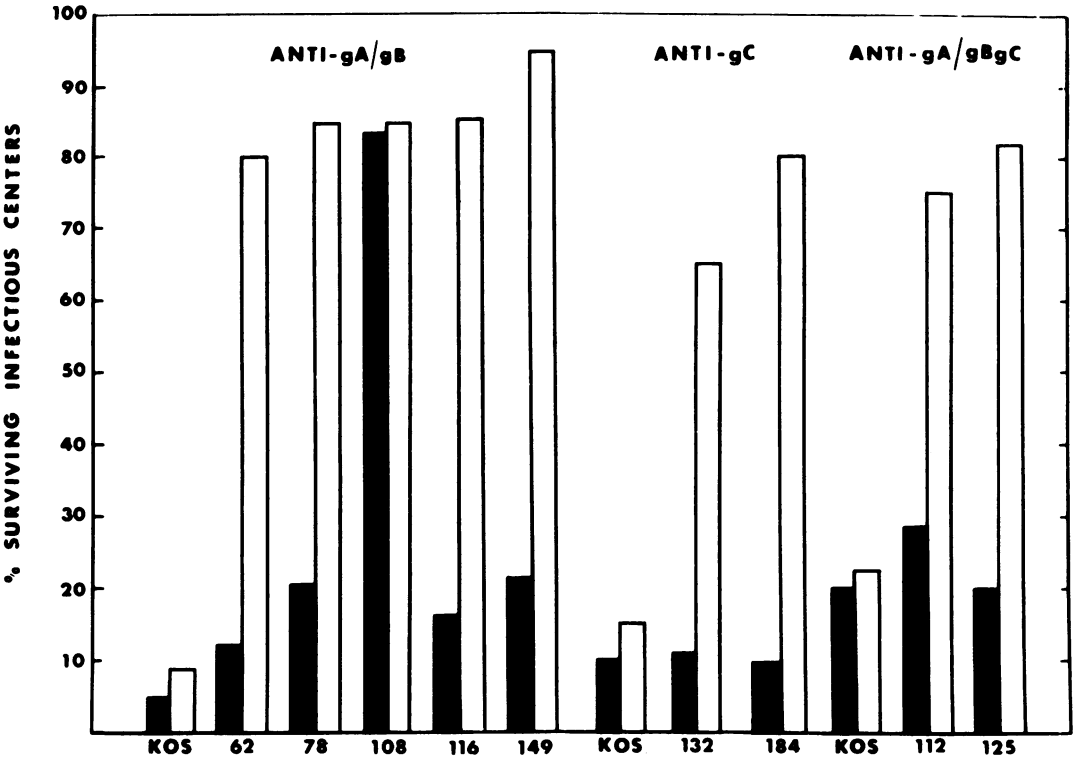


FIG. 1. Immune cytolysis tests of cells infected with *ts* mutants selected for their inability to induce cytolysis mediated by monospecific rabbit antisera to HSV-1 glycoproteins and complement at the nonpermissive temperature. A total of 10^5 HEL cells were infected with wild-type virus or each of the nine *icr ts* mutants. Infected cells were incubated for 18 h at 34 (□) or 39.6°C (■) and were subjected to immune cytolysis with the same monospecific rabbit antiserum used in the selection of each mutant.

icr ts116. The *ts* mutation in *icr ts*116 was rescued in two experiments only by the cloned *Eco*RI fragment A (0.493 to 0.636). The results of one of these experiments are shown in Fig. 2. Three separate attempts were also made to rescue the mutation in this virus with cloned *Bgl*II fragments D and G to map it more finely. No ts^+ virus was observed, however, suggesting the possibility that *icr ts*116 may possess two closely linked mutations (one in *Bgl*II fragment D and one in *Bgl*II fragment G) or that a single mutation may lie near the junction of *Bgl*II fragments D and G.

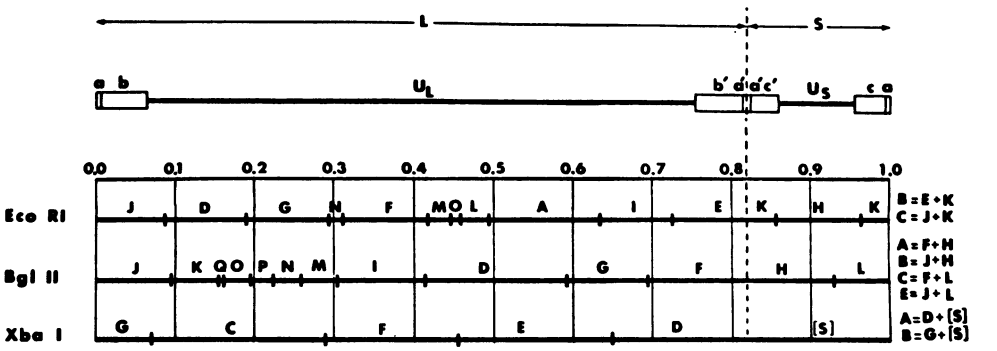
icr ts62. The mutation in *icr ts*62 was rescued in two independent experiments only by cloned *Eco*RI fragment B (which contains sequences in fragments E and K; Fig. 2). Since cotransfection with fragment C (containing sequences in fragments J and K) did not produce ts^+ progeny and since fragments B(EK) and C(JK) share *ab* sequences in the terminal repeats of fragment L as well as those in *Eco*RI fragment K, the map limits of the mutation in *icr ts*62 are most likely 0.724 to 0.772, which constitute unique sequences in fragment L immediately to the left of *b'a'* in fragment E (Fig. 2).

icr ts78. Results of three marker rescue tests conducted with *icr ts*78 DNA are shown in Fig.

TABLE 3. Marker rescue of *icr ts* mutations with total KOS DNA cleaved with *Eco*RI or *Bgl*II or individual fragments of KOS DNA

Mutant	Rescuing DNA cleaved with[a]:		
	*Eco*RI	*Bgl*II	Fragments
*icr ts*149	+	+	*Eco*RI-O
*icr ts*116	+	+	*Eco*RI-A
*icr ts*62	+	+	*Eco*RI-B(EK)
*icr ts*78	+	+	*Eco*RI-C(JK) and F; *Bgl*II-I; *Xba*I-G
*icr ts*132	+	NT[b]	–
*icr ts*112	+	+	–
*icr ts*125	+	+	–
*icr ts*108	–	–	NT
*icr ts*184	–	–	NT

[a] +, Mutation rescued; –, mutation not rescued.
[b] NT, Not tested.



Eco RI	C(JK)	D	G	N	F	M	O	L	A	I	B(EK)	H
Bgl II					I				D	G		
Xba I	G											
icr ts149												
Eco RI	*0.9*	*0.9*	*0.3*	*0.7*	*0.5*	*0.03*	70	*0.04*	*0.7*	*5.7*	*0.08*	*1.5*
icr ts116												
Eco RI	*0.01*	*0.02*	*0.01*	*0.01*	*0.2*	*0.3*	*0.005*	*0.01*	71	*0.02*	*0.01*	*0.002*
Bgl II									*0.03*	*0.1*		
icr ts62												
Eco RI	*0.1*	*0.03*	*0.1*	*0.03*	*0.06*	*0.03*	*0.06*	*0.02*	*0.03*	*0.08*	1171	*<0.1*
icr ts78												
Eco RI	3500	*0.01*	*0.06*	*0.005*	284	*0.3*	*0.1*	*0.1*	*0.1*	*0.004*	*0.01*	*0.2*
Bgl II					500							
Xba I	100											

FIG. 2. Results of marker rescue tests with *icr ts*149, 116, 62, and 78. The physical maps of *Eco*RI, *Bgl*II, and *Xba*I fragments have been described by others (7, 16, 42). *Eco*RI and *Bgl*II KOS DNA fragments were derived from cloned recombinant plasmids; *Xba*I fragment G was isolated from gels. The efficiency of rescue of *ts* mutations by individual viral DNA fragments is expressed as $(PFU_{39.6°C}/PFU_{34°C}) \times 10^4$.

