Expression and Regulation of Glycoprotein C Gene of Herpes Simplex Virus 1 Resident in a Clonal L-Cell Line

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Ltk⁻ cells were transfected with a plasmid containing the entire domain of glycoprotein C (gC), a true γ or γ_2 gene of herpes simplex virus 1 (HSV-1) and the methotrexate-resistant mouse dihydrofolate reductase mutant gene. The resulting methotrexate-resistant cell line was cloned; of the 39 clonal lines tested only 1, L3153$_{28}$, expressed gC after infection with HSV-1(MP), a gC⁻ mutant, and none expressed gC constitutively. The induction of gC was optimal at multiplicities ranging between 0.5 and 2 PFU per cell, and the quantities produced were equivalent to or higher than those made by methotrexate-resistant gC⁻ L cells infected with wild-type (gC⁺) virus. The gC gene resident in the L3153$_{28}$ cells was regulated as a β gene inasmuch as (i) the amounts of gC made in infected L3153$_{28}$ cells exposed to concentrations of phosphonoacetate that inhibited viral DNA synthesis were higher than those made in the absence of the drug, (ii) gC was induced at both permissive and nonpermissive temperatures by the DNA⁻ mutant tsHA1 carrying a lesion in the gene specifying the major DNA-binding protein and which does not express γ_2 genes at the nonpermissive temperature, and (iii) gC was induced only at the permissive temperature in cells infected with ts502 containing a mutation in the α4 gene. The gC induced in L3153$_{28}$ cells was made earlier and processed faster to the mature form than that induced in a gC⁻ clone of methotrexate-resistant cells infected with wild-type virus. Unlike virus stocks made in gC⁻ cells, HSV-1(MP) made in L3153$_{28}$ cells was susceptible to neutralization by anti-gC monoclonal antibody.

In this paper we report on the properties of a cell line carrying an inducible gene specifying glycoprotein C (gC) of herpes simplex virus 1 (HSV-1). Relevant to this report are the following.

The HSV-1 genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (14, 15). The α genes are expressed first (14, 15, 30). Functional α gene products are required to express β genes whose known products are involved in nucleotide metabolism and viral DNA synthesis (4, 6, 14, 15, 20, 24, 30, 41). β gene products are required to effect the expression of γ genes. The γ genes, mostly structural proteins of the virus, are inhomogeneous. Whereas the expression of the subset designated as β_γ, or γ_1 (40), is reduced but not abolished in the absence of viral DNA synthesis, the true γ, or γ_2, genes stringently require viral DNA synthesis for their expression (4, 12, 17).

The HSV-1 genes are transcribed in the nucleus by RNA polymerase II (5) probably in association with host and viral factors. To investigate the mechanism by which the transcriptional machinery of the infected cell differentiates among α, β, and γ genes, Post et al. (28) constructed chimeric genes consisting of the transcription initiation site and upstream sequences of α genes fused to the 5' transcribed noncoding and coding sequences of the thymidine kinase gene. These chimeric genes behaved as authentic α genes when introduced into the viral genome, and the fundamental properties of the α gene regulation were retained when the chimeric genes were stably integrated into

the cellular genome. An entirely different situation arose with chimeric genes consisting of the transcription initiation site and upstream sequences of a γ_2 gene fused to the 5' transcribed noncoding and coding sequences of the thymidine kinase gene. In this instance, the chimeric gene was regulated as an authentic γ_2 gene when recombined into the viral genome and as a β gene when resident in the environment of the host genome (35).

One possible explanation of these results is that determinants of viral gene regulation may also be contained within the transcribed coding and noncoding domains of the thymidine kinase, a β gene, notwithstanding the observation that in the environment of the viral genome the chimeric gene was regulated as an authentic viral γ_2 gene. To test this hypothesis, we constructed a recombinant plasmid containing a selectable maker and the entire domain of the gC gene of HSV-1. In this paper we report the selection and properties of the cell line and the regulation of the resident gC gene.

gC is one of the major glycoproteins specified by HSV-1 (38, 39). Originally designated as virion protein no. 7.5, it is the only viral glycoprotein currently known not to be essential for infectivity and virus growth (10, 39). The earliest-characterized mutant incapable of expressing gC is a spontaneous mutant designated as HSV-1(MP) (11). This mutant produces no detectable gC in infected cells, and none is found in purified virions (9, 18, 38). Furthermore, in contrast to wild-type virus containing gC, HSV-1(MP) is not neutralized by antiserum to gC. We selected gC for these studies because (i) the gene was mapped (31) and sequenced (8), and therefore its entire domain is known, (ii) because it is regulated as a γ_2 gene, and (iii) because a well-characterized gC⁻ mutant, HSV-1(MP), is available for study of induction
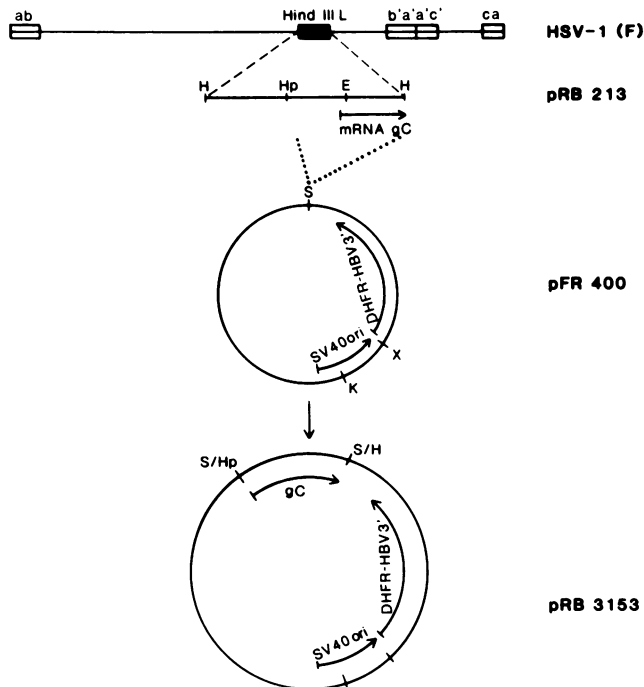
FIG. 1. Construction of plasmid pRB3153. The top line shows the sequence arrangement of the HSV-1 genome and the location of the gC gene. The HpaI-HindIII subfragment of the HindIII L fragment shown in the second line was cloned into the SalI site of pFR400. The transcription of the gC gene is in opposite orientation relative to that of the dhfr gene as shown by arrows. Abbreviations: E, EcoRI; H, HindIII; Hp, HpaI; K, KpnI; S, SalI; X, XbaI.

of the gC gene resident in the environment of the cellular genome.

MATERIALS AND METHODS

Cells and viruses. The Ltk⁻ cell line (19) was obtained from S. Kit and maintained in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal bovine serum. The L3153$_0$ cells and their clonal derivatives were grown in the same medium but containing 440 nM methotrexate.

