JOURNAL OF VIROLOGY, Nov. 1983, p. 396–404
0022-538X/83/110396-09$02.00/0

Vol. 48, No. 2

Insertion Mutants of Herpes Simplex Virus Have a Duplication of the Glycoprotein D Gene and Express Two Different Forms of Glycoprotein D

MARYLOU G. GIBSON AND PATRICIA G. SPEAR*

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

Received 10 May 1983/Accepted 9 August 1983

We produced insertion mutants of herpes simplex virus (HSV) that contain two functional copies of genes encoding different forms of glycoprotein D (gD). These viruses have the gene for HSV type 2 (HSV-2) gD at the normal locus and the gene for HSV-1 gD inserted into the thymidine kinase locus. Results of immunoprecipitation experiments done with monoclonal antibodies revealed that both gD genes were expressed by these viruses, regardless of orientation of the inserted HSV-1 gD gene, and that maximal synthesis of both glycoproteins depended on viral DNA replication. This apparently normal expression of the inserted HSV-1 gD gene was from a DNA fragment (*Sac*I fragment, 0.906 to 0.924 map units) containing nucleotide sequences extending from approximately 400 base pairs upstream of the 5′ end of the gD mRNA to about 200 base pairs upstream of the 3′ end. The glycoproteins expressed from both genes were incorporated into the surfaces of infected cells. Electrophoretic analyses of purified virions and neutralization studies suggest that both glycoproteins were also incorporated into virions. This nonpreferential utilization of both gene products makes these viruses ideal strains for the generation and characterization of a variety of mutations.

Glycoprotein D (gD) is one of several envelope glycoproteins specified by herpes simplex virus (HSV) (39). The two serotypes of HSV (HSV-1 and HSV-2) produce related, but antigenically and structurally differentiable, forms of gD (1, 4, 6, 13, 29, 30, 35, 36, 42). Little is known about the physiological role of this glycoprotein except that it can elicit the production of neutralizing antibodies (4, 30, 35, 42), a property shared with other HSV glycoproteins, and that anti-gD antibodies can block virus-induced cell fusion (A. G. Noble, G. T.-Y. Lee, R. Sprague, M. L. Parish, and P. G. Spear, Virology, in press).

The gene for gD was mapped to the S component of HSV DNA by analyses of HSV-1 × HSV-2 recombinant viruses (23, 34) and, later, to a 2.9-kilobase (kb) *Sac*I fragment of HSV-1 DNA (Fig. 1) by selection and translation in vitro of gD mRNA (16, 44). Nucleotide sequence determinations, coupled with construction of a fused gene that expresses a λ *cro*-gD hybrid protein in bacteria, have permitted precise localization of the coding sequence for the gD polypeptide of HSV-1 (44). The requirements for adjacent nucleotide sequences upstream and downstream of the coding sequence for gD to be expressed in productively infected cells have not previously been explored, although it has been shown that gD can be expressed from the *Bam*HI J DNA fragment (0.89 to 0.93 map units) injected into oocytes (43).

Insertion of appropriate HSV-1 DNA fragments into the thymidine kinase (TK) gene of a gC$^-$ HSV-1 mutant permitted us to monitor expression of gC from the inserted DNA sequences of selected thymidine arabinoside-resistant (AraT^r) progeny and thereby to define the boundaries of the region necessary for expression of the gC polypeptide in productively infected cells (17). In this report we describe a similar approach for identification of sequences necessary for expression of the gD polypeptide in productively infected cells. In the process we have also generated viruses that contain duplications of the gD gene. They express HSV-1 gD (gD-1) from the inserted DNA in the TK gene and HSV-2 gD (gD-2) from the normal genomic locus. We demonstrate expression and apparently normal regulation of both genes as well as utilization of both products in virion morphogenesis and transport to the cell surface.

MATERIALS AND METHODS

Cells and viruses. Vero (African green monkey kidney) cells, HEp-2 (human epidermoid carcinoma) cells, 143 (human TK$^-$) cells, and rabbit skin cells were used in this study. The 143 cells (2), produced by C. Croce and K. Huebner, and the rabbit skin cells, isolated by J. McLaren, were obtained from B. Roiz-