2. In the test which utilized all the cloned *Eco*RI fragments, cotransfections with both *Eco*RI fragments C(JK) and F gave rise to ts^+ virus of wild-type plaque size at 39.6°C. Because two fragments rescued the *ts* mutation in *icr ts*78 efficiently, additional tests were conducted not only with *Eco*RI fragments C(JK) and F, but also with gel-purified *Xba*I fragment G and cloned BglII fragment I. As shown by the maps at the top of Fig. 2, these last two fragments overlap major portions of *Eco*RI fragments C(JK) and F, respectively. Rescue was observed in five of five tests with cloned *Eco*RI fragment F and two of two tests with cloned *Bgl*II fragment I, placing the limits of the mutation between 0.315 (the *Eco*RI N/F cleavage site) and 0.415 (the *Bgl*II I/D cleavage site). In three of five tests with *Eco*RI fragment C(JK) and four of four tests with *Xba*I fragment G, efficient rescue was also observed. Because cloned *Eco*RI fragment B(EK) failed to rescue in three of three tests, whereas fragment C(JK) rescued, and because fragments B(EK) and C(JK) share sequences in fragment K as well as terminal *ab* sequences, the sequences in *Eco*RI fragment C and *Xba*I fragment G capable of rescuing the *ts* mutation in *icr ts*78 more than likely lie in unique sequences in fragment L immediately to the right of the *ab* sequences (0.058) and to the left of the *Xba*I G/C cleavage site (0.074) (Fig. 2). Importantly, the plaque size of all ts^+ rescuants of *icr ts*78 was wild type. Collectively, these results demonstrate an unusual case of rescue by sequences in two distinct regions of the genome. Restriction enzyme analysis of the DNA from the two clones which contain *Eco*RI fragments C and F has not revealed evidence that either is a mixed clone. Work is in progress to investigate further by cross-hybridization the possibility that the two may possess one or more sequences of homology not previously detected or described for HSV-1.

***icr ts* mutants that could not be mapped.** We were unable to localize the *ts* mutations in the

other five *icr ts* mutants to a single viral DNA fragment using the mapping procedures described above. These mutants can be subdivided into two classes (Table 3): (i) those bearing *ts* mutations rescuable by total KOS DNA cleaved with either *Eco*RI or *Bgl*II (*icr ts*132, 112, and 125); and (ii) those containing mutations which were not rescued by total cleaved KOS DNA (*icr ts*108 and 184). Although the *ts* mutations of *icr ts*132, 112, and 125 were rescued efficiently by total cleaved KOS DNA, attempts to rescue these mutations with individual cloned *Eco*RI DNA fragments proved unsuccessful. The failure of a single fragment to rescue when total cleaved KOS DNA rescued efficiently implies that either (i) the mutant possesses more than one mutation, or possibly (ii) the cloned fragments used in rescue tests were altered in some way, i.e., rearranged or deleted. Although one or both of these two explanations is most likely responsible for the failure of single cloned fragments to rescue, precedent for the latter possibility has been reported by Spaete and Frenkel (43) and Weller et al. (49), specifically with regard to sequences contained within the cloned *Eco*RI fragment F. Spontaneous deletions in other cloned fragments have not been reported, but this potential problem should be considered in evaluating negative marker rescue data.

The *ts* mutations of mutants *icr ts*108 and 184 were not rescued by total cleaved KOS DNA. The inability to rescue with a mixture of total cleaved viral DNA implies similarly that more than one mutation is present, that the *ts* mutation(s) may lie near a cleavage site, or that the mutant possesses a mutation affecting, or a deletion sufficiently large to inhibit, homologous recombination and, hence, rescue.

Complementation tests. As predicted by the divergent physical map locations of their *ts* mutations (Fig. 2), and despite the fact that *icr ts*149, 116, 62, and 78 were selected originally with monospecific rabbit antisera prepared against the gA/gB glycoprotein complex, all four mutants complemented each other efficiently and hence are defective in different viral functions (Table 4).

Complementation tests were next conducted with the four *icr ts* mutants and other *ts* mutants whose mutations mapped in or near sequences overlapping those of the *icr ts* mutations (Table 5).

Efficient complementation occurred between *icr ts*149 (coordinates 0.448 to 0.457) and six *ts* mutants in five complementation groups which possess mutations mapping between coordinates 0.408 and 0.520 (Table 5).

Complementation indices greater than 10 were also obtained in tests of *icr ts*116 (coordinates 0.493 to 0.636) and five *ts* mutants representing four complementation groups whose mutations mapped between coordinates 0.410 and 0.594.

TABLE 4. Results of complementation tests among the four *icr ts* mutants mapped by marker rescue

Mutant	Complementation index[a]			
	*icr ts*149	*icr ts*116	*icr ts*62	*icr ts*78
*icr ts*149		61	72	214
*icr ts*116			56	33
*icr ts*62				260
*icr ts*78				

[a] Complementation tests were performed as described in Schaffer et al. (39). Complementation indices of 10 or greater were considered positive.

The mutations of only two mutants, *ts*LG4 (coordinates 0.779 to 0.855 [16]) and *icr ts*62 (coordinates 0.724 to 0.772), have been mapped physically to the right-hand end of L. The mutations in both mutants were rescued by *Eco*RI fragment B, and in three of three tests *icr ts*62 and *ts*LG4 failed to complement each other, indicating that these two mutants are defective in the same essential gene. The fact that the physical map limits of the *ts*LG4 and *icr ts* 62 mutations do not overlap suggests that this gene must include the 1.1-kilobase sequence between 0.772 (the right-hand-most coordinate of *icr ts*62) and 0.779 (the left-hand-most coordinate of *ts*LG4).

Because the *ts* mutation in *icr ts*78 was rescued by two distinct DNA fragments, complementation tests were conducted with *ts* mutants possessing mutations which mapped within or near coordinates 0.058 to 0.074 and 0.315 to 0.415. Complementation was observed in all pair-wise tests of *icr ts*78 and six *ts* mutants representing four complementation groups whose mutations mapped between coordinates 0.086 and 0.220 (Table 5). Complementation indices greater than 10 were also observed in tests of *icr ts*78, with 10 of 11 *ts* mutants whose mutations mapped in or near coordinates 0.315 to 0.415.

Thus, with the possible exception of *ts*LB7, which was not available for testing, *icr ts*78 was shown to be functionally distinct from any of the HSV-1 genes known to map in this region of the genome. We next tested *icr ts*78 with two HSV-2 *ts* mutants representing a new late HSV-2 gene (10). These tests were conducted because (i) *icr ts*78 is DNA-positive (Table 6) and thus is presumably defective in a late gene and (ii) a new HSV-2 late gene represented by *ts*39 and *ts*201, for which there currently is no known HSV-1 equivalent, has recently been identified and mapped to coordinates 0.356 to 0.381 of the HSV-2 genome (10, 44). In three separate tests, *icr ts*78 failed to complement *ts*201 (complemen-

TABLE 5. Complementation between *icr ts*149, 116, 62, and 78 and *ts* mutants whose mutations map nearby

Cytolysis-resistant *ts* (*icr ts*) mutant (map coordinates)	Test mutant	Map coordinates (reference)[a]	Complementation group[b]	Complementation Index[c]
*icr ts*149 (0.448–0.457)	*ts*C4	0.408–0.440 (6)	1–3	22
	*ts*D9	0.422–0.427[d]	1–4	86
	*ts*N20	0.445–0.448[d]	1–13	12
	*ts*O22	0.422–0.448[e]	1–14	47
	*ts*LB5	0.410–0.520 (17)	1–22	77
	*ts*B7	0.501–0.503 (2)	1–20	350
*icr ts*116 (0.493–0.636)	*ts*LB5	0.410–0.520 (17)	1–22	57
	*ts*B7	0.501–0.503 (2)	1–20	1,125
	*ts*10	0.490–0.520 (14)	ND[f]	19
	*ts*T36	0.530–0.594 (7)	1–27	23
	*ts*V37	0.530–0.594 (7)	1–29	28
*icr ts*62 (0.724–0.772)	*ts*LG4	0.779–0.855 (16)	ND	
*icr ts*78 (0.058–0.074)	*ts*F18	0.086–0.103 (33)	1–6	185
	*ts*K13	0.095–0.108[e]	1–10	788
	*ts*M19	0.095–0.108[e]	1–10[g]	5,702
	*ts*G3	0.103–0.186 (33)[h]	1–7	2,387
	*ts*E6, E5	0.120–0.220 (27)[h]	1–5	1,006; 384
(0.315–0.415)	Glasgow *ts*F	0.320–0.420 (34)	1–11	81
	*ts*1201	0.329–0.337 (34)	ND	62
	*ts*J12	0.357–0.360[h]	1–9	718
	Glasgow *ts*A	0.380–0.400 (34)	1–16	34
	*ts*LB7	0.390–0.410 (17)	ND	ND
	*ts*A1, A15, A42	0.385–0.398 (49)	1–1	1,091; 147; 89
	*ts*A24	0.398–0.413 (49)	1–1	11
	*ts*C4, C7	0.408–0.440 (6)	1–3	361; 23
	*ts*201, 39[i]	0.356–0.381 (44)	ND	