The properties of the wild-type viruses HSV-1(F) and HSV-2(G) (7) and the spontaneous syn⁻ mutant HSV-1(MP) (11) have been previously described. The stock viruses were titrated in Vero cells.

Plasmids. Plasmid pRB132 carries the BamHI C fragment of HSV-1(F) cloned into the BamHI site of pBR322. BamHI-C contains the entire HindIII O fragment. Plasmid pFR400 was obtained from C. Simonsen (Genentech Inc.); it contains the simian virus 40 origin of replication and the early and late promoters and the methotrexate-resistant mouse dhfr gene (36). Plasmid pRB213 contains the HindIII L fragment of HSV-1(F) cloned into the HindIII site of pACYC177. Plasmids were propagated in Escherichia coli C600 cells, and DNA has prepared by the cleared lysate method as previously described (27).

Construction of plasmid pRB3153. Plasmid pRB213 was digested with restriction endonucleases HindIII and HpaI. The 5,000-base-pair fragment containing the HSV-1 gC gene was isolated from agarose gel slices and cloned into the SalI site of pFR400. The resulting plasmid was designated as

pRB3153. The transcriptional orientation of the gC gene in pRB3153 is opposite to that of the mouse dhfr gene (Fig. 1).

Transfection and selection of methotrexate-resistant cell lines. Monolayers of Ltk⁻ cells at 60 to 80% confluency were transfected with 2.5, 5, or 10 μg of closed circular plasmid DNA by the calcium phosphate precipitation method (42). After 24 h the medium was replaced with medium containing 110 nM methotrexate, and after 3 days the medium was replaced with medium containing 220 nM methotrexate. The foci of transformed cells were pooled and exposed to increasing concentrations of methotrexate (from 220 to 440 nM) in the course of six cell passages. The uncloned culture derived from the trasfected cultures was designated as the L3153$_0$ cell line. After the preliminary tests for gC expression described in the text below, the cells were cloned out by serial dilution, and 39 clones were isolated. All clones were maintained in 440 nM methotrexate.

Infection and radiolabeling of the clonal cell lines. Because the expression of the gC gene resident in L3153 cells was highly sensitive to the multiplicity of infection, the virus stocks were titrated in Vero cells as the reference standard. In most experiments reported here, the cells were exposed for 90 min to an inoculum yielding multiplicities of 0.25 to 3 PFU per cell as indicated in the text, and the time of exposure of cells to virus is the zero hour. Except as stated in the legends to the figures, the cells were labeled with [³⁵S]methionine (New England Nuclear Corp., Boston, Mass.; specific activity, 1,100 Ci/mmol; 25 μCi/ml of medium containing 1/10 the normal concentration of unlabeled methionine) from the end of virus adsorption to the time the cells were harvested.

Assays for the induction of gC. The initial screening of the L3153$_0$ cells and their clonal derivatives was done by a modification of the method of Holland et al. (13). Briefly, cells in a 24-well dish (Falcon; Becton Dickinson Labware, Oxnard, Calif.) were mock infected or infected with 2 to 5 PFU of HSV-1(MP) per cell. After 12 to 15 h the cells were incubated for 60 min in medium containing 20% horse serum, rinsed with serum-free medium, fixed for 15 min with methanol at 4°C (except where stated otherwise), rinsed three times, and incubated with mouse monoclonal antibody HC1 to gC (kindly provided by L. Pereira [25]) diluted 1:20 for 1 h at 37°C. Staining was done with the avidin-biotin amplified immunoperoxidase kit from Vector Laboratories, Burlingame, Calif., as detailed previiously (21). In the absence of gC cell monolayers remained unstained, whereas cells expressing gC appeared stained. This testing procedure is suitable for rapid screening, but it may not detect low levels of gC expression. The definitive characterization of the clones was done by testing lysates of cell clones, both uninfected and infected with either HSV-1(MP) or HSV-2(G), in immunoprecipitation tests with monoclonal antibody HC1. Immunoprecipitations were performed as previously described (33). Briefly, the cells were extracted for 10 min at 4°C in solution A containing 1% Nonidet P-40, 1% sodium deoxycholate in phosphate-buffered saline lacking Mg²⁺ and Ca²⁺ ions, and 10⁻⁵ M each of tolylsulfonyl phenylalanyl chloromethyl ketone and α-tosyl-L-lysine chloromethylketone (Sigma Chemical Co., St. Louis, Mo.). After centrifugation at 100,000 × g for 40 min the supernatant fluids were preadsorbed with 5 mg of protein A-Sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden) and then mixed with 2 to 4 μl of HC1 monoclonal antibodies for 2 h at 4°C. The immunocomplexes were adsorbed for 1 h at 4°C on protein A-Sepharose beads coated with rabbit anti-mouse immunoglobulin G (Miles Laborato-

ries, Naperville, Ill.). The beads were then washed four times with solution A and once in 50 mM Tris hydrochloride (pH 7.4) plus 150 mM NaCl. Immunoprecipitates were eluted by boiling for 3 min in disruption buffer (2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 50 mM Tris hydrochloride [pH 7]).

Presence of gC on the surfaces of L3153 cells infected with HSV-1(MP). Infected and mock-infected cells were reacted unfixed with the monoclonal antibody HC1 and assayed by the biotin-avidin enhanced-surface immunoassay as described previously (21).

Polyacrylamide gel electrophoresis and autoradiography. Samples denatured in disruption buffer were subjected to electrophoresis in 8.5% acrylamide gels cross-linked with N-N' diallyltartardiamide as previously described (24). The molecular weight markers (Radiochemical Centre, Amersham, England) contained myosin, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme with apparent molecular weights of 200,000, 100,000 to 92,000, 69,000, 46,000, 32,000, and 14,300, respectively. Gels were fixed in 10% acetic acid and 20% isopropanol and soaked for 15 min in Amplify (Radiochemical Centre). Dried gels were exposed to Kodak X-Omat films for fluorography.

Reconstruction of gene copy number. DNA was isolated from cells by lysis in 0.5% Nonidet P-40 in 10 mM Tris (pH 7.6) plus 1 mM EDTA and subsequent treatment with 0.1 mg of RNase A (Sigma) per ml, followed by digestion with 0.5 mg of proteinase K (Sigma) per ml in 0.5% sodium dodecyl sulfate for 2 h at 37°C. The samples were then extracted twice with phenol equilibrated in 10 mM Tris (pH 7.6) plus 1 mM EDTA, followed by three extractions with water-saturated ether to remove residual phenol. The DNA was ethanol precipitated and quantitated spectrophotometrically at 260 nm. For copy-number determination, 10 μg of cell DNA was digested with restriction endonuclease HindIII. Reconstructions were done by mixing 10 μg of untransformed Ltk⁻ DNA with appropriate amounts of pRB3153 or pRB132 DNAs and subsequently digesting with HindIII. The digested DNAs were separated on 0.9% agarose gels and transferred to nitrocellulose sheets by the procedure of Southern (37). The DNA probes, labeled by nick translation with [^{32}P-α]dCTP were a 1.5-kilobase-pair HindIII fragment from pFR400 containing the dhfr gene and the 3.0-kilobase-pair HindIII O fragment of HSV-1(F) from pRB132. Approximately 1 × 10⁷ cpm (Cerenkov) of the dhfr probe and 2 × 10⁷ cpm (Cerenkov) of the HindIII-O probe were used in the hybridization reaction. The specific activities of the two probes were approximately 2 × 10⁷ cpm/μg of DNA (Cerenkov). The conditions for hybridization were as previously described (27).