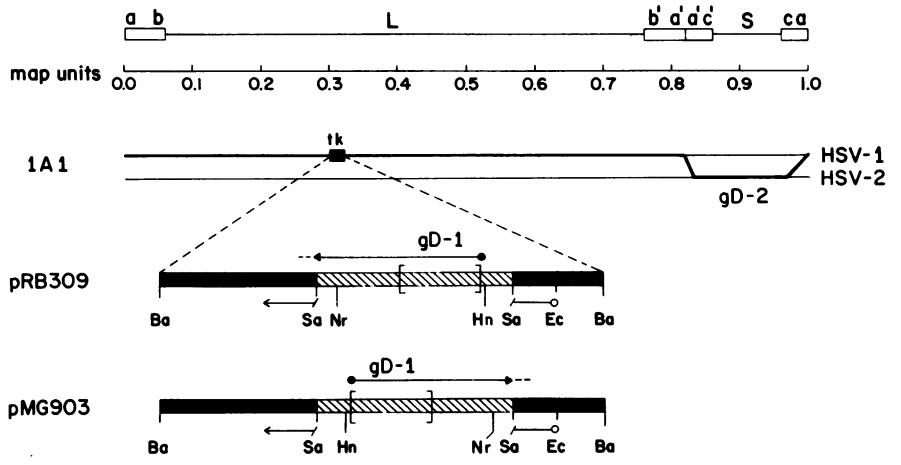

FIG. 1. Viral DNA sequence arrangements of HSV strain 1A1, of plasmids used, and of insertion mutants resulting from recombination between 1A1 and the plasmids. Strain 1A1, the derivation of which is described in the text, is a TK^+ $AraT^s$, HSV-1 × HSV-2 recombinant virus; the regions of the genome derived from HSV-1 and HSV-2 are indicated as reported by Tognon et al. (40). Location of the *Bam*HI fragment Q of 1A1 is indicated by the solid bar near 0.3 map units. The TK gene maps within *Bam*HI fragment Q (24, 41), and the gD-2 gene maps within the short segment of the recombinant genome (34) as shown. Plasmid pRB309 (25) contains a *Sac*I fragment of HSV-1(F) DNA (striped bar; map coordinates 0.906 to 0.924) inserted into the single *Sac*I site of *Bam*HI fragment Q of HSV-1(F) DNA (solid bar), interrupting the coding sequence of the TK gene (24, 41). We reversed the orientation of the *Sac*I fragment with respect to the TK gene to obtain the new plasmid pMG903. The gD-1 gene has been mapped to this *Sac*I fragment (16, 44). On the basis of nucleotide sequence analyses and other results, Watson et al. (43, 44) located the gD-1 coding sequence to the region enclosed within brackets and reported that the sequence homologous to gD-1 mRNA spans from near the *Hin*dIII site (5' end) to the *Sac*I site at 0.925 map units; the 3' end of the mRNA lies approximately 200 bp beyond this *Sac*I site. Of the two *Nru*I sites reported to be in this *Sac*I fragment of HSV-1(Patton) DNA (44), only the one shown is present in HSV-1(F) DNA. Sequences homologous to uninterrupted TK mRNA are also indicated along with the direction of transcription (24, 41). Recombination between either of the plasmids shown and strain 1A1 DNA resulted in the generation of insertion mutants that were $AraT^r$. Abbreviations used for restriction endonucleases are: Ba, *Bam*HI; Ec, *Eco*RI; Hn, *Hin*dIII; Nr, *Nru*I; Sa, *Sac*I.

man. An HSV-1 × HSV-2 recombinant virus, designated R50BG13 (also obtained from B. Roizman) and isolated by Tognon et al. (40), was modified for use as the parental virus in these studies. This virus, originally TK^- and resistant to AraT, was made TK^+ and AraT-sensitive ($AraT^s$) by rescue with the intact cloned TK gene (pRB103). Selection for the TK^+ $AraT^s$ virus was performed in 143 cells in the presence of HAT medium (32). The resultant virus, designated R50BG13-1A1, will hereafter be called 1A1. HSV-1(F) (8) and HSV-2(G) (8) were also used in these studies.

Plasmid and viral DNAs. The recombinant plasmids pRB103 [*Bam*HI fragment Q of HSV-1(F) DNA cloned into pBR322] (31) and pRB309 (25) (described in Fig. 1) were provided by B. Roizman; pMG903 was generated as described in the legend to Fig. 1, and pSKS309 contains a *Sac*I fragment (0.906 to 0.924 map units) of HSV-1(F) DNA cloned into the single *Sac*I site of pSKS106 (S. K. Shapira, J. Chou, F. V. Richard, and M. J. Casadaban, Gene, in press). Preparation and determination of authenticity of each plasmid DNA were performed as described (16).

*Bam*HI digests of genomic HSV DNAs prepared from infected Vero cells were separated on 0.6% agarose gels by electrophoresis. Southern transfer of DNA and hybridization with appropriate ^{32}P-labeled probes were performed (31).

Construction of recombinant viruses. Rabbit skin cells were cotransfected with mixtures of 1A1 viral DNA and pRB309 or pMG903 DNA at various concentrations of each. Calcium phosphate precipitates of DNA were prepared, containing 50 to 200 ng of *Bam*HI-digested plasmid DNA, 8 or 12 μg of cytoplasmic DNA from 1A1-infected Vero cells, and 12 μg of carrier DNA per 0.6 ml. Rabbit skin cell cultures were treated with this DNA as described (46). Progeny from the transfected cells were plated on Vero cells in the absence or presence of AraT (Raylo Chemical, Edmonton, Alberta, Canada) at 100 μg/ml (33), and $AraT^r$ isolates were screened for gD-1 production by immunoprecipitation with a monoclonal antibody specific for gD-1 (anti-gD-1 [II436]). Isolates of interest were plaque purified twice.

Isotopic labeling of cells and virions. (i) Continuous labeling with [^{35}S] methionine. HEp-2 cells infected wtih HSV at a multiplicity of 10 PFU per cell were labeled from 4 to 22 h after infection with [^{35}S]methionine (10 μCi/ml, 1,084 Ci/mmol; New England Nuclear Corp.) for the preparation of extracts for immunoprecipitation. Purified virions were prepared as previously described (3) from HEp-2 cells infected at a multiplicity of 3 PFU per cell and labeled at 34°C from 5 h after infection with [^{35}S]methionine (5 μCi/ml) until cytopathic effect was complete. Purified virions were

pelleted, and proteins were solubilized in extraction buffer for immunoprecipitation.

(ii) Pulse-chase with [^{35}S]methionine. Pulse-chase experiments were performed at 4.5 h after infection by labeling cells for 7 min with [^{35}S]methionine (50 μCi/ml) in methionine-deficient medium. Some cultures were harvested immediately after the pulse, whereas others were incubated for an additional 3 h in nonradioactive medium containing five times the usual levels of methionine.

(iii) Cell surface labeling with Na^{125}I. Intact HEp-2 cell monolayers infected at a multiplicity of 10 PFU per cell were iodinated at room temperature at 24 h after infection according to the procedure of Smith and Brown (38) with Na^{125}I (250 μCi per 4 × 10^6 cells, 17.4 Ci/mg; New England Nuclear Corp.). Post-iodination washes of the labeled monolayer were performed with phosphate-buffered saline plus 10 mM NaI. Soluble proteins were extracted as described below.

Antibodies and immunoassays. Monoclonal antibodies directed against HSV glycoproteins were produced by M. F. Para in this laboratory (Noble et al., in press; M. F. Para, R. Sprague, A. G. Noble, K. M. Zezulak, M. L. Parish, and P. G. Spear, manuscript in preparation). The antibodies selected for use in this study were II436, specific for gD-1 (anti-gD-1); III255 and III114, both of which react with gD-1 and gD-2 (anti-gD-1/2); and II73, specific for gC-1.