[a] References describe the narrowest published map limits.
[b] Complementation groups 1–1 through 1–23 were described by Schaffer et al. (39); groups 1–24 through 1–29 were described by Chu et al. (7).
[c] Complementation tests were performed as described in Schaffer et al. (39). Complementation indices of 10 or greater were considered positive.
[d] Coen et al., submitted for publication.
[e] Weller et al., submitted for publication.
[f] ND, Not determined.
[g] Based on physical mapping data and reevaluation of complementation results, the complementation group assignment of *ts*M19 has been changed from 1–12 to 1–10 (Weller et al., submitted for publication).
[h] Coordinates given in references are for mutants *ts*G8 and *ts*E6. The coordinates for *ts*J12 are those of N. DeLuca and S. Person (personal communication).
[i] *ts* mutants of HSV-2 (10).

tation indices: 0.87, 0.70, and 1.1) and *ts*39 (complementation indices: 0.89, 0.93, and 0.72) (Table 5), whereas efficient complementation was observed in the same tests with *icr ts*62 (complementation indices: 15, 367, and 22) and *ts*H9, an HSV-2 mutant in the major DNA-binding protein (10) (complementation indices: 12, 44, and 55).

These results indicate that *icr ts*78 is the HSV-1 counterpart of a late HSV-2 gene which lies immediately to the left of the gene for the major DNA binding protein (10). Experiments are currently in progress to map the *ts* mutation of *icr ts*78 more finely. Sequences which lie between the gene specifying glycoproteins gA and gB and the gene for the major DNA-binding protein are

TABLE 6. Summary of mutant phenotypes at 39.6°C

Virus	Viral DNA phenotype (% of wild type)[a]	Viral glycoprotein synthesis[b]				Presence of viral glycoproteins at the cell surface[c]			Phenotype at 34°C[d]
		gA	gB	gC	gD	gB	gC	gD	
KOS	+ (100)	+	+	+	+	+	+	+	+
*icr ts*149	+ (43)	+	±	±	+	+	+	+	+
*icr ts*116	± (4)	+	±	±	+	±	±	±	+
*icr ts*62	± (7)	−	−	−	−	−	−	−	GP
*icr ts*78	+ (59)	+	+	+	+	±	±	±	+
*icr ts*132	+ (29)	+	+	+	+	−	+	±	+
*icr ts*184	± (3)	+	±	±	+	+	±	+	+
*icr ts*108	± (2)	+	±	±	+	±	±	±	R
*icr ts*112	± (6)	+	±	±	+	±	±	±	+
*icr ts*125	± (10)	+	−	−	+	±	±	±	+

[a] [^{3}H]thymidine incorporation into viral DNA was measured as described by Aron et al. (1). +, >20% of wild-type levels; ±, 1 to 20% of wild-type levels, −, no viral DNA synthesis detected.

[b] Synthesis of [^{14}C]glucosamine-labeled viral glycoproteins in mutant-infected cells relative to that synthesized in wild-type virus-infected cells. +, Like wild-type virus; ±, less than in wild-type virus; −, not detected.

[c] Immune precipitable ^{125}I-labeled viral glycoproteins on the surfaces of mutant-infected cells relative to that on the surfaces of wild-type virus-infected cells. ±, Like wild-type; +, less than on wild-type virus-infected cells; −, not detected.

[d] +, Viral DNA synthesis, glycoprotein synthesis, expression of glycoproteins at the cell surface, and sensitivity of infected cells to immune cytolysis were like wild-type virus at 34°C; R, infected cells were resistant to immune cytolysis at 34°C; GP, glycoprotein defect was also evident at 34°C.

roughly 0.368 to 0.385 or approximately 2.6 kilobases—sufficient information to encode a protein with an approximate molecular weight of 100,000 daltons.

Tests of *icr ts*149, 116, 62, and 78 with representatives of unmapped mutants in 14 complementation groups yielded indices greater than 10 (data not shown). Thus, the four *icr ts* mutants represent new complementation groups and have been designated 1-30 (*icr ts*149), 1-31 (*icr ts*116), 1-32 (*icr ts*62), and 1-33 (*icr ts*78). As members of the KOS series of complementation groups they have been designated *ts*W44, *ts*X45, *ts*Y46, and *ts*Z47, respectively.

No effort was made to assign unmapped *icr ts* mutants to complementation groups because of our suspicion that each contains more than one *ts* mutation.

(iii) Phenotypic analysis of *icr ts* mutants. Viral DNA phenotypes. The viral DNA phenotypes of *icr ts* mutants at 39.6°C were next determined. Based on incorporation of [^{3}H]thymidine into viral DNA from 4 to 24 h postinfection (1), cells infected with *icr ts* mutants synthesized from 2 (*icr ts*108) to 59% (*icr ts*78) of wild-type levels of viral DNA (Table 6). All nine *ts* mutants produced wild-type levels of viral DNA at 34°C. In accordance with accepted precedent (1), mutants *icr ts*78, 132, and 149 have been designated DNA$^+$, and mutants *icr ts*62, 108, 116, 184, 112, and 125 have been designated DNA$^\pm$.

Viral glycoprotein phenotypes. Because we anticipated that resistance to immune cytolysis would reflect altered glycoprotein synthesis, maturation, or insertion into cell membranes, two types of phenotypic analyses were performed to characterize the glycoprotein species synthesized during infection and to identify those exposed on the plasma membranes of infected cells.

Viral glycoprotein synthesis. The viral glycoprotein species designated gA, gB, gC, and gD in Fig. 3A were identified by immune precipitation of lysates of wild-type virus-infected cells with monoclonal antibodies to each of these glycoproteins followed by SDS-PAGE analysis of precipitates (not shown). The identity of glycoprotein gE was based solely on comparison of the molecular weight determination made in this study with published values (3). However, because gE represented a very minor species in KOS-infected HEp-2 cells labeled continuously from 4 to 24 h with [^{14}C]glucosamine, we were unable to evaluate gE synthesis in mutant-infected cells.

At 39.6°C, HEp-2 cells infected with mutants *icr ts*149 and 116 synthesized all the major viral glycoprotein species; however, the mature forms of gB and gC were under-represented (Fig. 3A). No virus-specific glycoproteins were detected in cells infected with *icr ts*62 at the nonpermissive temperature such that the gel profile of cells infected with this mutant at 39.6°C was similar to that of mock-infected HEp-2 cells labeled with [^{14}C]glucosamine. In cells infected with *icr ts*78, on the other hand,

the glycoprotein profile was similar to that of cells infected with the wild-type virus. At 34°C, the glycoproteins synthesized by all mutants except *icr ts*62 were similar to those synthesized by wild-type virus. In *icr ts*62-infected cells, however, significantly reduced levels of gC were evident compared with KOS-infected cells at 34°C.

Although their *ts* mutations could not be mapped physically, the other five *icr ts* mutants exhibited glycoprotein phenotypes which may prove useful in studies of the roles of viral glycoproteins in immunity to HSV. Cells infected with four of these mutants, *icr ts*108, 184, 112, and 125, exhibited greatly reduced levels of both glycoproteins gB and gC at 39.6°C (Fig. 3B). In contrast, all the major glycoprotein species in amounts indistinguishable from KOS-infected cells were detected in cells infected with *icr ts*132 (data not shown).