Neutralization test. L3153$_{28}$ and L3153$_{34}$ cells were infected with HSV-1(MP) at a multiplicity of 1 PFU per cell. At the end of virus adsorption the monolayers were washed three times with medium containing pooled human gamma globulins to neutralize unadsorbed virus and three times with medium without gamma globulins, overlaid with the same medium, and frozen either immediately or after incubation for 24 h at 37°C. The ratio of infectious virus contained in cells harvested after virus adsorption and virus yield at 24 h was about 1:1,000. Approximately 80 PFU of each progeny virus in 0.2 ml was reacted with 20 μl of monoclonal antibody HC1 or phosphate-buffered saline at room temperature for 2 h and then plated on Vero cell monolayers. After virus adsorption, the inoculum was removed, and the monolayers were rinsed three times with medium containing

pooled human gamma globulins and incubated in the same medium. Flaques were scored 48 h after infection.

RESULTS

Selection of clonal line L3153$_{28}$. As described in Materials and Methods, Ltk⁻ cells were transfected with the pRB3153 plasmid DNA containing the HSV-1(F) gC gene and the methotrexate-resistant dhfr gene described elsewhere (36). The progeny of the transfected cells resistant to methotrexate, designated as cell line L3153$_0$, was tested for constitutive and induced expression of gC. gC was not detected in lysates of uninfected cells, and only trace amounts of gC were observed in cells infected with the mutant HSV-1(MP), which does not express gC, or with HSV-2(G) (data not shown). To determine whether the low level of gC induced by HSV-1(MP) reflected heterogeneity in the cell population, 39 clonal lines were established from the parental L3153$_0$ cells. Each of the clonal lines was tested for constitutive and induced expression of gC as detailed in Materials and Methods. As described below, only one of the clonal lines, designated as L3153$_{28}$, expressed gC after infection with HSV-1(MP) but not constitutively. Inasmuch as this cell line is methotrexate resistant and may not be directly comparable with the parental L cells, for the comparative studies described below we arbitrarily selected another methotrexate-resistant clonal cell line (L3153$_{34}$) that did not express gC under any of the conditions tested.

Properties of the cell line L3153$_{28}$. Because of differences in growth rate, not all of the clonal lines were tested at the same time. Fig. 2 illustrates the results of one of a series of experiments. The autoradiographic image of the material immune precipitated with monoclonal antibody HC1 from lysates of a series of clonal cell lines labeled with [^{35}S]methionine from 1.5 to 14 h post-mock-infection or post-infection with HSV-1(MP) indicated that only the clonal cell line L3153$_{28}$ produced gC after infection with HSV-1(MP). None of the clones tested produced gC constitutively. Clonal lines L3153$_{28}$ and L3153$_{34}$ have been passaged at least 30 times in methotrexate-containing media without change in the observed pattern of expression of gC.

Requirements and temporal pattern of induction of gC in L3153$_{28}$ cells. Studies on the requirements for the induction of gC in L3153$_{28}$ cells revealed the following.

(i) Induction of gC was optimal in cells infected with HSV-1(MP) at relatively low multiplicities of infection (0.5 to 2 PFU per cell) and diminished at higher multiplicities. gC could not be detected in cultures infected at 10 PFU per cell (Fig. 3).

(ii) gC was detected in lysates of L3153$_{28}$ cells labeled with [^{35}S]methionine from 1.5 to 3.5 h postexposure to 2 PFU of HSV-1(MP) per cell. The quantities of labeled gC were highest in extracts of cells labeled from 11.5 to 13.5 h postinfection and were drastically diminished in extracts of cells labeled from 16.5 to 18.5 h postinfection. In contrast, gC was not detected in extracts of L3153$_{34}$ cells infected with HSV-1(F) at the same multiplicity and labeled from 3 to 5 h postinfection. Moreover, significant quantities of gC were labeled between 16.5 to 18.5 h postinfection (Fig. 4). The accelerated appearance of gC directed by the gene resident in the cellular genomic environment relative to that directed by the gene resident in the viral genome was reproducible from one experiment to another.

Effect of PAA on induction of gC in L3153$_{28}$ cells. Previous studies have shown that γ₂ genes resident in cellular genomes can be induced in the presence of phosphonoacetate (PAA) at concentrations that inhibit viral DNA
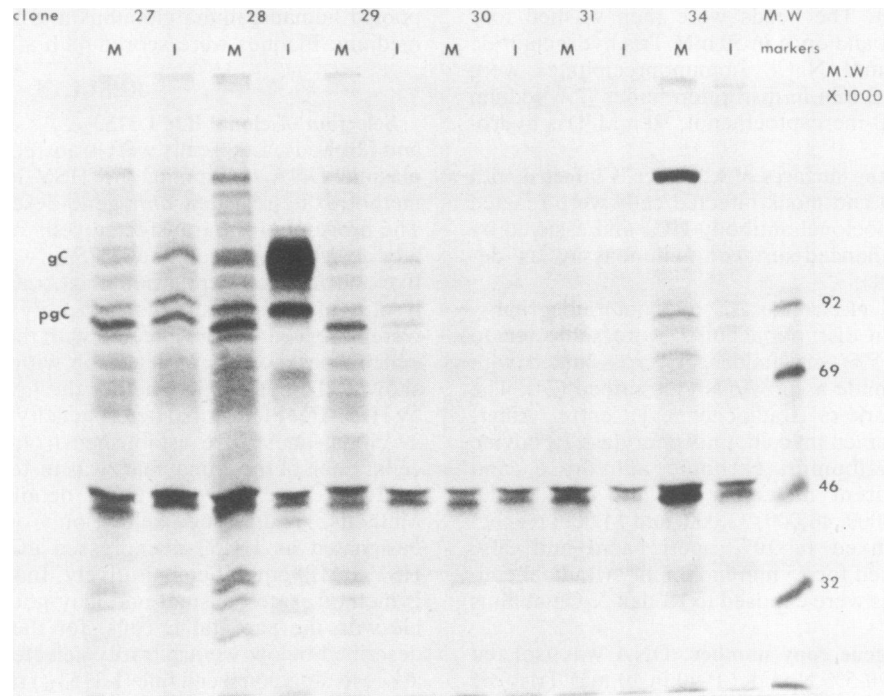
FIG. 2. Autoradiographic image of immune precipitates electrophoretically separated on polyacrylamide gels. The immune precipitates were obtained by mixing monoclonal antibody HC1 with lysates of L3153 clones 27 to 34 derived from the $L3153_0$ cell line. The L-cell lines were mock infected (M) or infected with HSV-1(MP) (I) and labeled with [^{35}S]methionine from 1.5 to 14 h postexposure of cells of virus. The precursor and mature forms of gC are indicated as pgC and gC, respectively. The right lane contains labeled molecular weight (M. W.) markers in thousands ($\times$ 1000).

