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Evidence for Translational Regulation of Herpes Simplex Virus Type 1 gD Expression

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We compared the rates of synthesis of herpes simplex virus type 1 glycoproteins C and D and quantitated the accumulation of translatable mRNA for each glycoprotein at various times after infection. The rate of synthesis of gD increased sharply early in the infection, peaked by 4 to 6 h after infection, and declined late in the infection. In contrast, the rate of synthesis of gC increased steadily until at least 15 h after infection. The levels of mRNA for both of these glycoproteins, as detected by hybridization and by translation in vitro, continued to increase until at least 15 or 16 h after infection. Synthesis of both gC and gD and their respective mRNAs was found to be sensitive to inhibition of viral DNA replication with phosphonoacetic acid. The finding that reduced amounts of gD were synthesized late in the replicative cycle, whereas gD mRNA continued to accumulate in the cytoplasm, argues that the synthesis of gD is regulated, in part, at the level of translation.

The temporal control of herpes simplex virus (HSV) gene expression during the replicative cycle depends at least in part upon the regulation of transcription of various classes of mRNAs (α, β, and γ) (reviewed in references 29 and 30 and by E. K. Wagner [*in* B. Roizman, ed., *The Herpesviruses, Vol. 3*, in press]). The polypeptides produced immediately after infection (immediate-early or α polypeptides) are required for transcription of other segments of the genome expressed later in infection (β and γ polypeptides). The synthesis of late or γ polypeptides, many of which are structural polypeptides, requires the synthesis of β polypeptides and the replication of viral DNA, and these γ polypeptides are characteristically made at increasing rates until 15 to 20 h postinfection. In contrast, the rates of synthesis of α and β polypeptides decline after early and intermediate times of infection. Evidence has been presented that the shutoff of α polypeptide synthesis is mediated by a posttranscriptional cytoplasmic process that results presumably in either accelerated turnover of or less efficient translation of α mRNA (9, 28). Only limited information is available as to the relationship between declining rates of α and β polypeptide synthesis and changes in levels of functional α and β mRNAs. Sharp et al. (31) recently reported that the levels of HSV thymidine kinase mRNA decrease late in infection, a fact consistent with the reduced rates of thymidine kinase activity observed by others (12).

The HSV glycoproteins are structural proteins, and some clearly belong to the γ class. For example, gC is not synthesized early in infection and is not produced at maximal rates until late in infection (1, 8). In addition, the 2.5-kilobase (kb) mRNA encoding gC appears after the initiation of DNA replication, and synthesis of both the mRNA and the polypeptide is very sensitive to inhibitors of DNA replication (10, 11, 13, 27). However, other glycoproteins, such as gD, are detected very early after infection, and maximal rates of synthesis occur at 4 to 6 h, after which the rate of synthesis declines (1, 8). Inhibitors of DNA replication permit some gD synthesis, but only at reduced levels (13, 27). Accumulation of gD mRNA is also inhibited under these conditions (17). Therefore, gD does not fit into the β or γ categories. HSV polypeptides of this kind have been designated βγ polypeptides (E. K. Wagner, in press).

We have looked closely at the kinetics of synthesis of HSV type 1 (HSV-1) gD and gC and at the accumulation of the mRNAs which encode these polypeptides. We found a discordance between the rates of synthesis of gD and the levels of translatable, polyadenylated gD mRNA which accumulate in the cytoplasm of infected cells. Rates of synthesis of gD peaked early in the infection and declined dramatically late in the infection even though levels of translatable, polyadenylated gD mRNA continued to increase in the cytoplasm of infected cells until late in the infection. In contrast, the rate of synthesis of gC more closely paralleled the accumulation of gC mRNA in the cytoplasm of infected cells. Our results indicate that some form of regulation of the translation of gD mRNA occurs in infected cells.

MATERIALS AND METHODS

Cells and virus. HEp-2 cells and African green monkey kidney cells (Vero) were grown as monolayer cultures in Dulbecco modified Eagle minimal essential medium supplemented with 10% fetal calf serum, both from KC Biologicals, Lenexa, Kans. The virus strain used was HSV-1(HFEM), obtained from A. Buchan (University of Birmingham, Birmingham, England), and it was passaged at low multiplicity on HEp-2 cells.