Viral glycoproteins in plasma membranes. Three major glycoprotein species (gB, gC, and gD) were precipitable by hyperimmune rabbit antiserum from lysates of KOS-infected HEp-2 cells surface labeled with ^{125}I before lysis 20 h after infection at 34 and 39.6°C (Fig. 4A). The identities of these glycoproteins were confirmed by immune precipitation with monoclonal antibodies (data not shown). Two observations concerning wild-type virus-infected cells are noteworthy. (i) Glycoprotein gA was noticeably absent, or was present in minor amounts, in surface-labeled cells at both temperatures, a finding which is in agreement with the observations of Baucke and Spear (3), Glorioso et al. (15), and Norrild and Pedersen (28). (ii) Although gB, gC, and gD were readily detected in surface membranes of infected cells incubated at 39.6°C, these species were less efficiently labeled than when cells were incubated at 34°C, probably reflecting the temperature-sensitive nature of glycoprotein processing or insertion of glycoproteins into cell membranes. The failure to detect other species precipitable from metabolically labeled cells at 34 and 39.6°C probably reflects the failure of these glycoproteins to be inserted into surface membranes in a labelable and precipitable form as they were clearly synthesized in KOS-infected cells at both temperatures (Fig. 3A).

Bands corresponding to wild-type amounts of gB, gC, and gD were detected in ^{125}I-surface-labeled *icr ts*149-infected cells at 39.6°C, whereas these glycoproteins were found to be either greatly reduced in *icr ts*116- and *icr ts*78-infected cells and undetectable on the surface of *icr ts*62-infected cells (Fig. 4A). The failure to detect viral glycoproteins on *icr ts*62-infected cell membranes was not surprising as no viral glycoprotein synthesis was detected in these cells (see above and Fig. 3A). In *icr ts*116-infected cells, gD was also greatly reduced or absent at 39.6°C (Fig. 4A).

Compared with KOS-infected cells at 39.6°C, reduced levels of gB, gC, and gD were detected on the surface of HEp-2 cells infected with *icr ts*108, 112, and 125 (Fig. 4B). Cells infected with *icr ts*184 exhibited reduced levels of surface-associated gC yet greater than wild-type levels of gB. A glycoprotein species which comigrated with gC was detected on the surface of *icr ts*132-infected cells; however, no gB was detected. That the predominant species were indeed gA/gB-related in *icr ts*184-infected cells and gC-related in *icr ts*132-infected cell lysates was confirmed by selective immune precipitation with monoclonal antibodies to gA/gB and gC and SDS-PAGE analysis (data not shown).

Glycoprotein phenotypes of ts^+ revertant and rescuant virus. To confirm the association between the altered glycoprotein phenotypes of the four *icr ts* mutants whose mutations had been mapped and their respective temperature-sensitive mutations, rare ts^+ revertants and recombinants (rescuants) were isolated, and the patterns of glycoprotein synthesis and insertion into surface membranes in cells infected with ts^+ virus were examined. In all cases, the patterns of viral glycoprotein synthesis and insertion into plasma membranes were identical to those of wild-type virus. Thus, the altered glycoprotein phenotypes of these four mutants, and presumably their inability to render cells susceptible to immune cytolysis at 39.6°C, are attributable to their respective *ts* mutations.

DISCUSSION

We have described herein the genetic and biochemical properties of nine *ts* mutants isolated after mutagenesis of HSV-1 (strain KOS) with 2-aminopurine and selected by complement-mediated immune cytolysis with monospecific rabbit antisera directed against KOS glycoproteins gA, gB, or gC (25). Because of the manner in which they were selected, we anticipated that new mutants would be found with mutations in previously unidentified genes and that these mutants would possess defects in the synthesis, processing, or insertion of viral glycoproteins into infected cell membranes.

Physical map locations of *icr ts* mutations relative to the locations of glycoprotein structural genes. Attempts to map the *ts* mutations of *icr ts* mutants revealed the following: (i) the *ts* mutations of only four of nine *icr ts* mutants were rescued by single cloned *Eco*RI DNA fragments; (ii) the four mutants whose mutations could be mapped were selected with monospecific rabbit antiserum to glycoproteins gA and gB; and (iii) the *ts* mutations of two mutants (*icr ts*149 and

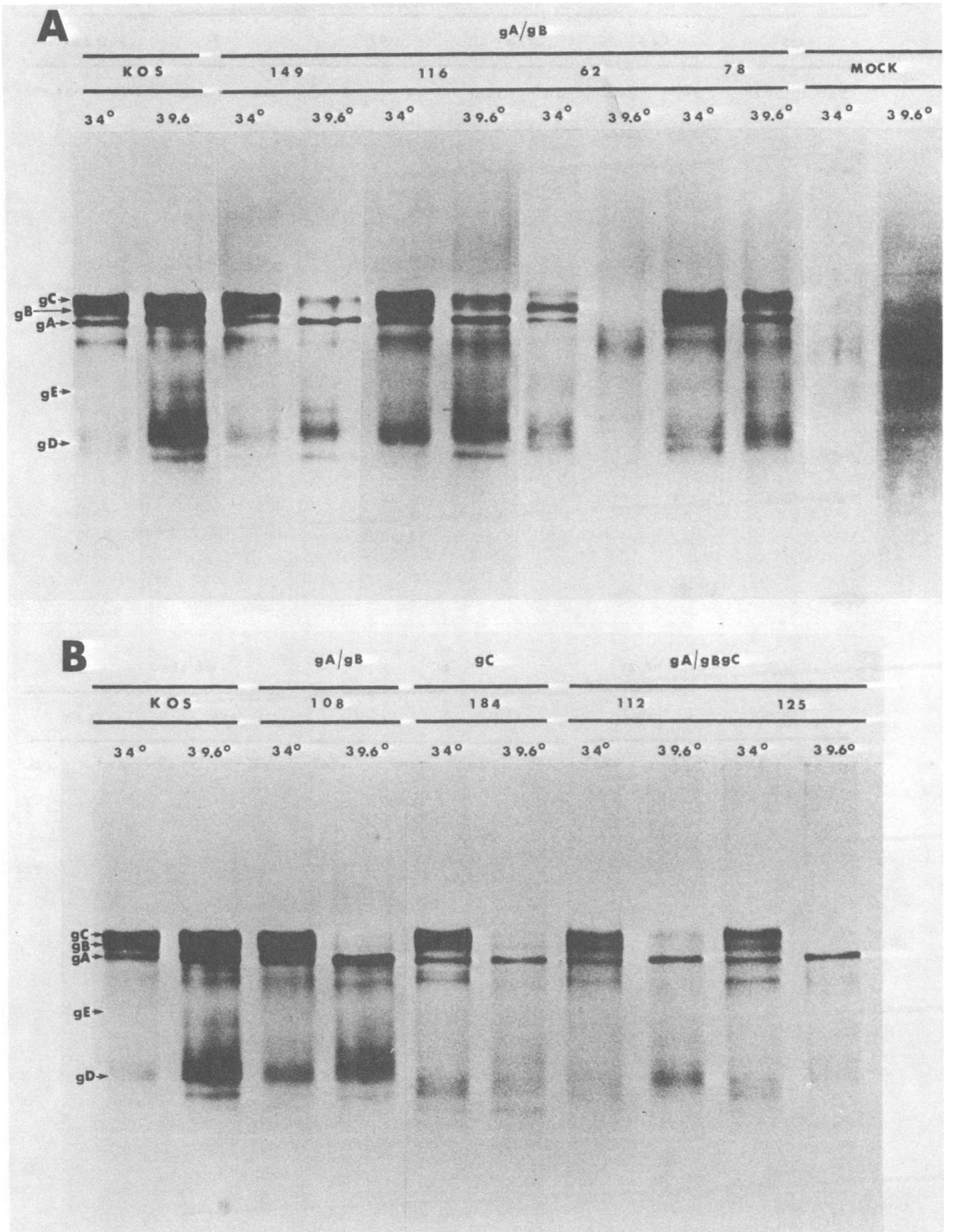


FIG. 3. Viral glycoproteins synthesized in mutant and wild-type virus-infected cells at 34 and 39.6°C. A total of 1×10^6 to 3×10^6 HEp-2 cells were infected with wild-type (KOS) or mutant virus at multiplicities of infection ranging from 10 to 30 PFU/cell. Infected cells were incubated at 34 or 39.6°C. D-[1-^{14}C]glucosamine (1 to 2 μCi per culture) was added at 4 h postinfection, and incubation was continued at 34 or 39.6°C for an additional 20 h. Infected cell lysates were subjected to SDS-PAGE as described in the text. Bands corresponding to glycoproteins gA, gB, gC, pgC, and gD were identified by immune precipitation of cell lysates with monoclonal antibodies to these glycoproteins and by gel electrophoresis of precipitated glycoproteins. (A) Gel profiles produced by the four mutants whose mutations were mapped physically; (B) profiles produced by four of the five mutants whose mutations were not mapped. The designations gA/gB (A) and gA/gB, gC, and gA/gBgC (B) refer only to the glycoprotein-specific antisera used in mutant selection and not to the mutant glycoprotein phenotypes.