synthesis even though the drug inhibits the expression of the corresponding genes contained in the genome of the infecting virus (35). Inasmuch as the gC gene resident in the viral genome is a γ_2 gene sensitive to PAA, it was of interest to determine the PAA sensitivity of the induction of the gC gene resident in $L3153_{28}$ cells by HSV-1(MP). The expression of gC was higher in infected cells exposed to PAA (300 µg/ml) than in untreated infected cells (Fig. 5). Moreover, in treated cells, the quantities of gC labeled between 15.5 and
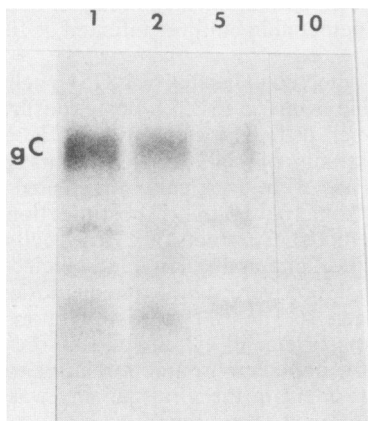

FIG. 3. Autoradiographic images of electrophoretically separated gC precipitated by HC1 from $L3153_{28}$ cells infected with HSV-1(MP) at the indicated multiplicities of infection (in PFU per cell).

17.0 h postexposure to virus was higher than in untreated infected cultures.

Induction of gC resident in $L3153_{28}$ cells by temperature-sensitive mutants. Failure of PAA to inhibit the induction of gC by HSV-1(MP) suggested that the gC gene resident in the $L3153_{28}$ clonal cell line is not regulated as a γ_2 gene. To explore this observation further, we examined the induction of gC in $L3153_{28}$ cells infected with two temperature-sensitive mutants, tsHA1 (4) and ts502 (28). The temperature-sensitive lesion in ts502 is in the $\alpha4$ gene, and at the nonpermissive temperature (39°C) the transition from α to viral gene groups expressed later in infection does not ensue. The tsHA1 mutant carries a temperature-sensitive lesion in the gene specifying the major DNA-binding protein ICP8 (4), and at the nonpermissive temperature (39°C), DNA synthesis is blocked and the transition from β and γ_1 to γ_2 genes does not ensue.

In this series of experiments replicate cultures of $L3153_{28}$ and $L3153_{34}$ cells were infected with ts502 at 0.5 and 2 PFU per cell and tsHA1 at 0.25, 0.75, and 2 PFU per cell and incubated at 33 or 39°C. The cells were labeled with [^{35}S]methionine from 1.5 h to the time of harvest at 10 h postinfection. The autoradiographic images of the gC precipitated with monoclonal antibody HC1 and electrophoretically separated in polyacrylamide gels are shown in Fig. 6 and 7. The experimental design chosen in these studies was based on the observation that at the multiplicities of infection effective for the induction of the gC gene resident in $L3153_{28}$ cells, little or no gC specified by the gene resident in the viral genome was detected by 10 h postinfection.

The quantities of gC induced by ts502 in $L3153_{28}$ cells at the permissive temperature was far in excess of the amounts induced in $L3153_{28}$ at nonpermissive temperature or in

L3153$_{34}$ cells at either temperature (Fig. 6). With tsHA1, gC was induced in L3153$_{28}$ cells at both permissive and nonpermissive temperatures, and the quantities of gC induced in these cells were greater that those induced in L3153$_{34}$ cells (Fig. 7). These results indicated that gC requires a functional α4 gene product but that neither DNA synthesis nor functional ICP8 was required for the induction of gC in L3153$_{28}$ cells.

Effect of HSV-1(MP) infection on the copy number of the recombinant plasmid resident in L3153$_{28}$ cells. Because the quantities of gC produced in L3153$_{23}$ cells infected with HSV-1(MP) were at least as high as those observed in L3153$_{34}$ cells infected with HSV-1(F), it was of interest to determine whether the copy number of the resident plasmid contained in the L3153$_{28}$ cells changed significantly during infection with HSV-1(MP). In this series of experiments, L3153$_{28}$ cells were infected with HSV-1(MP) at multiplicities of 1, 3, or 10 PFU per cell and maintained in the presence and absence of PAA. The cells were harvested at 3 and 10 h postinfection, and the DNA was extracted, digested with HindIII, electrophoretically separated in an agarose gel, transferred to a nitrocellulose sheet and hybridized with two ^{32}P-labeled probes. Because the gC gene resident in the cellular genome could not be differentiated by hybridization from the silent gC gene in HSV-1(MP), a DNA fragment containing largely the dhfr gene was used as a probe to measure the copy number of the recombinant plasmid resident in the cellular genome. The HindIII O fragment of
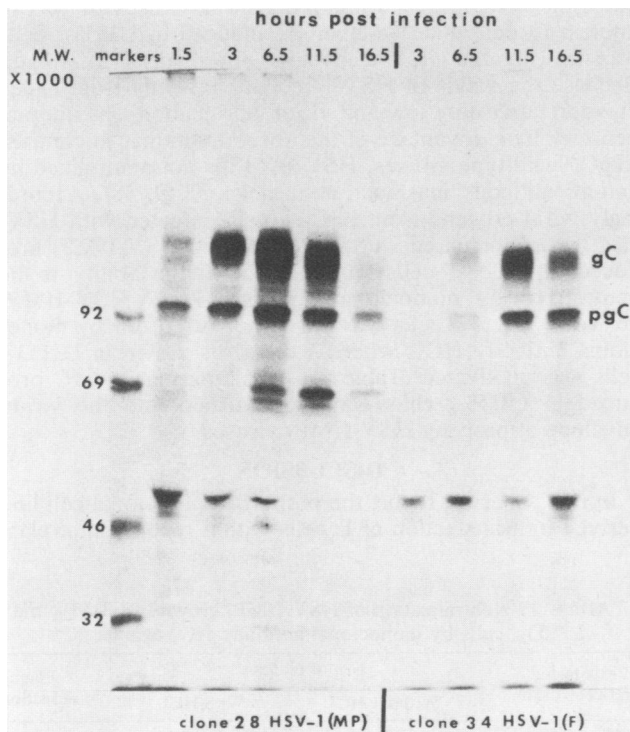