Infection of cells and preparation of [^{35}S]methionine-labeled lysates. Monolayer cultures of HEp-2 cells growing in plastic dishes (25 cm^2) were infected with HSV-1(HFEM) at 20 PFU per cell. Virus was adsorbed in phosphate-buffered saline (10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$ [pH 7.4]) containing 0.1% (wt/vol) glucose and 1% calf serum. The virus was removed after 1.5 h, and medium 199 supplemented with 1% calf serum (199V) was added. Phosphonoacetic acid (PAA) (disodium salt; Abbott Laboratories, North Chicago, Ill.) was prepared as a stock solution at 30 mg/ml and added to cells at 300 μg/ml 2 h before the infection and during the infection. Infected cell proteins were labeled by incubating cells for 10 min with [^{35}S]methionine (100 μCi/ml, 1,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) in 199V without nonradioactive methionine and containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic

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acid), pH 7.4. The cells were washed twice with Tris-saline (50 mM Tris-hydrochloride [pH 7.5], 100 mM NaCl) and then lysed in Tris saline containing 1% Nonidet P-40 (Gallard-Schlesinger Corp., Carle Place, N.Y.), 0.5% sodium deoxycholate (Calbiochem-Behring, La Jolla, Calif.), and ovalbumin at 1 mg/ml (5×10^6 infected cells per ml of extraction buffer at each time point). Extracts were kept on ice for 15 min with occasional mixing and then sonicated for 30 to 60 s before storage at −70°C. Immediately before immunoprecipitation, the extracts were thawed and cleared by centrifugation at 25,000 rpm in an SW27.1 rotor for 1 h.

Immunoprecipitation and electrophoresis of radiolabeled viral glycoproteins. Monoclonal antibodies II-436, specific for gD, and II-474, specific for gC, have been described previously (18, 19). Monoclonal antibody II-886 is specific for gD and was isolated and characterized by M. Para in this laboratory. A rabbit antiserum specific for HSV-1 gC, designated R#24, was prepared by M. Sarmiento-Batterson by the isolation of gC extracted from purified HSV-1 virions on preparative polyacrylamide gels and the injection of this material into rabbits. This antibody was found to be useful in immunoprecipitating in vitro-translated gC (21). Monoclonal antibodies C3, C4, and C5, specific for HSV-1 gC, were kindly provided by J. Glorioso and M. Levine (University of Michigan, Ann Arbor), and monoclonal antibody HD1, specific for HSV-1 gD, was kindly provided by L. Pereira (California Department of Public Health, Berkeley). HSV-1 glycoproteins were isolated by immunoprecipitation (18) and electrophoresed on 8.5% polyacrylamide gels cross-linked with *N,N'*-diallyltartardiamide (Bio-Rad Laboratories, Richmond, Calif.) as previously described (14). Gels were infused with 2,5-diphenyloxazole by the procedure of Bonner and Laskey (6) and then dried and placed in contact with Cronex medical X-ray film. To quantitate viral glycoproteins, the bands corresponding to the glycoproteins in the dried polyacrylamide gels were localized with fluorograms, and each band was cut out, dissolved in 2% periodic acid, and counted in aqueous scintillation fluid (Beckman Redisolve HP).

Preparation of viral mRNA. For each time point, equal numbers of infected HEp-2 cells (2×10^8) were washed twice with cold phosphate-buffered saline, scraped off the growth surface (32-oz [ca. 907.2-g] glass bottles), and lysed in a solution containing 0.5% Nonidet P-40, 100 mM NaCl, 25 mM HEPES-HCl, (pH 7.5), and 10 mM vanadyl-ribonucleoside complexes (5) or RNasin at 500 U/ml (Biotec, Inc., Madison, Wis.). The lysate was incubated for 5 min with shaking and then centrifuged at $800 \times g$ for 7 min. An equal volume of a solution containing 7 M urea, 1% sodium dodecyl sulfate, 0.3 M NaCl, and 25 mM HEPES (pH 7.4) was added. The suspension was extracted twice and 50% phenol–50% chloroform (vol/vol) and twice with chloroform (22), and then it was precipitated with ethanol. Polyadenylated RNA was isolated by oligodeoxythymidylate cellulose (Collaborative Research, Inc., Waltham, Mass.) chromatography (22).