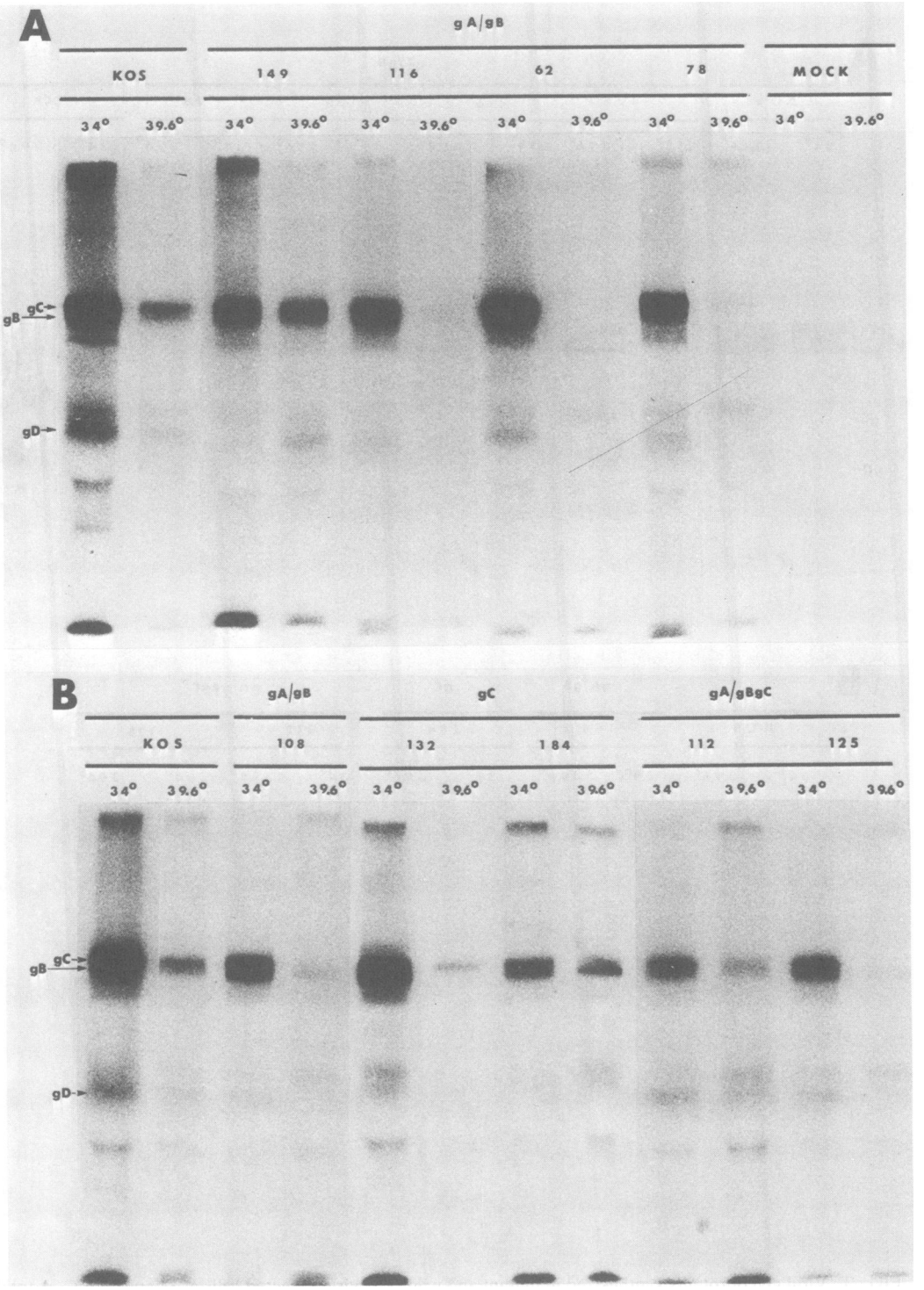


FIG. 4. ^{125}I-labeled HSV-specific glycoproteins in plasma membranes of mutant-infected cells incubated at 34 and 39.6°C. A total of 1×10^6 to 3×10^6 HEp-2 cells were infected with wild-type (KOS) or *icr ts* mutant virus at a multiplicity of infection of 10 to 30 PFU per cell and were incubated at 34 or 39.6°C for 20 h. Intact cells were iodinated and lysed, and lysates were immune precipitated with hyperimmune rabbit antiserum to HSV-1 (KOS)-infected cells. Immune precipitates were analyzed by SDS-PAGE. Glycoprotein species related to gB, gC, and gD were identified by immune precipitation with monoclonal antibodies. (A) Composite fluorograph of SDS-PAGE analysis of lysates of cells infected with *icr ts*149, 116, 62, and 78; (B) composite fluorograph of the unmapped *icr ts*108, 132, 184, 112, and 125. The designations gA/gB (A) and gA/gB, gC, and gA/gBgC of (B) refer only to the glycoprotein-specific antisera used in mutant selection and not to the mutant glycoprotein phenotypes.

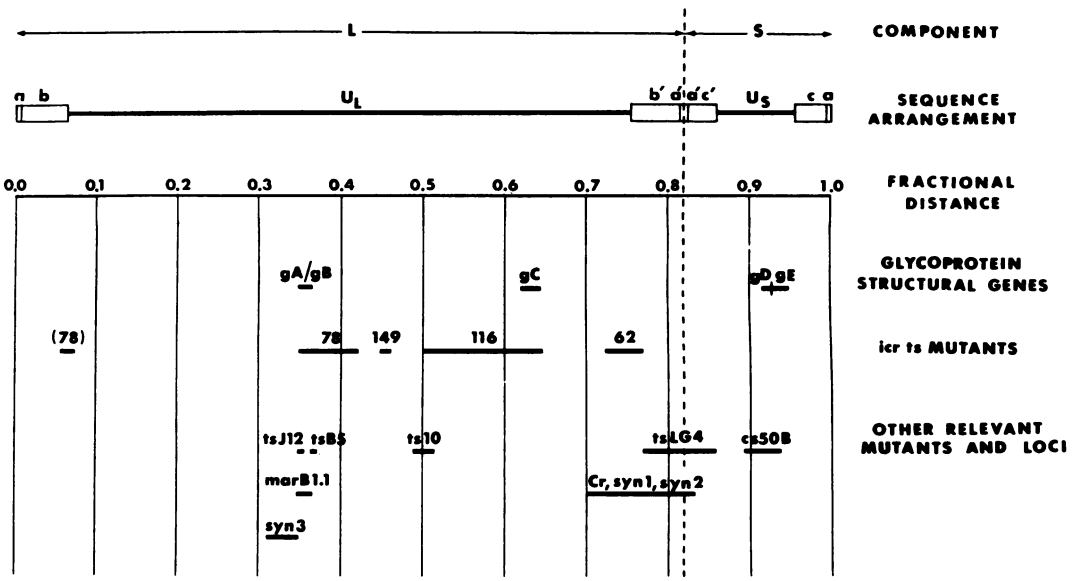


FIG. 5. Physical map locations of HSV-1 glycoprotein structural genes and of mutations affecting viral glycoprotein synthesis or the incorporation of viral glycoproteins into the plasma membrane of infected cells. See text for references.