FIG. 4. Autoradiographic images of electrophoretically separated gC precipitated by HCl from L3153$_{28}$ and L3153$_{34}$ cell lines infected with HSV-1(MP) and HSV-1(F), respectively, under identical conditions and labeled with [^{35}S]methionine for 2 h, beginning at the times indicated in the figure. The cells were harvested and lysed for immune precipitation at the end of the labeling interval. Molecular weight (M. W.) markers are shown in thousands (× 1000) at the left.
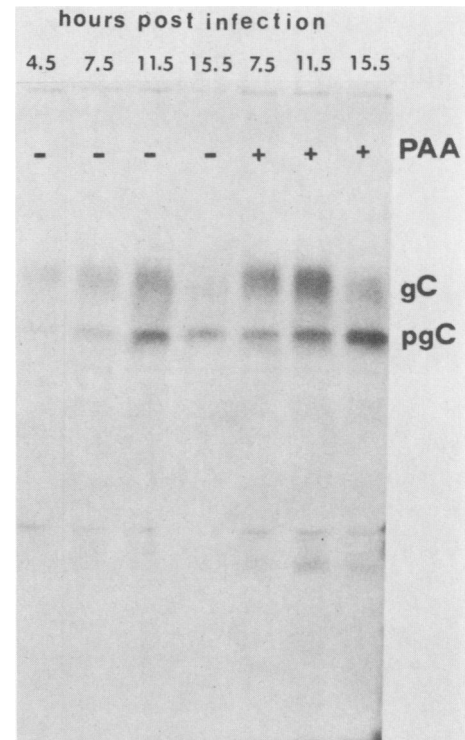

FIG. 5. Autoradiographic images of electrophoretically separated gC precipitated by HCl from lysates of replicate cultures of L3153$_{28}$ cells infected with HSV-1(MP) and maintained in the presence (+) or absence (−) of PAA. The cells were labeled with [^{35}S]methionine for 90 min, beginning at various times postexposure of cells to virus as shown in the figure, and harvested for immune precipitation immediately after the labeling interval.

HSV-1(F) was used as a probe to verify the effect of PAA on the superinfecting virus. The dhfr gene was present in approximately 100 copies per cell, and the copy number did not increase between 3 and 10 h postexposure of cells to HSV-1(MP) or with increasing multiplicities of infection (Fig. 8). Furthermore, the dhfr copy number was not affected by PAA. In contrast, in the presence of PAA the copy number of the HindIII-O sequences at 10 h postinfection was identical to that observed at 3 h postinfection and considerably lower than that seen at 10 h postinfection in DNA extracted from untreated infected cells. It should be noted that in HSV-1(MP) the HindIII cleavage site between the HindIII O and I fragments is absent; this explains the higher mobility of the viral DNA band containing sequences homologous to the HindIII-O DNA probe.

Processing of gC induced in L3153$_{28}$ cells infected with HSV-1(MP). Two series of experiments were done to characterize the products of the gC gene resident in L3153$_{28}$ cells. In the first, replicate cultures of L3153$_{28}$ cells were pulse-labeled with [^{35}S]methionine for 20 min at 8 h postinfection with HSV-1(MP). One culture was harvested immediately after the pulse, whereas the others were incubated further in nonradioactive medium containing unlabeled methionine for 30, 60, 90, 120, and 150 min (chase). An identical experiment was carried out concurrently with L3153$_{34}$ cells infected with HSV-1(F). Comparisons of the immune precipitates shown in Fig. 9 indicate that the lysates of cells harvested after a pulse contained a lower-molecular-weight precursor that was replaced during the chase by a higher-molecular-weight band. Two apparent differences were
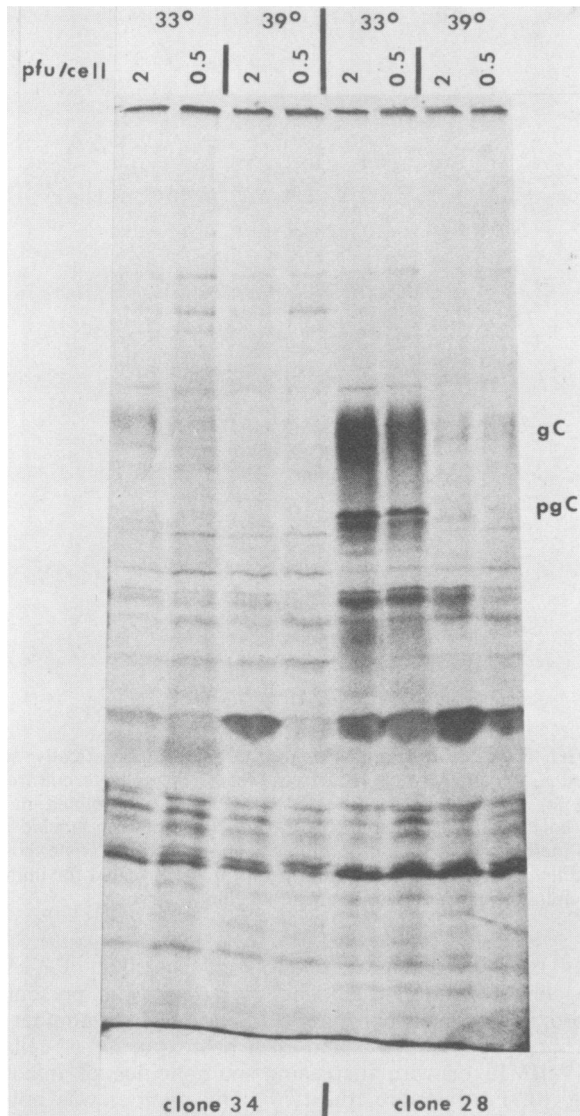
FIG. 6. Autoradiographic images of immune precipitates obtained with HC1 from lysates of cells infected at the permissive (33°C) and nonpermissive (39°C) temperatures with $ts502$. Replicate cultures of $L3153_{28}$ and $L3153_{34}$ cells were infected with $ts502$ at the indicated multiplicities of infection and incubation at 33 or 39°C. The cells were labeled from 1.5 to 10 h postexposure of cells to virus and harvested at the end of the labeling interval.

noted. First, the gC made in $L3153_{28}$ cells was processed more rapidly inasmuch as the precursor band was barely detectable in lysates of $L3153_{28}$ cells 60 min after the chase but was prominent in HSV-1(F)-infected lysates of $L3153_{34}$ cells at that time. In addition, the electrophoretic mobility of the gC produced in the $L3153_{28}$ cells was slightly lower than that of gC made in $L3153_{34}$ cells.