Sequential immunoprecipitations were performed on extracts prepared from infected HEp-2 cells. Labeled infected-cell monolayers were washed once in phosphate-buffered saline and lysed in extraction buffer consisting of 140 mM Tris-hydrochloride buffer (pH 7.4), 20 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, bovine serum albumin (1 mg/ml), and aprotinin (100 U/ml; Mobay Chemical Corp., New York, N.Y.). Cell extracts were cleared of nonsolubilized material by centrifugation at 178,000 × *g* in a Beckman airfuge. Cell extracts were mixed with an appropriate amount of antibody; after 30 min on ice, Formalin-fixed *Staphylococcus aureus* (14) was added for 10 min, and immune complexes were collected by centrifugation. Antibody-treated supernatants from one reaction were usually mixed with a second aliquot of antibody, or with a different antibody, for second and subsequent rounds of immunoprecipitation. Precipitated proteins were eluted from *S. aureus* after washing as described (16) and subjected to electrophoresis on 8.5% polyacrylamide gels linked with *N,N'*-diallyltartardiamide (10). Fluorography was performed as described previously (15).

Antibody-mediated neutralization of viral infectivity in the presence of complement was tested by a plaque reduction assay. Mixtures containing 500 PFU of the virus, the ascites form of the monoclonal antibody at a final concentration of either 1/50 or 1/500, and complement at a final concentration of 1/45 in 1 ml of total volume were incubated for 1 h at 37°C and then added to Vero cell monolayers as described (27). Percent neutralized virus was calculated. Total transfection yields were assayed for the percentage of progeny expressing gD-1 by a slightly modified version (17) of a plaque immunoassay developed by Holland et al. (11).

RESULTS

Selection and characterization of insertion mutants expressing gD-1 and gD-2. The procedure for construction of the insertion mutants is detailed above and in the legend to Fig. 1. The 2.9-kb *Sac*I fragment containing the gD-1 coding sequence was inserted in both orientations into the TK gene of 1A1 virus by recombination occurring after cotransfection of cells with 1A1 viral DNA and pRB309 or pMG903. The percentage of progeny resistant to AraT was significantly higher from cells transfected with 1A1 DNA plus plasmids than with 1A1 viral DNA alone (Table 1). Data are also presented showing that, of 63 AraT^r plaque isolates obtained from transfections with plasmid and viral DNA, 62 (98%) were found to express gD-1 by immunoprecipitation analysis. Four isolates obtained from transfection with viral DNA alone were all gD-1$^-$. A total of 87% of 24 additional AraT^r isolates from other transfections (data not

TABLE 1. Analyses of progeny virus obtained from cells cotransfected with 1A1 viral DNA and plasmid DNAs

Transfection no.[a]	Plasmid[b]	Amt of DNA used		PFU[c]		No. of gD-1$^+$ isolates/no. of AraT^r mutants tested[d]
		Plasmid (ng)	1A1 (μg)	% AraT^r	% gD-1$^+$	
4	pRB309	50	8	22.3	20.5	20/20
7	pRB309	50	12	0.7	2.8	18/19
13	pRB309	50	8	3.1	ND[e]	12/12
3	pMG903	200	8	5.7	ND	12/12
20	None		12	0.057	0	0/4

[a] Plaque isolates mentioned in the text were obtained from these transfections. The first number in the designation of each AraT^r isolate denotes the transfection from which it was obtained.

[b] See Fig. 1 and the text for derivation of plasmids.

[c] Virus titrations were done on Vero cells in the absence and presence of AraT (100 μg/ml). Titers in the absence of AraT ranged from 20 × 10^6 to 8.8 × 10^7 PFU/ml. Yields of some transfections were screened for gD-1 expression by the in situ plaque immunoassay mentioned in the text and described elsewhere (11, 17).

[d] Virus isolated from plaques formed in the presence of AraT was used to infect Vero cells in a 24-well dish. Each culture was labeled with [^{35}S]methionine before immunoprecipitation with anti-gD-1.

[e] ND, Not done.

shown) also expressed gD-1. These findings are consistent with the expectation that gD-1 sequences were inserted into the TK gene, rendering the virus AraT^r, and that the gD-1 gene is expressed from the inserted gene. Immunoassay of two transfection yields in situ indicated that 20.5 and 2.8%, respectively, of progeny virus expressed gD-1. In the first case, this percentage was the same as the percentage of AraT^r progeny; in the second case, 10-fold more progeny expressed gD-1 than were AraT^r. This would indicate that marker rescue as well as marker insertion occurred to a variable extent during transfection, possibly due to variations in DNA concentrations or ratios.

Genomic analyses of AraT^r progeny were performed to determine whether the *Sac*I sequence containing the gD-1 gene was joined to the *Bam*HI Q sequence containing the TK gene. Immobilized *Bam*HI restriction fragments of DNAs of representative AraT^r progeny (Fig. 2) were hybridized with either ^{32}P-labeled pRB103 (TK probe) or pSKS309 (gD-1 probe). DNAs from all three AraT^r gD-1$^-$ isolates, 4-1, 3-3, and 7-13, have a novel 6.5-kb fragment homologous both to the TK probe and to the gD-1 probe. These isolates did not have the 3.6-kb *Bam*HI Q fragment characteristic of the parental 1A1 strain. All isolates contained a fragment of 4.6 kb which is derived from the HSV-2 DNA sequence in the S component of 1A1 and derivatives and which hybridizes to the gD-1 probe. These results indicate that most AraT^r isolates have an insertion of the gD-1 gene into the TK gene. One AraT^r isolate (7-17) identified as gD-1$^-$ did not have an insertion in the TK gene, indicating that it is a spontaneous TK$^-$ mutant.