62) were mapped to regions of the HSV-1 genome other than those known to encode the structural genes for gA and gB or the genes for other viral glycoproteins (Fig. 5).

Unexpectedly, the *ts* mutation of *icr ts*78 was rescued by sequences at the left end of U_L (0.074 to 0.085, a region not known to encode a viral glycoprotein gene) as well as by sequences between coordinates 0.315 and 0.415, which contain the structural gene for glycoproteins gA and gB (coordinates 0.348 to 0.369 [19; D. Bzik, N. DeLuca, and S. Person, personal communication]; Fig. 5). Recent studies have demonstrated that mutations in the gene for gA and gB can result in (i) temperature-sensitive synthesis of glycoprotein gB (e.g., *ts*B5, *ts*J12 [23, 37]); (ii) temperature-independent formation of syncytia (with no apparent glycoprotein defect) (e.g., *syn*3 [9]); and (iii) antigenic variation (e.g., marB1.1 [19]) (see Fig. 5). However, the observations that *icr ts*78 complements a mutant, *ts*J12, known to possess a *ts* mutation in the structural gene for gA and gB (23), exhibits no apparent defect in the synthesis of gA or gB at high temperature, and does not induce the formation of syncytia in Vero, HEL, RK, or HEp-2 cells (unpublished results) strongly suggest that the *ts* mutation of *icr ts*78 is not in the gene for gA and gB. This hypothesis was confirmed by the failure of *icr ts*78 to complement HSV-2 *ts* mutants in a newly identified late gene which maps immediately to the right of the gene for gA and gB. The DNA^+ phenotype and wild-type profile of *icr ts*78 with respect to glycoprotein synthesis at 39.6°C also reflect the fact that this mutant is defective in a late function.

The *ts* mutation in *icr ts*116 also lies outside the structural gene for gA and gB (Fig. 5). Whether the *ts* mutation in *icr ts*116 (map coordinates 0.493 to 0.636) lies within the structural gene for gC (0.630 to 0.646 [13]) is currently unknown as all attempts to date to map this mutation more finely have proven unsuccessful. Thus, none of the four *icr ts* mutations lies in the structural gene for gA and gB, and with the possible exception of *icr ts*116, three of the four *icr ts* mutations have been mapped to sequences other than those which encode any of the known HSV-1 glycoprotein structural genes (gA/gB and gC, see above; gD, 0.911 to 0.924 [22]; gE, 0.924 to 0.951 [22]) (Fig. 5).

We were unable to map the *ts* mutations of a fifth mutant selected with antiserum to gA/gB (*icr ts*108), two mutants selected with antiserum to gC (*icr ts*132 and 184), and two mutants selected with antiserum to gA/gB and gC (*icr ts*112 and 125). We suspect that these mutants may possess more than one mutation because rescue could not be demonstrated with a single viral DNA fragment and because of the stringency of the procedure used to select *icr ts*112 and 125 (i.e., selection for failure to synthesize gA, gB, and gC or to insert these glycoproteins into infected cell membranes).

Complementation tests with *icr ts* mutants: identification of new complementation groups. Because of the constraints of the selection procedure used in the isolation of *icr ts* mutants, we

anticipated that mutants representing new functional complementation groups would be identified. This indeed proved to be the case. Mutants *icr ts*149 and 116 complemented all mutants whose *ts* mutations mapped near the *icr ts* mutations in these two mutants, as well as representatives of all unmapped complementation groups. Thus, *icr ts*149 and 116 represent new groups which have been designated 1-30 and 1-31. Because *icr ts*62 failed to complement *ts*LG4, because both mutants contain *ts* mutations rescued by the same cloned fragment, and because *icr ts*62 complemented mutants representing all groups whose members have not yet been mapped, *icr ts*62 and *ts*LG4 constitute a third new complementation group which has been designated 1-32. Based on marker rescue and complementation data, *icr ts*78 appears to represent the HSV-1 counterpart of a new late HSV-2 gene and hence a new HSV-1 cistron which has been designated 1-33. Thus, the four *icr ts* mutants whose mutations were rescued with a single viral DNA fragment constitute four previously unrecognized HSV-1 complementation groups.

Viral glycoprotein phenotypes of *icr ts* mutants: molecular basis for resistance of mutant-infected cells to immune cytolysis. The nine *icr ts* mutants differ markedly in their ability to induce the synthesis of viral glycoproteins and to promote their insertion into cell membranes in such a way as to render infected cells susceptible to immune cytolysis (Table 6).

***icr ts*149.** Cells infected with *icr ts*149 at 39.6°C contained all the major viral glycoprotein species, although gB and gC were underrepresented compared with wild-type virus-infected cells as detected with [^{14}C]glucosamine. Of interest was the observation that the kinds and amounts of viral glycoproteins labeled at the surface of *icr ts*149-infected cells were indistinguishable from those labeled at the surface of wild-type virus-infected cells, yet *icr ts*149-infected cells were resistant to cytolysis with antiserum to gA/gB, and wild-type virus-infected cells were fully susceptible. Apparently, gB (and perhaps other viral glycoproteins) is either antigenically or structurally altered and hence unrecognizable by antiserum to gA/gB; or perhaps this glycoprotein is improperly inserted into surface membranes, which would make it inaccessible to antibody and complement and hence resistant to cytolysis but would allow it to be labeled with ^{125}I and precipitated by certain hyperimmune and monoclonal antibodies. In either case, the mutant appears to be defective in a late stage of glycoprotein processing as all glycoprotein species reach the cell surface. Among the acivities thought to occur in the Golgi and on surface membranes are the final stages of glycosylation mediated by specific glycosyl transferases. Recently, Olofsson et al. have shown that sialyl and galactosyl transferase activities are altered after infection with HSV (30). Whether the virus encodes its own transferase(s) or modifies cellular transferese(s) is currently unknown. Mutant *icr ts*149, however, is an interesting subject for further investigation of this question.

***icr ts*116.** Like *icr ts*149, cells infected with *icr ts*116 at 39.6°C contain all major viral glycoprotein species, yet gB and gC are underrepresented compared with wild-type virus-infected cells. In contrast to *icr ts*149, these glycoproteins either fail to be inserted into surface membranes or they are inserted in a form not recognizable by antibody to gA/gB and complement and incapable of being labeled with ^{125}I and precipitated. Whether the problem lies in a final stage of glycosylation or at an earlier intracellular stage is currently unknown. Clearly, however, resistance of *icr ts*116-infected cells at 39.6°C appears to result from aberrant presentation of viral glycoproteins at the cell surface.

***icr ts*62.** The phenotypic properties of *icr ts*62 at 39.6°C indicate that this mutant is defective in an early function essential for viral DNA synthesis. Thus, *icr ts*62-infected cells are characterized by greatly reduced synthesis of viral DNA and by the absence of all of the major viral glycoproteins. Moreover, cells infected with *icr ts*62 at 34°C also exhibited reduced levels of gC, although the synthesis of viral DNA and of the other viral glycoproteins appeared to be normal at this temperature. Obviously, the failure of *icr ts*62 to induce the synthesis of all viral glycoproteins at 39.6°C accounts for the resistance of mutant-infected cells to immune cytolysis at this temperature.

The identity of the specific function defective in *icr ts*62 is another question, however. The mutations in *icr ts*62 and the other member of complementation group 1-32, *ts*LG4, respresent the only essential viral gene identified to date at the far right-hand end of L. In addition to the two *ts* mutations, two syncytial loci (*syn*1 and *syn*2) and a locus affecting the expression of gC (the Cr locus) have been mapped to this region of the genome (coordinates 0.700 to 0.782) (35; Fig. 5). Interestingly, the 2-kilobase mRNA encoding ICP27, an immediate early protein with a molecular weight of 63,000, also maps to these sequences (coordinates 0.741 to 0.754) (48). The possibility that the *ts*, *syn*, and gC regulatory mutations may all lie in the gene for ICP27 should certainly be considered as the phenotypic properties of *icr ts*62 are consistent with this hypothesis. Thus, *icr ts*62 is defective in a very early function and hence synthesizes little or no late gene products (i.e., viral glycoproteins), and *icr ts*62 is specifically defective in the expression

of gC (a nonessential gene product) at 34°C. This mutant does not, however, induce the formation of syncytia. Mutant *icr ts*62 is currently being studied in greater detail in an effort to determine whether it possesses a single or multiple closely linked mutation(s), whether the expression of ICP27 and gC is coordinately regulated, and whether such coordinate expression (if it occurs) is associated with the *ts* defect in this mutant.