To compare the products of the viral and cellular gC genes copies more directly, we compared the electrophoretic mobility in polyacrylamide gels of gC precipitated from lysates of $L3153_{28}$ cells infected with HSV-1(MP) with that of the mature form of gC purified by affinity chromatography on monoclonal antibody HC1 linked to Sepharose from lysates of BHK cells infected with HSV-1(F). The apparent molecular weights of precursor and mature gC expressed from

HSV-1(MP)-infected $L3153_{28}$ cells were 98,000 and 120,000, respectively (Fig. 10). The latter value was slightly higher than that of gC purified from lysates of BHK cells infected with HSV-1(F) (118,000). The results of these experiments suggest that the product of the gC gene resident in $L3153_{28}$ cells undergoes posttranslational modifications and is glycosylated in a fashion similar to that of the gC product of HSV-1(F) in $L3153_{34}$ cells. The slight differences in the apparent molecular weight of gC observed in the above experiments may reflect differences in the glycosyl transferases contained in the three cell lines. This conclusion rests on experiments with mutant cells defective in specific glycosyl transferases showing host-dependent variations in the electrophoretic mobility of HSV-1 glycoproteins and in their oligosaccharide composition (2, 3, 32).

Transport of gC to the surface of $L3153_{28}$ cells infected with HSV-1(MP). In light of the observation that gC made in $L3153_{28}$ cells infected with HSV-1(MP) is processed to a form comigrating with the mature form of gC, it was of interest to determine whether gC in these cells is transported to the plasma membranes. In these studies, $L3153_{28}$ and $L3153_{34}$ cells were infected with HSV-1(MP) at a multiplicity of 3 PFU per cell and tested by the biotin-avidin enhanced surface immunoassay with HC1 monoclonal antibody (13, 21). The procedure stained infected $L3153_{28}$ cells but not the infected $L3153_{34}$ cells, suggesting that the gC produced in $L3153_{28}$ cells is transported to the cell surface as in normal infection.

Neutralization of HSV-1(MP) grown in $L3153_{28}$ cells by monoclonal antibody to gC. Inasmuch as gC is a component of virion envelopes of wild-type HSV-1 viruses, it was of interest to determine whether gC induced in $L3153_{28}$ cells was incorporated into the envelope of progeny HSV-1(MP). Because the yields of HSV infectious progeny from L-cell line derivatives are low and virion purification was impractical, we took advantage of the observation that in contrast to gC$^+$ wild-type viruses, HSV-1(MP) is not neutralized by anti-gC antibody inasmuch as it lacks gC (9, 38). Accordingly, viral progeny from $L3153_{28}$ cells infected with HSV-1(MP) was compared with HSV-1(F) and HSV-1(MP) produced in $L3153_{34}$ cells with respect to the ability to be neutralized by monoclonal antibody HC1. HSV-1(MP) grown in $L3153_{28}$ cells was partially neutralized by monoclonal antibody HC1, whereas the virus grown in $L3153_{34}$ cells was unaffected (Table 1), indicating that the gC produced in $L3153_{28}$ cells was incorporated into the virion envelope of progeny HSV-1(MP) viruses.

DISCUSSION

In this paper we report the properties of a clonal cell line derived by transfection of L cells with a recombinant plas-

TABLE 1. Neutralization of HSV-1(MP) grown in $L3153_{28}$ and $L3153_{34}$ cells by monoclonal antibody HC1 against gC[a]

Virus tested and cells used	No. of plaques[b]		% Plaque reduction
	Without HC1	With HC1	
HSV-1(MP) $L3153_{28}$	60.0	36.3	40
$L3153_{34}$	60.0	61.0	0
HSV-1(F) Vero	29.6	2.6	91

[a] Each virus was reacted with monoclonal HC1 or phosphate-buffered saline at room temperature for 2 h and then plated in replicate Vero cell monolayers for the plaque assay, as detailed in Materials and Methods.

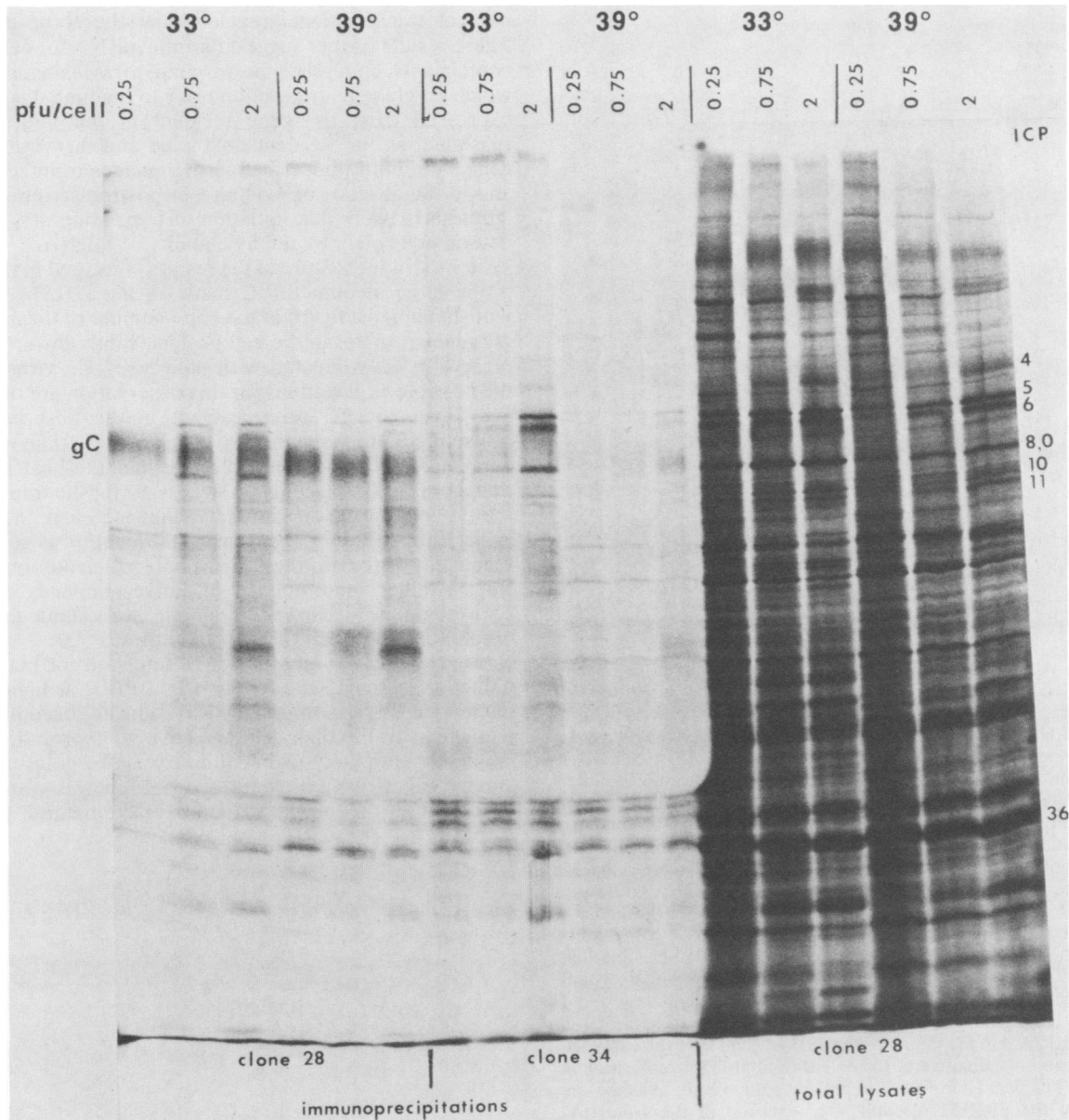[b] Data represent the average number of plaques scored in three dishes.