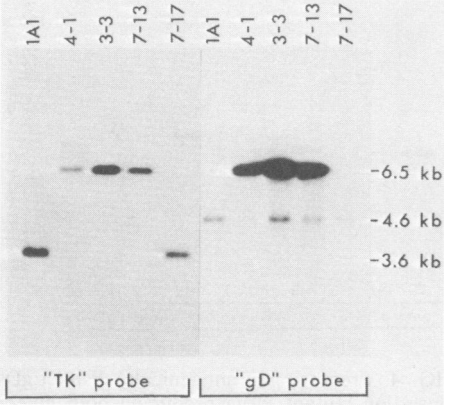

FIG. 2. Analysis of the genome structures of AraT^r progeny obtained from cells cotransfected with strain 1A1 DNA and either pRB309 or pMG903. *Bam*HI restriction fragments of genomic DNAs of the parental virus (1A1), three AraT^r gD-1$^+$ isolates (4-1, 3-3, 7-13), and one AraT^r gD-1$^-$ isolate (7-17) were separated on a 0.6% agarose gel by electrophoresis and transferred to nitrocellulose. Isolates 4-1 and 7-13 came from two different transfections with pRB309 DNA, whereas isolate 3-3 was from a transfection with pMG903 DNA. Fragments containing sequences homologous to pRB103 (TK probe) or pSKS309 (gD probe) were identified by hybridization with the indicated labeled probes.

This same genomic analysis was performed on 35 AraT^r gD-1$^+$ isolates before the second cycle of plaque purification. All of these isolates had an identical insertion of the *Sac*I fragment into the *Bam*HI Q fragment (data not shown). Of four AraT^r gD-1$^-$ isolates obtained from cells transfected with pRB309 and 1A1 DNA, none had insertions, nor did four other AraT^r gD-1$^-$ isolates obtained from transfection with 1A1 DNA alone (Table 1).

Sequential immunoprecipitation experiments (Fig. 3) of infected cell extracts showed that mutant viruses 4-1, 7-13, and 3-3 expressed the inserted gD-1 gene as well as retained the ability to express gD-2. Data are also presented in Fig. 3 showing that the parental virus 1A1, the AraT^r gD-1$^-$ isolate 7-17, and HSV-2(G) expressed only gD-2, whereas HSV-1(F), the strain from which the cloned gD-1 gene was obtained, expressed only gD-1. Apparent differences in the amounts of gD-1 and gD-2 precipitated may be due at least in part to differences in immunoprecipitating efficiencies of the antibodies used. When both antibodies were tested against samples of the same antigen preparation from HSV-1(F)-infected cells, anti-gD-1/2 precipitated less gD-1 than did anti-gD-1.

The inserted gD-1 gene was expressed regardless of its orientation within the TK gene, indicating that transcription did not require the viral TK promoter. The genes for gD-1 and TK are transcribed in the same direction in isolates 4-1 and 7-13, whereas transcription is in opposite directions in isolate 3-3 (Fig. 1).

All of the insertion mutants analyzed in this study replicated in HEp-2 cells to titers comparable to those obtained with the 1A1 strain, HSV-1(F), or HSV-2(G). There were no indications that replication of the mutants was in any way impaired by the insertion.

Processing of gD-1 and gD-2 by the insertion mutants. Pulse-chase experiments were performed to compare the immature and mature forms of gD-1 and gD-2 made by an insertion mutant with those made by other HSV strains (Fig. 4). Infected-cell extracts (pulse and pulse-chase) were sequentially and exhaustively precipitated, first with anti-gD-1 and then with anti-gD-1/2. The precursor and product forms of gD-1 produced by the insertion mutant 7-13 were indistinguishable by electrophoresis from those made by HSV-1(F). Similarly, the precursor and

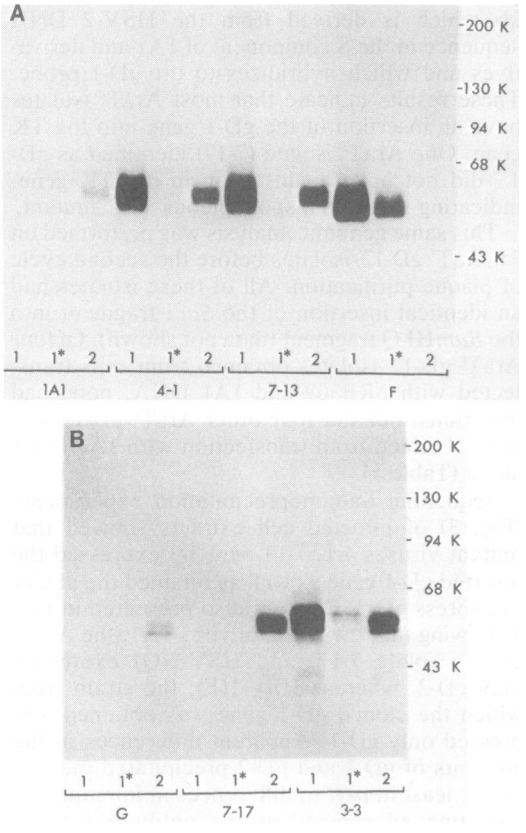

FIG. 3. Production of gD-1 and gD-2 by AraT^r insertion mutants. HEp-2 cells infected with plaque-purified individual isolates were labeled with [^{35}S]methionine from 4 to 22 h after infection, and the extracts obtained were sequentially and exhaustively immunoprecipitated, first with anti-gD-1 and then with anti-gD-1/2 (III255). The resulting precipitates were analyzed by polyacrylamide gel electrophoresis. This autoradiogram of the gel shows the first precipitates obtained with anti-gD-1 (lanes designated 1), the second precipitates obtained with anti-gD-1 (lanes designated 1*), and the third precipitates obtained with anti-gD-1/2 (lanes designated 2). Results obtained with the parental strain 1A1, HSV-1(F), and HSV-2(G) are shown for comparison with the results obtained with the AraT^r isolates 4-1, 3-3, 7-13, and 7-17 (previously described in the legend to Fig. 2). (A) 1A1, 4-1, 4-13, HSV-1(F); (B) HSV-2(G), 7-17, 3-3.

product forms of gD-2 expressed by strain 7-13 could not be distinguished from the gD-2 of 1A1 and HSV-2(G). As previously noted (3, 6, 23, 34), both forms of gD-2 have slightly faster electrophoretic mobilities than those of gD-1.