***icr ts*78.** Although cells infected with *icr ts* mutants 78 and 116 exhibited similar glycoprotein profiles at the cell surface (all major viral glycoproteins were only minimally represented), they exhibited slightly different profiles of glycoprotein synthesis based on incorporation of [^{14}C]glucosamine. Mature forms of gB and gC were underrepresented in *icr ts*116-infected cells, whereas wild-type amounts of all the viral glycoproteins were found in cells infected with *icr ts*78 (Fig. 3A and 4A; Table 6). Mutant *icr ts*78 is, in all probability, a late mutant based on its viral DNA phenotype at 39.6°C and its inability to complement two late mutants of HSV-2, indicating that it represents the HSV-1 equivalent of this late gene.

Thus, resistance of cells infected with *icr ts*149, 116, 62, and 78 to cytolysis mediated by complement and antibody to gA/gB can be correlated with (i) failure to synthesize viral glycoproteins (*icr ts*62); (ii) insertion of reduced levels of viral glycoproteins into surface membranes of infected cells (*icr ts*116 and 78); and (iii) failure of membrane-associated glycoproteins to react with antibody and complement in a manner resulting in cytolysis (*icr ts*149). Presumably, the latter phenomenon reflects altered conformation, aberrant glycosylation, improper insertion of viral glycoproteins into the plasma membrane, or alterations in the membrane itself. Because the mutations in each of these four mutants could be mapped to a single viral DNA fragment and because the glycoprotein profiles of cells infected with ts^+ revertants and rescuants were identical to that of wild-type virus-infected cells, it is likely that the complex phenotypes of these mutants are the consequence of single *ts* mutations in genes affecting synthesis, maturation, transport, or insertion of viral glycoproteins into the plasma membrane. We cannot rule out the possibility, however, that these phenotypes result from two or more closely linked mutations.

Coordinate expression of gB and gC. The following evidence suggests that the maturation of at least two viral glycoproteins (gB and gC) is coordinately regulated. (i) All four mutants whose mutations were mapped exhibited altered synthesis or membrane insertion not only of gB but also gC, despite the fact that these mutants were selected with antiserum to gA/gB and not to gC (Fig. 3 and 4; Table 6). (ii) Moreover, cells infected with four of the five mutants whose mutations were not mapped exhibited reduced synthesis and exposure of both gB and gC on cell surface membranes; the one-to-one correlation in the phenotypes of gB and gC in the cases of *icr ts*112 and 125 is understandable in that these two mutants were selected with antisera to gA/gB and gC. (iii) Indeed, comparison of the two phenotypes (i.e., synthesis of gB and gC and of the presence of these glycoproteins at the infected-cell surface) (Table 6) reveals a correlation between the two phenotypes for all mutants but two (*icr ts*132 and 184). (iv) The disparity in the phenotypes of gB and gC in cells infected with *icr ts*184 probably reflects the fact that this mutant was selected with antiserum only to gC. (v) Although *icr ts*132 was also selected with antiserum to gC, gC was clearly synthesized in a form that could also be inserted into surface membranes of cells infected at the nonpermissive temperature, labeled with ^{125}I, and precipitated by either hyperimmune antisera or monoclonal antibodies. Although gB was synthesized, this glycoprotein species was absent from the cell surface. We cannot rule out the possibility that this mutant possesses a mutation in the gC structural gene that could result in altered conformation and inability of cell-surface exposed gC to interact with antibody and complement, resulting in resistance to cytolysis. Alternatively, effective cytolysis by antibody to gC may require not only the presence of gC in surface membranes but also of gB. This latter contention is supported by immune cytolysis tests of cells infected with the gB-deficient mutant, *ts*J12. Cells infected with this mutant synthesized wild-type levels of gC at the nonpermissive temperature, yet *ts*J12-infected cells were resistant to cytolysis both with monospecific antisera to gA/gB and to gC (25). Interestingly, three of six members of complementation group 1-9 exhibit defects not only in the synthesis of gB at the nonpermissive temperature but also of gC (N. DeLuca, personal communication). Further studies will be required to determine the stage(s) in glycoprotein maturation (glycosylation, transport, or membrane insertion) in which regulation of these two glycoproteins is being exerted and the mechanism(s) of this phenomenon. Whether control of synthesis and membrane insertion of glycoprotein gD is also coordinately regulated cannot be readily assessed from the results of this study. It is notable, however, that gD was either not detected or was present in reduced amounts in plasma membranes of cells infected with *icr ts*116, 62, 78, and 132 at 39.6°C.

ACKNOWLEDGMENTS

The authors thank Patricia Temple and Lori Sandner for excellent technical assistance. We are indebted to Rozanne

Sandri-Goldin and Valerie Preston for providing *ts* mutants and to Neal DeLuca and Stan Person for permitting us to quote unpublished information and for helpful discussions. We also thank our colleagues, Sandra Weller, Donald Coen, and Richard Respess, for useful suggestions and assistance. We gratefully acknowledge the assistance of Madelon Datz in the preparation of this manuscript.

This work was supported by research grants PCM-8115594 from the National Science Foundation and by Public Health Service grants CA20260 and CA21082 from the National Cancer Institute. B.A.P. was the recipient of fellowship CA06034 from the National Cancer Institute and of funds from Biomedical Research Support grant RR05526-18 and from American Cancer Society grant IN-118A for biomedical research.