FIG. 7. Autoradiographic images of total cell lysates and immune precipitates obtained with HC1 from lysates of cells infected at the permissive (33°C) and nonpermissive (39°C) temperatures with tsHA1. Replicate cultures of L3153$_{28}$ and L3153$_{34}$ cells were infected with tsHA1 at the indicated multiplicities of infection and incubated at 33 or 39°C. The cells were labeled from 1.5 to 10 h postexposure of cells to virus and harvested at the end of the labeling interval. The autoradiograms of the total cell lysates show that the amounts of viral proteins made in L3153$_{28}$ cells by HSV-1(MP) at 10 h postinfection are small or barely detectable as would be expected from the very low multiplicities of infection used.

mid containing a $dhfr$ gene resistant to methotrexate and the entire domain of the gC gene of HSV-1. The salient features of the results presented in this report are as follows.

(i) None of the cell clones tested produced gC constitutively as determined by a variety of assays designed to detect gC. Furthermore, only a small fraction, i.e., 1 of 39 clones tested, expressed significant quantities of gC upon infection of the methotrexate-selected cells with HSV-1(MP). These results suggest that the plasmid introduced into the parent cells may have undergone considerable rearrangement in cells selected for methotrexate resistance, but they neither support nor refute the hypothesis that the selection of methotrexate-resistant cells incapable of expressing gC con-

stitutively reflects the toxicity of the protein. An alternative hypothesis for the lack of isolation of clonal lines expressing gC constitutively is that the γ_2 promoter must be induced by viral $trans$-acting factors to be expressed efficiently. It should be noted that Berman et al. (1) reported the selection of a cell line constitutively expressing glycoprotein D of HSV-1. In that instance, the cells were also transfected with a plasmid containing the $dhfr$ gene and selected for methotrexate resistance. The significant difference, however, was the use of a simian virus 40 promoter in place of the authentic gD promoter.

(ii) The gC gene resident in the L3153$_{28}$ cell line was regulated as a β gene rather than as a γ_2 gene. This
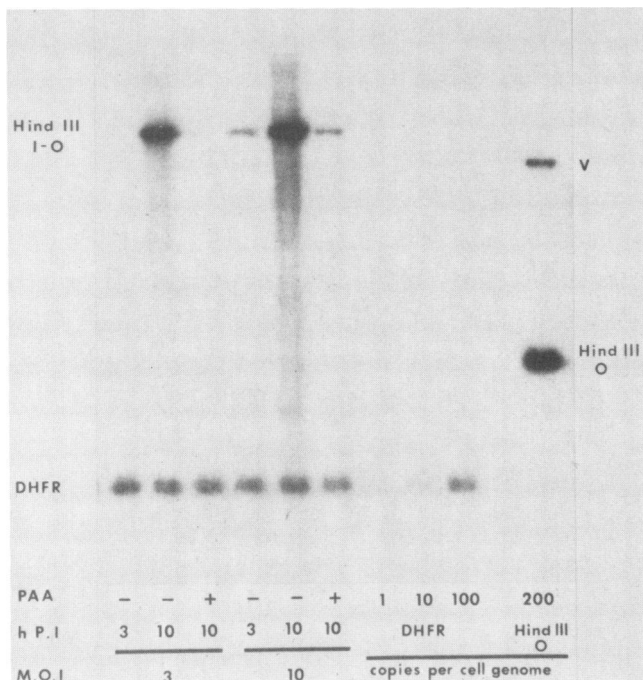
FIG. 8. Autoradiographic image of HindIII digests of pRB3153, pRB132, and L3153₂₈ DNAs electrophoretically separated in agarose gels, transferred to nitrocellulose, and hybridized with a ³²P-labeled HindIII fragment from pRB3153 containing the dhfr gene and the HindIII O fragment from pRB132. The L3153₂₈ cell DNA was isolated as described in Materials and Methods from cells either mock infected or infected with HSV-1(MP) at the multiplicities of infection (M.O.I.) (PFU per cell) shown in the figure, in the presence (+) or absence (−) of PAA (300 μg/ml) at 3 or 10 h postinfection (h PI) as indicated in the figure. The reconstructions were done by mixing appropriate amounts of pRB3153 or pRB132 DNA with Ltk⁻ DNA prior to HindIII digestion. The dhfr fragment in the L3153₂₈ cell line that hybridizes to the dhfr probe is 1,500 base pairs and is identical in size to the piece contained in the pRB3153 plasmid that was used to transform these cells to the methotrexate resistant phenotype. Note that in HSV-1(MP) DNA the HindIII O and I fragments are fused. The band that hybridizes to the HindIII-O probe is indicated in the figure as Hind III I-O and is of the expected size; this band is indicative of the extent of viral DNA replication in the cells. The band labeled V represents the vector plasmid sequences of the pRB132 plasmid after excision of the HindIII-O sequences and it hybridizes to the 350 base pairs of the plasmid vector sequences contained in the DNA fragment carrying the dhfr gene.

conclusion is based on three observations. First, the gC gene resident in L3153₂₈ cells was expressed in detectable amounts significantly earlier and was turned off earlier than the gC gene resident in the HSV-1(F) genome. Second, the results of the studies with the ts502 mutant indicate that a functional α4 gene product is required, and the results of the experiments with tsHA1 excluded the requirement for viral DNA synthesis. Third, concentrations of PAA that completely block viral DNA synthesis (300 μg/ml, Fig. 5) caused an increase rather than a decrease in the production of gC in L3153₂₈ cells infected with HSV-1(MP). These results are in accord with similar observations on a chimeric gene comprising the promoter regulatory region of a γ₂ gene fused to the 5′ transcribed noncoding and coding domains of the thymidine kinase gene (35) and suggest that the 5′ transcribed coding and noncoding sequences are not a determi-

nant of transcriptional regulation of the tk or gC genes. These results further suggest that the mRNA for gC does not contain cis-acting sites or respond to trans-acting signals which preclude its translation prior to the time it is normally expressed from the gene resident in the viral genome. Inasmuch as the resident host gene and the viral genomic gene were both transcribed in the nucleus of infected cells, and in the absence of evidence of posttranscriptional regulation, it is likely that initiation of translation of γ₂ genes is determined solely by the availability of mRNA.