Effects of a DNA synthesis inhibitor on gD-1 and gD-2 accumulation. To determine whether the amounts of gD-1 and gD-2 made or accumulated by the insertion mutants depended on viral DNA replication, cells were infected and labeled in the presence of phosphonoacetic acid (PAA), a specific inhibitor of herpesvirus DNA replication (22, 26). Treatment of insertion mutant-infected cells (7-13, 3-3) with PAA significantly reduced the amounts of gD-1 and gD-2 that accumulated as compared to untreated infected cells (Fig. 5). The production of gD-1 by HSV-1(F) virus was also significantly inhibited by PAA, as was gD-2 production by HSV-2(G) virus. Similar findings were previously reported by Peake et al. (28).

The late glycoprotein gC, immunoprecipitable by ascites fluid II73, was undetectable in PAA-treated cells infected with insertion mutant 3-3. Extracts of untreated and PAA-treated infected cells are presented in Fig. 5 to show the differential effects of PAA on accumulation of various viral proteins.

Transport of gD-1 to the infected-cell surface. Lacto-peroxidase-catalyzed iodination of infected cells was performed to determine whether both gDs made by the insertion mutants were transported to the cell surface. Extracts of iodinated cells were sequentially immunoprecipitated as described. Analyses of these precipitates are presented in Fig. 6. Insertion mutants 4-1 and 7-13 displayed both gD-1 and gD-2 on the cell surface, whereas 7-17, an AraT^r gD-1$^-$ mutant

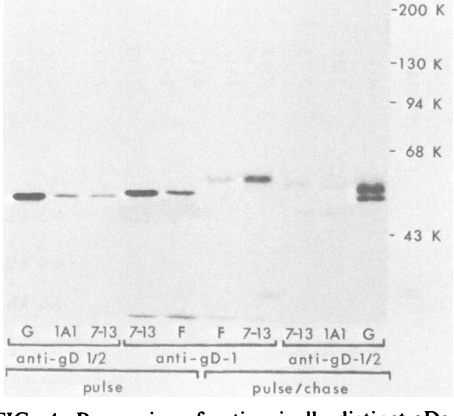

FIG. 4. Processing of antigenically distinct gDs by an insertion mutant virus producing both glycoproteins. HEp-2 cells infected with the mutant or with other viruses as indicated were labeled at 4.5 h after infection for 7 min with [^{35}S]methionine; extracts were prepared from the cells either immediately (pulse) or after 3 h of additional incubation in medium containing a fivefold excess of methionine (pulse-chase). For AraT^r insertion mutant 7-13, these extracts were sequentially precipitated with anti-gD-1 and then with anti-gD-1/2 (III114) as described in the legend to Fig. 3. Only the first and third precipitates are shown. For viruses expressing only one antigenic form of gD, the extracts were precipitated with anti-gD-1 in the case of HSV-1(F), or with anti-gD-1/2 (III114) in the cases of 1A1 and HSV-2(G).

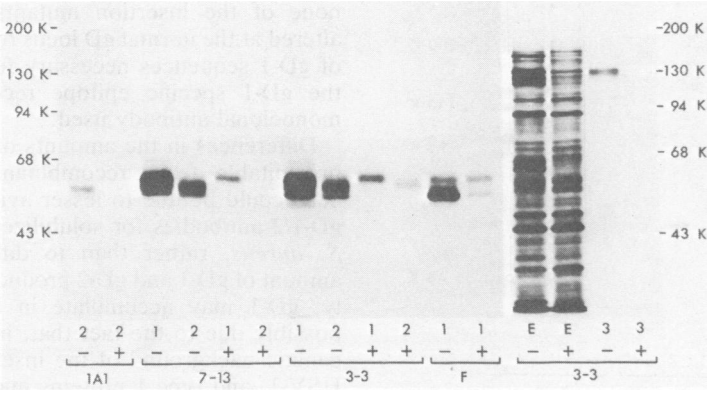

FIG. 5. Effects of PAA treatment on production of gD-1 and gD-2 by the insertion mutants 7-13 and 3-3 and by strains 1A1 and HSV-1(F). HEp-2 cell monolayers were pretreated with PAA (300 μg/ml) for 4 h before infection; this level of PAA was maintained throughout the infection and labeling period with [^{35}S]methionine (5 to 22 h postinfection) in treated cultures (+). Control cultures (−) were not exposed to PAA. Sequential immunoprecipitations as described in the legend to Fig. 3 were performed on extracts obtained from the cells, using first anti-gD-1 (lanes designated 1) and then anti-gD-1/2 (III114) (lanes designated 2). Extracts of control and treated cultures of insertion mutant 3-3 are also shown (E) along with immunoprecipitates obtained with anti-gC-1 (lanes designated 3).

with no insertion, displayed only gD-2. The viruses 1A1, HSV-1(F), and HSV-2(G) expressed either gD-1 or gD-2 only on infected-cell surfaces, as expected. Anti-gD-1 precipitated an additional iodinated protein of 125,000 daltons from viruses expressing gD-1. This high-molecular-weight material was not precipitable by anti-gB or anti-gC monoclonal antibodies (data not shown). It could be a dimeric form of gD-1, previously detected by Eisenberg et al. (7). Alternatively, the iodination procedure could have induced cross-linking of gD-1 to other cell surface components.

Incorporation of gD into the virion. [^{35}S]methionine-labeled virions of the insertion mutant 7-13 were purified on Dextran T10 gradients to determine whether both gDs were incorporated into the viral envelope. Electrophoretic analysis of immunoprecipitates (Fig. 7) demonstrated that both gD-1 and gD-2 were present in the purified virion preparations of the insertion mutant. The parental virus 1A1 incorporated only gD-2 into the virion, as expected.