LITERATURE CITED

1. **Aron, G. M., D. J. M. Purifoy, and P. A. Schaffer.** 1975. DNA synthesis and DNA polymerase activity of herpes simplex virus type 1 temperature-sensitive mutants. J. Virol. **16**:498–507.
2. **Batterson, W., D. Furlong, and B. Roizman.** 1983. Molecular genetics of herpes simplex virus. VIII. Further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. J. Virol. **45**:397–407.
3. **Baucke, R. B., and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. V. Identification of an Fc-binding glycoprotein. J. Virol. **32**:779–789.
4. **Brown, S. M., D. A. Ritchie, and J. H. Subak-Sharp.** 1973. Genetic studies with herpes simplex virus type 1: the isolation of temperature-sensitive mutants, their arrangement into complementation groups, and recombination leading to a linkage map. J. Gen. Virol. **18**:329–346.
5. **Carter, V. C., P. A. Schaffer, and S. S. Tevethia.** 1981. The involvement of herpes simplex virus type 1 glycoproteins in cell-mediated immunity. J. Immunol. **126**:1655–1660.
6. **Chartrand, P., C. S. Crumpacker, P. A. Schaffer, and N. M. Wilkie.** 1980. Physical and genetic analysis of the herpes simplex virus DNA polymerase locus. Virology **103**:311–326.
7. **Chu, C.-T., D. S. Parris, R. A. F. Dixon, F. E. Farber, and P. A. Schaffer.** 1979. Hydroxylamine mutagenesis of HSV DNA and DNA fragments: introduction of mutations into selected regions of the viral genome. Virology **98**:168–181.
8. **Courtney, R. J., P. A. Schaffer, and K. L. Powell.** 1976. Synthesis of virus-specific polypeptides by temperature-sensitive mutants of herpes simplex virus type 1. Virology **75**:306–318.
9. **DeLuca, N., D. J. Bzik, V. C. Bond, W. Snipes, and S. Person.** 1982. Genetic domains of the HSV-1 (KOS) genome affecting virus entry, cell fusion and the production of gB. Virology **122**:411–423.
10. **Dixon, R. A. F., D. J. Sabourin, and P. A. Schaffer.** 1983. Genetic analysis of temperature-sensitive mutants which define the genes for the major herpes simplex virus type 2 DNA-binding protein and a new late function. J. Virol. **45**:343–353.
11. **Eberle, R., and R. J. Courtney.** 1980. Preparation and characterization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein region of herpes simplex virus type 1. J. Virol. **35**:902–917.
12. **Esparza, J., M. Benyesh-Melnick, and P. A. Schaffer.** 1976. Intertypic complementation and recombination between temperature-sensitive mutants of herpes simplex virus types 1 and 2. Virology **70**:372–384.
13. **Frink, R. J., R. Eisenberg, G. Cohen, and E. K. Wagner.** 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. J. Virol. **45**:634–647.
14. **Glorioso, J. C., T. Holland, and E. E. Smiley.** 1981. A temperature-sensitive mutation in HSV-1 (KOS) affecting transport of viral glycoproteins to the cell surface, p. 102. *In* A. S. Kaplan, M. LaPlaca, F. Rapp, and B. Roizman (ed.), International Workshop on Herpesviruses. Esculapio Publishing Co., Bologna, Italy.
15. **Glorioso, J. C., M. Levine, T. C. Holland, and M. S. Szczesiul.** 1980. Mutant analysis of herpes simplex virus-induced cell surface antigens: resistance to complement-mediated immune cytolysis. J. Virol. **35**:672–681.
16. **Goldin, A. L., R. M. Sandri-Goldin, M. Levine, and J. C. Glorioso.** 1981. Cloning of herpes simplex virus type 1 sequences representing the whole genome. J. Virol. **38**:50–58.
17. **Halliburton, I. W.** 1980. Intertypic recombinants of herpes simplex viruses. J. Gen. Virol. **48**:1–23.
18. **Halliburton, I. W., R. E. Randall, R. A. Killington, and D. H. Watson.** 1977. Some properties of recombinants between type 1 and type 2 herpes simplex virus. J. Gen. Virol. **36**:471–484.
19. **Holland, T. C., R. M. Sandri-Goldin, L. E. Holland, S. D. Marlin, M. Levine, and J. C. Glorioso.** 1983. Physical mapping of the mutation in an antigenic variant of herpes simplex virus type 1 by use of an immunoreactive plaque assay. J. Virol. **46**:649–652.
20. **Knipe, D. M., W. Batterson, C. Nosal, B. Roizman, and A. Buchan.** 1981. Molecular genetics of herpes simplex virus. VI. Characterization of a temperature-sensitive mutant defective in the expression of all early viral gene products. J. Virol. **38**:539–547.
21. **Lawman, M. J. P., R. J. Courtney, R. Eberle, P. A. Schaffer, M. K. O'Hara, and B. T. Rouse.** 1980. Cell-mediated immunity to herpes simplex virus: specificity of cytotoxic T cells. Infect. Immun. **30**:451–461.
22. **Lee, G. T.-Y., M. F. Para, and P. G. Spear.** 1982. Location of the structural genes for glycoproteins gD and gE and for other polypeptides in the S component of herpes simplex virus type 1 DNA. J. Virol. **43**:41–49.
23. **Little, S. P., J. T. Jofre, R. J. Courtney, and P. A. Schaffer.** 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. Virology **115**:149–160.
24. **Little, S. P., and P. A. Schaffer.** 1981. Expression of the syncytial (*syn*) phenotype in HSV-1, strain KOS: genetic and phenotypic studies of mutants in two *syn* loci. Virology **112**:686–702.
25. **Machtiger, N. A., B. A. Pancake, R. Eberle, R. J. Courtney, S. S. Tevethia, and P. A. Schaffer.** 1980. Herpes simplex virus glycoproteins: isolation of mutants resistant to immune cytolysis. J. Virol. **34**:336–346.
26. **Markwell, M. A. K., and C. F. Fox.** 1978. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. Biochemistry **17**:4807–4817.
27. **Morse, L. S., T. G. Buchman, B. Roizman, and P. A. Schaffer.** 1977. Anatomy of herpes simplex virus DNA. IX. Apparent exclusion of some parental DNA arrangements in the generation of intertypic (HSV-1 × HSV-2) recombinants. J. Virol. **24**:231–248.
28. **Norrild, B., and B. Pedersen.** 1982. Effect of tunicamycin on the synthesis of herpes simplex virus type 1 glycoproteins and their expression on the cell surface. J. Virol. **43**:395–402.
29. **Norrild, B., S. L. Shore, and A. J. Nahmias.** 1979. Herpes simplex virus glycoproteins: participation of individual herpes simplex virus type 1 glycoprotein antigens in immunocytolysis and their correlation with previously identified glycopolypeptides. J. Virol. **32**:741–748.
30. **Olofsson, S., B. Khanna, and E. Lycke.** 1980. Altered kinetic properties of sialyl and galactosyl transferases associated with herpes simplex virus infection of GMK and BHK cells. J. Gen. Virol. **47**:1–9.
31. **Para, M. F., R. B. Baucke, and P. G. Spear.** 1982. Glycoprotein gE of herpes simplex virus type 1: effects of anti-gE on virion infectivity and on virus-induced Fc-binding receptors. J. Virol. **41**:129–136.
32. **Para, M. F., L. Goldstein, and P. G. Spear.** 1982. Similar-

ities and differences in the Fc-binding glycoprotein (gE) of herpes simplex virus types 1 and 2 and tentative mapping of the viral gene for this glycoprotein. J. Virol. **41**:137–144.

33. **Parris, D. S., R. A. F. Dixon, and P. A. Schaffer.** 1980. Physical mapping of herpes simplex virus type 1 ts mutants by marker rescue: correlation of the physical and genetic maps. Virology **100**:275–287.
34. **Preston, V. G., J. A. V. Coates, and F. J. Rixon.** 1983. Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. J. Virol. **45**:1056–1064.
35. **Ruyechan, W. T., L. S. Morse, D. M. Knipe, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. **29**:677–697.
36. **Sandri-Goldin, R. M., M. Levine, and J. C. Glorioso.** 1981. Method for induction of mutations in physically defined regions of the herpes simplex virus genome. J. Virol. **38**:41–49.
37. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B_2) in virion infectivity. J. Virol. **29**:1149–1158.
38. **Schaffer, P. A., G. M. Aron, N. Biswal, and M. Benyesh-Melnick.** 1973. Temperature-sensitive mutants of herpes simplex virus type 1: isolation, complementation and partial characterization. Virology **52**:57–71.
39. **Schaffer, P. A., V. C. Carter, and M. C. Timbury.** 1978. Collaborative complementation study of temperature-sensitive mutants of herpes simplex virus types 1 and 2. J. Virol. **27**:490–504.
40. **Schaffer, P. A., R. J. Courtney, R. M. McCombs, and M. Benyesh-Melnick.** 1971. A temperature-sensitive mutant of herpes simplex virus defective in glycoprotein synthesis. Virology **46**:356–368.
41. **Showalter, S. D., M. Zweig, and B. Hampar.** 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP 4. Infect. Immun. **34**:684–692.
42. **Skare, J., and W. C. Summers.** 1977. Structure and function of herpesvirus genomes. II. *Eco*RI, *Xba*I, and *Hin*dIII endonuclease cleavage sites on herpes simplex virus type 1 DNA. Virology **76**:581–595.
43. **Spaete, R. R., and N. Frenkel.** 1982. The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. Cell **30**:295–304.
44. **Spang, A. E., P. J. Godowski, and D. M. Knipe.** 1983. Characterization of herpes simplex virus 2 temperature-sensitive mutants whose lesions map in or near the coding sequences for the major DNA-binding protein. J. Virol. **45**:332–342.
45. **Spear, P. G.** 1976. Membrane proteins specified by herpes simplex viruses. I. Identification of four glycoprotein precursors and their products in type 1-infected cells. J. Virol. **17**:991–1008.
46. **Tognon, M., D. Furlong, A. J. Conley, and B. Roizman.** 1981. Molecular genetics of herpes simplex virus. V. Characterization of a mutant defective in ability to form plaques at low temperatures and in a viral function which prevents accumulation of coreless capsids at nuclear pores late in infection. J. Virol. **40**:870–880.
47. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. U.S.A. **76**:615–619.
48. **Watson, R. J., C. M. Preston, and J. B. Clements.** 1979. Separation and characterization of herpes simplex virus type 1 immediate-early mRNA's. J. Virol. **31**:42–52.
49. **Weller, S. K., K. J. Lee, D. J. Sabourin, and P. A. Schaffer.** 1983. Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex virus 1 DNA-binding protein. J. Virol. **45**:354–356.