Recombinant plasmid probes. Recombinant plasmid pCB68 was derived from plasmid pDJ19, which contains the 2.6-kb *Pst*I-*Bam*HI fragment (0.627 to 0.643 map unit) of HSV-1(F) DNA cloned into M13mp9/pUC9 (24). Sequences from the *Pst*I site (0.627 map unit) to a position ca. 3 base pairs upstream of the translation initiation codon for the gC polypeptide were removed by cleaving pDJ19 with *Pst*I, digesting with the nuclease Bal31, and religating the plasmid to yield the derivative pCB68.

Plasmid pMG25, kindly provided by M. G. Gibson, was constructed by deleting the 300-base-pair *Nru*I-*Sac*I fragment from plasmid pSKS309 (13), which contains the 2.9-kb *Sac*I fragment (0.906 to 0.924 map unit) of HSV-1(F) DNA.

RNA electrophoresis, transfer to nitrocellulose, and hybridization. For each time point, polyadenylated cytoplasmic RNA extracted with 2×10^8 infected cells was denatured in 2.2 M formaldehyde and 50% (vol/vol) formamide, heated to 55°C for 15 min, and then separated on 1.5% agarose gels containing 2.2 M formaldehyde (22). The gels were soaked in 50 mM NaOH–10 mM NaCl for 45 min at room temperature and then blotted onto nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) in 3 M NaCl and 0.3 M trisodium citrate (20× SSC) (1× SSC is 0.15 M NaCl, plus 0.015 M sodium citrate) for at least 12 h. The filter was washed in 3× SSC, air dried, and baked for 4 h at 80°C. DNA probes were ^{32}P labeled by nick translation with [^{32}P]dCTP according to the recommendations of the manufacturer (New England Nuclear) and hybridized for 20 h at 45°C in a solution containing 50% formamide, 5× SSC, 5× Denhardt buffer (22), denatured salmon sperm DNA at 100 μg/ml, 0.25% sodium dodecyl sulfate, 5 mM EDTA, and 2×10^6 to 5×10^6 cpm of the denatured ^{32}P-labeled DNA probe per ml. Blots were washed at 65°C with 2× SSC, then 0.5× SSC, then 0.1× SSC, air dried, and placed in contact with X-ray film.

In vitro translation. In vitro translation by reticulocyte lysate (New England Nuclear) was carried out as specified by the manufacturer. The radioactive tracer used was [^{35}S]methionine (1,000 Ci/mmol, 50 μCi per 25-μl reaction). One microliter of the total reaction was removed for direct analysis of the total translation product by polyacrylamide gel electrophoresis, and the rest was immunoprecipitated.

RESULTS

Synthesis of gC and gD during the replicative cycle of HSV-1. The rates of synthesis of gC and gD were assessed at various times after infection with HSV-1(HFEM) by pulse-labeling HEp-2 cells for 10 min with [^{35}S]methionine, followed immediately by preparation of cell lysates. The glycoproteins were immunoprecipitated with monoclonal antibodies specific for gD (II-436) or gC (II-474) and then electrophoresed on polyacrylamide gels (Fig. 1). The amount of labeled polypeptide precipitated from each sample should reflect the rate of synthesis of each glycoprotein, assuming that there was little degradation of newly synthesized polypeptides within the 10-min labeling period and that the antibodies quantitatively precipitated gD and gC. It seems unlikely that during the replicative cycle these glycoproteins (labeled in a short pulse) are altered in antigenicity so as not to be recognized by one particular monoclonal antibody. However, to rule out this possibility, we utilized two additional monoclonal antibodies directed against gD, II-886 and HD1, and three additional monoclonal antibodies directed against gC, C3, C4, and C5, with results similar to those shown in Fig. 1.