Neutralization tests were done with selected insertion mutants to verify that gD-1 is on the virion surface and can serve as a target for immune recognition (Table 2). Viruses expressing both types of gD were neutralized by anti-gD-1 but not as efficiently as was HSV-1(F) virus, which expresses only gD-1. Although the extent of neutralization of insertion mutants and HSV-1(F) increased with increasing concentrations of anti-gD-1, it is unclear whether total neutralization might be achieved with this antibody under these conditions. Viruses expressing gD-1 alone, gD-2 alone, or both glycoproteins could be totally neutralized by anti-gD-1/2 at a dilution of 1/50.

DISCUSSION

We have isolated insertion mutants of HSV which contain two different forms of the gene for gD and express and utilize the products of both genes in a codominant fashion. These experi-

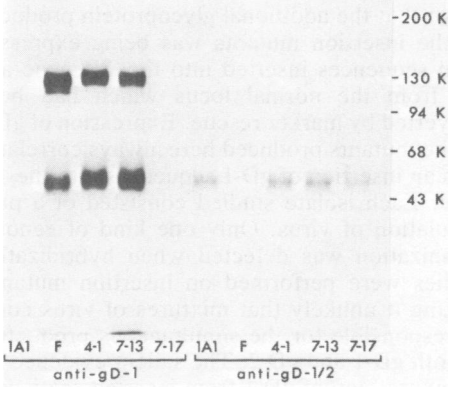

FIG. 6. Exposure of gD on the surfaces of cells infected with the parental virus 1A1, HSV-1(F), or the AraT^r isolates 4-1, 7-13, or 7-17. Iodination of infected cells was done as described in the text at 24 h after addition of virus. Extracts prepared from the cells were sequentially precipitated as described in the legend to Fig. 3. In this autoradiogram of the polyacrylamide gel only the first immunoprecipitates obtained with each monoclonal antibody are shown.

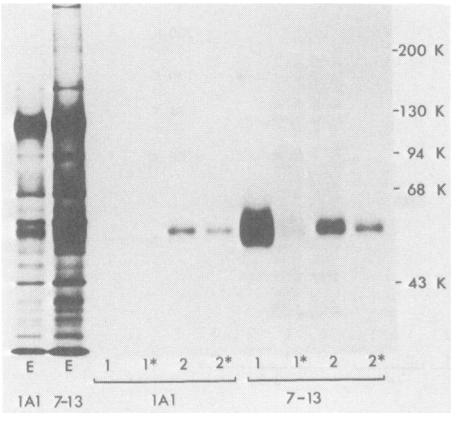

FIG. 7. Incorporation of gD-1 and gD-2 into virions produced by an insertion mutant. Extracts (E) prepared from [^{35}S]methionine-labeled parental (1A1) and recombinant (7-13) virions are shown. These extracts were sequentially immunoprecipitated, first with anti-gD-1 (lanes designated 1) and then again with anti-gD-1 (lanes designated 1*), followed by two successive precipitations with anti-gD-1/2 (III114, lanes designated 2 and 2*).

ments indicate that genetic information required in the vicinity of the gD-1 coding sequence for the expression and proper regulation of the gene is located within the *Sac*I fragment at map coordinates 0.906 through 0.924 on HSV-1 DNA and that this information is functional when inserted into the TK gene.

In previous studies from this laboratory, insertion mutants were produced in part to define the coding sequence for HSV-1 gC (17). It was pertinent in those studies as well as in these to prove that the additional glycoprotein produced by the insertion mutants was being expressed from sequences inserted into the TK gene and not from the normal locus which had been converted by marker rescue. Expression of gD-1 by the mutants produced here always correlated with an insertion of gD-1 sequences into the TK gene. Each isolate studied consisted of a pure population of virus. Only one kind of genome organization was detected when hybridization studies were performed on insertion mutants, making it unlikely that mixtures of virus could be responsible for the simultaneous production of both gD-1 and gD-2. The salient evidence for the expression of gD-1 from inserted sequences is that each clonally derived insertion mutant expressed two antigenically distinguishable forms of gD. After exhaustive immnoprecipitation and removal of gD-1 from lysates of infected cells, gD-2 could subsequently be immunoprecipitated. Consequently, although marker rescue was observed in some of our experiments (accounting for the AraT^s gD-1$^+$ progeny; Table 1), none of the insertion mutants analyzed was altered at the normal gD locus by the acquisition of gD-1 sequences necessary for expression of the gD-1 specific epitope recognized by the monoclonal antibody used.

Differences in the amounts of gD-1 and gD-2 precipitable from recombinant virus-infected cells could be due to lesser avidity of the anti-gD-1/2 antibodies for solubilized antigen or for *S. aureus*, rather than to differences in the amount of gD-1 and gD-2 produced. Alternatively, gD-1 may accumulate in larger amounts, possibly due to the fact that, in large part, the genetic background of the insertion mutants is HSV-1, and type 1 proteins may be selectively produced by these viruses or may selectively accumulate. Experiments different in design from those reported here must be done to quantitate the relative rates of synthesis and accumulation of gD-1 and gD-2 by the mutants.

Expression of a gD-1-related protein in bacterial cells was achieved by fusing part of the gD-1 coding sequence to the *cro* coding sequence of bacteriophage λ (44, 45). Expression of gD-1 protein upon injection of *Xenopus* oocytes with the *Bam*HI J fragment of HSV-1 DNA (map coordinates 0.89 to 0.93) has also been reported (43). Those results, coupled with nucleotide sequence analyses (44), have defined the coding sequence for gD-1 as well as determined that no additional viral functions coding outside of the *Bam*HI J fragment are necessary for expression of the gene in oocytes.