(iii) A striking feature of the results presented in this report is the large amount of gC made in the L3153₂₈ cells not withstanding the fact that the copy number of the competent gC genes resident in the cell is significantly lower than that present in cells infected with wild-type gC⁺ virus. Among the possible explanations for this observation are the following. First, the gC gene resident in the host genome is transcribed at a significantly higher rate than the gene contained in the viral genome; second, the host transcriptional or translational factors necessary for the translation of the gC mRNA are in greater abundance early in infection rather than at late times; third, the quantity of gC made is regulated; or fourth, the γ₂ genes are efficiently transcribed from a rather small number of active templates. Although each could be discussed at length, we cannot at present differentiate between these possibilities.

(iv) The optimal multiplicity of infection for maximal gC induction in the L3153₂₈ cells was 2 PFU; at higher multiplicities, the expression of gC was reduced, becoming undetectable at 10 PFU per cell. We have no explanation for the effect of multiplicity of infection except to point out that a similar effect of multiplicity of infection was observed by Leiden et al. (22) on the induction of the natural, β-tk gene
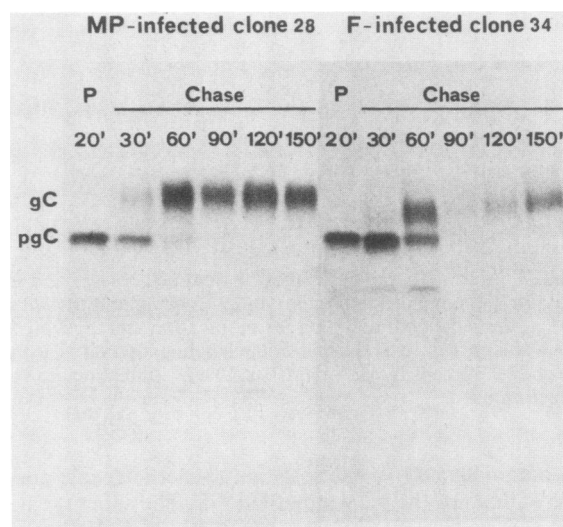

FIG. 9. Autoradiographic images of electrophoretically separated gC precipitated by HCl from lysates of infected cells harvested after a 20-min labeling interval and after a chase in medium containing unlabeled methionine. The L3153₂₈ cells and L3153₃₄ cells were infected with HSV-1(MP) (1 PFU per cell) and HSV-1(F) (10 PFU per cell), respectively. At 8 h postinfection, cells were pulse-labeled for 20 min with [³⁵S]methionine (P) (200 μCi/ml of medium containing unlabeled methionine), followed by chase for 30, 60, 90, 120, and 150 min in medium containing unlabeled methionine. The cells were harvested after the pulse or pulse and chase at the times indicated in the figure.

resident in L cells by TK⁻ virus. These authors suggested that at the higher multiplicity of infection the resident β gene was inhibited because of the more rapid transition from β to γ protein synthesis at higher multiplicities of infection.

(v) The gC induced in L3153$_{28}$ cells by HSV-1(MP) was processed to mature form and rendered the progeny HSV-1(MP) virus susceptible to neutralization by anti-gC monoclonal antibody. A striking feature of the results is the observation that the processing of gC induced by infection of L3153$_{28}$ cells infected with HSV-1(MP) was significantly faster than that of the gene product expressed by HSV-1(F) in L3153$_{34}$ cells. A similar observation was made by Johnson and Smiley (16) in cells which carryed the α4 gene and were
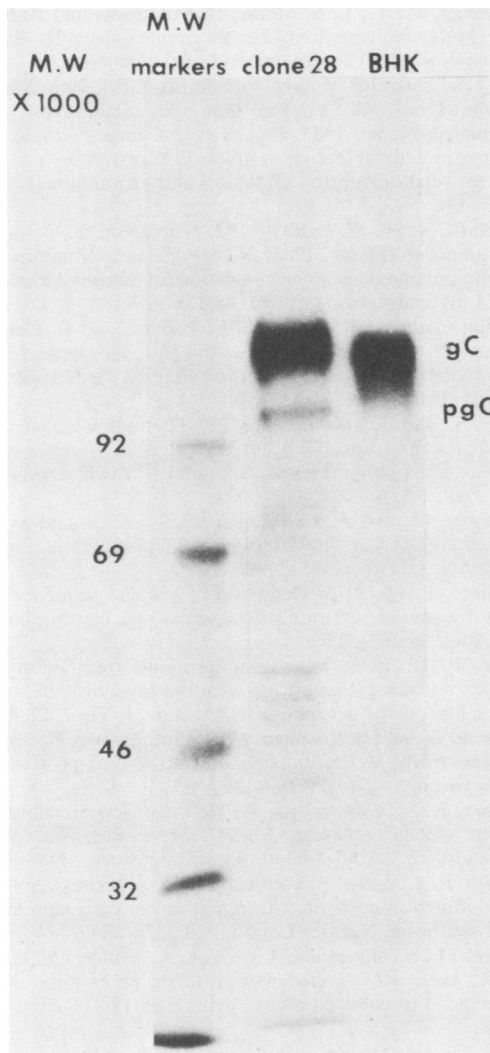

FIG. 10 Autoradiographic images of gC contained in precipitates from lysates of L3153$_{28}$ cells infected with HSV-1(MP) and of mature gC purified from BHK cells infected with HSV-1(F). The L3153$_{28}$ cells infected with HSV-1(MP) were labeled with [^{35}S]methionine from 1.5 h until harvested at 15 h after exposure of cells to virus. gC was precipitated with HCl. HSV-1(F)-infected BHK cells were labeled with [^{14}C]glucosamine from 7 to 18 h postinfection and chased for an additional 3 h in medium lacking radioactive precursors. The mature form of gC was purified by affinity chromatography on HCl monoclonal antibodies linked to Sepharose as previously described (34). Molecular weight (M. W.) markers are shown in thousands ($\times$ 1000) at the left.

transfected with a plasmid carrying the gD gene. Their interpretation was that the presence of viral gene products and, particularly, budding particles in nuclear membranes retarded the potentially rapid transport of gD to the Golgi apparatus. Since both L3153$_{28}$ and L3153$_{34}$ cells were methotrexate resistant and infected, the difference in the rates of processing cannot be attributed either to properties related to the selectable marker or to a difference in viral products and capsids associated with membranes. We have no explanation for this observation.

The L3153$_{28}$ cell line joins a growing number of cells expressing HSV genes either constitutively or upon introduction of virus or viral genes (1, 16, 23, 26, 28, 29, 35). These lines provide important tools for analyses of gene products and their function and regulation.

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