The synthetic rates of these two glycoproteins differed markedly, especially early and very late in the infection (Fig. 2). gD was first detected at 2.5 h after infection. Its rate of synthesis peaked at 4 h and then declined dramatically, as had been reported previously by others (1, 8). We also found, as did Balachandran et al. (1) for HSV type 2 gE, that rates of synthesis of HSV-1 gE peaked early (4 h) and then declined (data not shown). In contrast, gC was not detected until 4 h, and its rate of synthesis increased continuously until at least 15 h after infection, consistent with results reported by others (1, 8). We observed similar kinetics of synthesis for these two glycoproteins in Vero cells (data not shown).

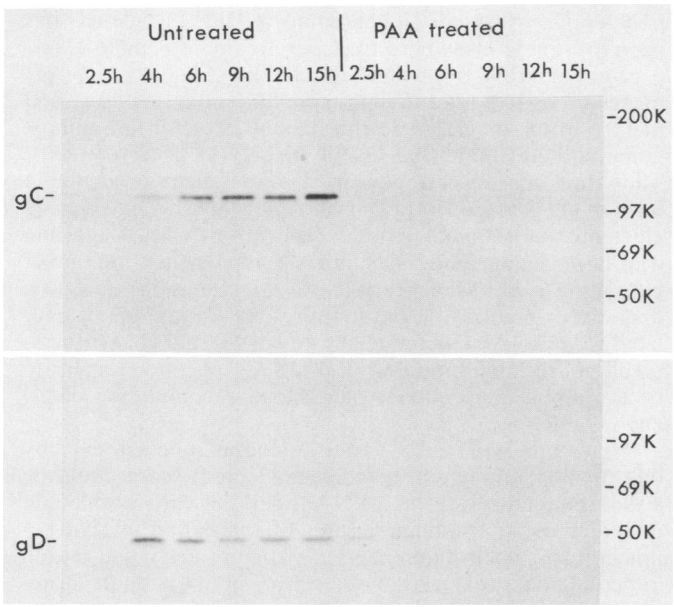

FIG. 1. Synthesis of gC and gD at various times after infection with HSV-1. HEp-2 cells were infected with HSV-1(HFEM) and pulse-labeled at various times (2.5, 4, 6, 9, 12, and 15 h) after infection for 10 min with [^{35}S]methionine. Cells exposed to PAA (300 μg/ml) were treated 2 h before infection and throughout the infection. Cell lysates were prepared immediately after the labeling period. gC was immunoprecipitated with monoclonal antibody II-474, gD was precipitated with monoclonal antibody II-436, and immunoprecipitates were analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. Molecular weight markers (in thousands [K]) were myosin (200), phosphorylase *b* (97), bovine serum albumin (69), and immunoglobulin G heavy chain (50).

When cells were treated with PAA (300 μg/ml) to inhibit viral DNA replication (16), the synthesis of gC was completely inhibited at all times after infection (Fig. 1). The synthesis of gD at very early times (2.5 h) was not noticeably affected by PAA treatment; however, at later times (4 h and after), gD synthesis was inhibited significantly, as had been previously reported (13, 27). It should be noted that in these experiments PAA-treated cells failed to produce most late proteins and synthesized HSV-1 early proteins as late as 15 h after infection at levels comparable to levels of synthesis in untreated cells at 2.5 h after infection (data not shown).

Expression of viral glycoprotein mRNAs. To determine whether the rates of synthesis of gC and gD reflected the amounts of their mRNAs present in the cytoplasm, we performed Northern blot analysis on cytoplasmic polyadenylated RNA extracted from infected cells at various times after infection (Fig. 3). The RNA was fractionated by electrophoresis in 1.5% agarose gels containing 2.2 M formaldehyde, transferred to nitrocellulose, and hybridized with ^{32}P-labeled DNA probes.