Our results extend these findings and set the boundaries for any *cis*-acting sequences adjacent to the gD-1 coding sequence that might be necessary for the appropriate expression and regulation of gD-1 in the infected cell. Apparent-

TABLE 2. Neutralization of infectivity of insertion mutants by monoclonal antibodies

Virus	gD expressed	PFU (% of control)[a]		
		Anti-gD-1 at concn:		Anti-gD-1/2 (1/50)
		1/50	1/500	
7-13	gD-1 + gD-2	23.2	35.6	<0.002
3-3	gD-1 + gD-2	25.3	32.3	<0.002
7-17	gD-2	86.4	106.0	<0.002
1A1	gD-2	81.0	89.8	<0.002
HSV-2(G)	gD-2	89.6	102.4	<0.003
HSV-1(F)	gD-1	6.8	11.3	<0.002

[a] Neutralization assays were performed as described in the text. The values given are derived from the numbers of PFU detected after treatment with antibody plus complement, expressed as a percentage of the PFU obtained in the presence of complement alone. Numbers of PFU in control cultures ranged from 368 to 556.

ly the *Sac*I fragment (coordinates 0.906–0.924), already known to contain the gD-1 coding sequence (16, 44), also contains any necessary promoter and regulatory signals for gD-1 expression. Several pieces of evidence support this conclusion. First, gD-1 is expressed by the insertion mutants in equivalent amounts regardless of orientation of the *Sac*I fragment within the TK gene. Second, the inserted gene (regardless of its orientation) is apparently expressed under the same kind of control as is the gD-2 gene at the normal locus. Experiments with PAA showed a significant decrease in the amounts of both gD-1 and gD-2 produced by insertion mutants as well as by conventional viruses in the presence of the drug, whereas expression of TK, an early or β product, is enhanced under these conditions (9, 12, 18). Therefore it seems likely that the 400-base-pair (bp) sequence upstream of the gD-1 mRNA initiation site (bounded on the left by a *Sac*I site; see Fig. 1) contains all promoter and regulatory signals necessary for expression of gD-1 under normal control in the infected cell. Mackem and Roizman (19–21) showed that regulatory signals influencing the expression of HSV α genes lie at least 110 to 140 bp upstream of the transcription initiation site and may even extend more than 330 bp upstream in some instances.

The 3′ terminus of gD-1 mRNA lies 200 bp 3′ to the *Sac*I site of the cloned gene (43, 44) and is therefore not contained in the sequences inserted into the TK gene. Transcription termination signals outside the *Sac*I fragment are presumably used for production of functional gD-1 mRNAs by the insertion mutants, as must be the case for the production of gC-1 mRNA by other insertion mutants (17). Thus there appears to be no strict requirement with respect to sequence of nucleotides in the 3′ noncoding region, at least for gD-1 and gC-1 mRNAs.

Several investigators have classified gD as an early or β polypeptide, based on its appearance by 2 h postinfection (1, 5) or its expression after a cycloheximide block or when DNA synthesis is inhibited (28), or based on quantitation of gD mRNA accumulated in the presence of cytosine arabinoside, an inhibitor of DNA synthesis (44). Our studies show that synthesis or accumulation of the gD polypeptide is significantly inhibited by PAA, a more effective inhibitor of viral DNA synthesis. Either gD is a late or γ polypeptide according to the original definitions proposed (synthesis inhibited by the inhibition of viral DNA replication; see reference 12), or translation of the gD mRNA (if actually made in significant quantities when viral DNA replication is completely blocked) to yield a stable product requires a factor made under γ control.

The findings that both copies of the gD gene are expressed in the insertion mutants under apparently normal controls for this gene, and that both glycoprotein products appear in the cell surface and in virions, make the experimental system described here especially attractive for certain kinds of studies of HSV gene expression. Mutated forms of a glycoprotein gene can be inserted into the TK gene to assess effects of the mutations on expression of the gene and on synthesis, posttranslational processing, intracellular transport, and incorporation into cell membranes and virions. The effects of lethal mutations can be studied because the product expressed from the normal genetic locus can supply all essential functions. The results presented here and earlier (17, 25, 32, 33, 37) show that HSV can be a cloning vector useful for the isolation and study of particular HSV regulatory sequences, genes, and their products in the environment of the HSV-infected cell. The virus may also be a suitable cloning vector for the study of certain non-HSV genes.

ACKNOWLEDGMENTS

Various aspects of this work were supported by grants from the National Cancer Institute (CA 21776 and CA 19264) and the American Cancer Society. M.G.G. was a trainee under Public Health Service grant 5 T32 CA 09241 from the National Institutes of Health and is now a fellow of the Leukemia Society of America.

LITERATURE CITED

1. **Balachandran, N., D. Harnish, W. E. Rawls, and S. Bacchetti.** 1982. Glycoproteins of herpes simplex virus type 2 as defined by monoclonal antibodies. J. Virol. **44**:344–355.
2. **Campione-Piccardo, J., W. E. Rawls, and S. Bacchetti.** 1979. Selective assay for herpes simplex viruses expressing thymidine kinase. J. Virol. **31**:281–287.
3. **Cassai, E. N., M. Sarmiento, and P. G. Spear.** 1975. Comparison of the virion proteins specified by herpes simplex virus types 1 and 2. J. Virol. **16**:1327–1331.
4. **Cohen, G. H., M. Katze, C. Hydrean-Stern, and R. J. Eisenberg.** 1978. Type-common CP-1 antigen of herpes simplex virus is associated with a 59,000-molecular-weight envelope glycoprotein. J. Virol. **27**:172–181.
5. **Cohen, G. H., D. Long, and R. J. Eisenberg.** 1980. Synthesis and processing of glycoproteins gD and gC of herpes simplex virus type 1. J. Virol. **36**:429–439.
6. **Eisenberg, R. J., M. Ponce de Leon, and G. H. Cohen.** 1980. Comparative structural analysis of glycoprotein gD of herpes simplex virus types 1 and 2. J. Virol. **35**:428–435.
7. **Eisenberg, R. J., M. Ponce de Leon, L. Pereira, D. Long, and G. H. Cohen.** 1982. Purification of glycoprotein gD of herpes simplex virus types 1 and 2 by use of monoclonal antibody. J. Virol. **41**:1099–1104.
8. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. J. Gen. Virol. **3**:357–364.
9. **Garfinkle, B., and B. McAuslan.** 1974. Regulation of herpes simplex virus induced thymidine kinase. Biochem. Biophys. Res. Commun. **58**:822–829.
10. **Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J. Virol. **14**:640–651.
11. **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.

12. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14**:8–19.
13. **Honess, R. W., and D. H. Watson.** 1974. Herpes simplex virus-specific polypeptides studied by polyacrylamide gel electrophoresis of immune precipitates. J. Gen. Virol. **22**:171–185.
14. **Kessler, S.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody absorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115**:1617–1624.
15. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:335–341.
16. **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.
17. **Lee, G. T.-Y., K. L. Pogue-Geile, L. Pereira, and P. G. Spear.** 1982. Expression of herpes simplex virus glycoprotein C from a DNA fragment inserted into the thymidine kinase gene of this virus. Proc. Natl. Acad. Sci. U.S.A. **79**:6612–6616.
18. **Leiden, J. M., R. Buttyan, and P. G. Spear.** 1976. Herpes simplex virus gene expression in transformed cells. I. Regulation of the viral thymidine kinase gene in transformed L cells by products of superinfecting virus. J. Virol. **20**:413–424.
19. **Mackem, S., and B. Roizman.** 1982. Differentiation between promoter and regulator regions of herpes simplex virus 1: the functional domains of a movable α regulator. Proc. Natl. Acad. Sci. U.S.A. **79**:4917–4721.
20. **Mackem, S., and B. Roizman.** 1982. Regulation of α genes of herpes simplex virus: the α 27 gene promoter-thymidine kinase chimera is positively regulated in converted L cells. J. Virol. 43:1015–1023.
21. **Mackem, S., and B. Roizman.** 1982. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. **44**:939–949.
22. **Mao, J. C.-H., E. E. Robishaw, and L. R. Overby.** 1975. Inhibition of DNA polymerase activity from herpes simplex virus-infected Wi-38 cells by phosphonoacetic acid. J. Virol. **15**:1281–1283.
23. **Marsden, H. S., N. D. Stow, V. G. Preston, M. C. Timbury, and N. M. Wilke.** 1978. Physical mapping of herpes simplex virus-induced polypeptides. J. Virol. **28**:624–642.
24. **McKnight, S. L.** 1980. The nucleotide sequence and transcript map of the herpes simplex virus type 1 thymidine kinase gene. Nucleic Acids Res. **8**:5949–5964.
25. **Mocarski, E. S., L. E. Post, and B. Roizman.** 1980. Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. Cell **22**:243–255.
26. **Overby, L. R., E. E. Robishaw, J. B. Schleicher, A. Reuter, N. L. Shipkowitz, and J. C.-H. Mao.** 1974. Inhibition of herpes simplex virus replication by phosphonoacetic acid. Antimicrob. Agents Chemother. **6**:360–365.
27. **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.
28. **Peake, M. L., P. Nystrom, and L. I. Pizer.** 1982. Herpesvirus glycoprotein synthesis and insertion into plasma membranes. J. Virol. **42**:678–690.
29. **Pereira, L., D. V. Dondero, D. Gallo, V. Devlin, and J. D. Woodie.** 1982. Serological analysis of herpes simplex virus types 1 and 2 with monoclonal antibodies. Infect. Immun. **35**:363–367.
30. **Pereira, L., T. Klassen, and J. R. Baringer.** 1980. Type-common and type-specific monoclonal antibody to herpes simplex virus type 1. Infect. Immun. **29**:724–732.
31. **Post, L. E., A. J. Conley, E. S. Mocarski, and B. Roizman.** 1980. Cloning of the reiterated and non-reiterated herpes simplex virus 1 sequences as BamHI fragments. Proc. Natl. Acad. Sci. U.S.A. **77**:4201–4205.
32. **Post, L. E., S. Mackem, and B. Roizman.** 1981. Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. Cell **24**:555–565.
33. **Post, L. E., and B. Roizman.** 1981. A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. Cell **25**:227–232.
34. **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**:667–697.
35. **Showalter, J. D., M. Zweig, and B. Hampar.** 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP4. Infect. Immun. **34**:684–692.
36. **Sim, C., and D. Watson.** 1973. The role of type specific and cross reacting structural antigens in the neutralization of herpes simplex virus types 1 and 2. J. Gen. Virol. **19**:217–233.
37. **Smiley, J. R., B. S. Fong, and W.-C. Leung.** 1981. Construction of a double-jointed herpes simplex virus DNA molecule: inverted repeats are required for segment inversion and direct repeats promote deletions. Virology **11**:345–362.
38. **Smith, J. F., and D. T. Brown.** 1977. Envelopment of Sindbis virus: synthesis and organization of proteins in cells infected with wild type and maturation-defective mutants. J. Virol. **22**:662–678.
39. **Spear, P. G.** 1980. Herpesviruses, p. 709–750. *In* H. A. Blough and J. M. Tiffany (ed.), Cell membranes and viral envelopes. Academic Press, London.
40. **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.
41. **Wagner, M. J., J. A. Sharp, and W. C. Summers.** 1981. Nucleotide sequence of the thymidine kinase gene of herpes simplex virus type 1. Proc. Natl. Acad. Sci. U.S.A. **78**:1441–1445.
42. **Watson, D. H., and P. Wildy.** 1969. The preparation of "monoprecipitin" antisera to herpes virus specific antigens. J. Gen. Virol. **4**:163–168.
43. **Watson, R. J., A. M. Colberg-Poley, C. J. Markus-Sekura, B. J. Carter, and L. W. Enquist.** 1983. Characterization of the herpes simplex virus type 1 glycoprotein D mRNA and expression of this protein in Xenopus oocytes. Nucleic Acids Res. **11**:1507–1522.
44. **Watson, R. J., J. H. Weis, J. S. Salstrom, and L. W. Enquist.** 1982. Herpes simplex virus type 1 glycoprotein D gene: nucleotide sequence and expression in Escherichia coli. Science **218**:381–384.
45. **Weis, J. H., L. W. Enquist, J. S. Salstrom, and R. J. Watson.** 1983. An immunologically active chimaeric protein containing herpes simplex virus type 1 glycoprotein D. Nature (London) **302**:72–74.
46. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **76**:1373–1376.