A single 2.5-kb mRNA was observed when blots were probed with labeled plasmid pCB68, which includes sequences extending from 3 bases upstream of the translation start codon for gC to the *Bam*HI site just downstream of the translation termination codon for gC (Fig. 4). Frink et al. (11) previously reported that this 2.5-kb mRNA is the major transcription product from this region, encoding a polypeptide immunoprecipitated by anti-gC antibodies and identical to authentic HSV-1 gC by comparison with tryptic peptides. Other less abundant, spliced mRNAs were also shown to be derived from transcripts of the same region. Accumulation of the 2.5-kb mRNA in infected cells closely paralleled the synthesis of gC during infection. Maximal levels for gC mRNA were detected late in infection, in keeping with the maximal rates of gC synthesis observed at these times.

When Northern blots were probed with plasmid pMG25, which includes the 2.6-kb *Sac*I-*Nru*I fragment within which the coding sequence of gD is located (34), a major RNA species with an estimated size of 3.0 kb and minor species, both larger and smaller, were detected (Fig. 3). Watson et al. (33), using a probe containing the 2.9-kb *Sac*I fragment, found a very similar pattern of RNAs, with a major species of 3.0 kb encoding gD. A smaller (1.6-kb) mRNA which does not contain the gD coding sequence was found to be 3′ coterminal with the 3.0-kb mRNA (Fig. 4). Ikura et al. (17) reported that gD mRNA produced in adenovirus-transformed cells is spliced as indicated in Fig. 4 and is about 2.3 kb long. In our experiments, the 3.0-kb gD mRNA was detected earlier than was the 2.5-kb gC mRNA, at 2.5 h after infection, and the level of this RNA increased throughout the infection. This result was not expected, given the declining synthetic rates of gD after 4 h of infection.

PAA treatment of infected cells inhibited the appearance of the 2.5-kb gC mRNA and drastically reduced the amount of the 3.0-kb gD mRNA that accumulated (Fig. 3).

In vitro translation of glycoprotein mRNAs. To determine whether the gC and gD mRNAs that accumulated in infected cells were functional, especially late in the infection, we isolated polyadenylated RNA at 4, 9, and 15 h after infection with HSV-1 and translated the mRNA in vitro with commercial reticulocyte lysate (Fig. 5). Rabbit anti-gC serum (R#24) immunoprecipitated a single polypeptide of ca. 74,000 daltons, and monoclonal antibody II-436 (anti-gD) immunoprecipitated a polypeptide of ca. 50,000 daltons, corresponding to the unglycosylated forms of these glycoproteins (11, 20, 21, 23).

The amounts of both gD and gC translated in vitro from the mRNAs extracted at various times after infection paralleled the relative amounts of mRNA detected by Northern blot analysis. The high levels of both gD and gC mRNAs which accumulated late in infection were reflected in high levels of in vitro-translated gD and gC. Lee et al. (20) also noted that more gD could be translated in vitro from mRNAs extracted at 12 or 14 h after infection than from those

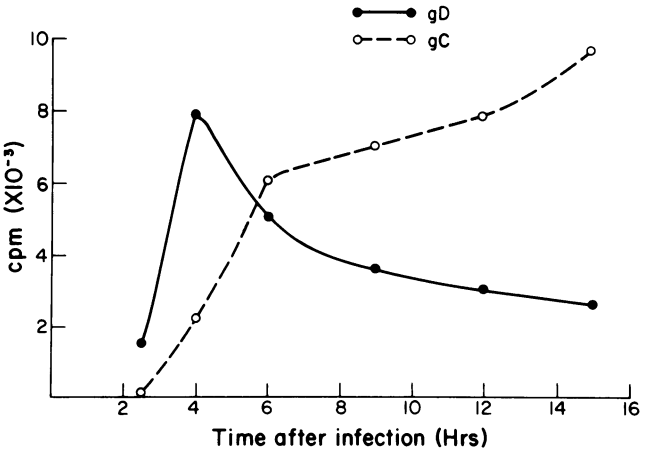

FIG. 2. Quantitation of gC and gD synthesized at various times after infection with HSV-1. Radiolabeled bands corresponding to gC and gD in the polyacrylamide gel shown in Fig. 1 were localized with the fluorogram, excised, and dissolved in 2% periodic acid, and the radioactivity was counted.

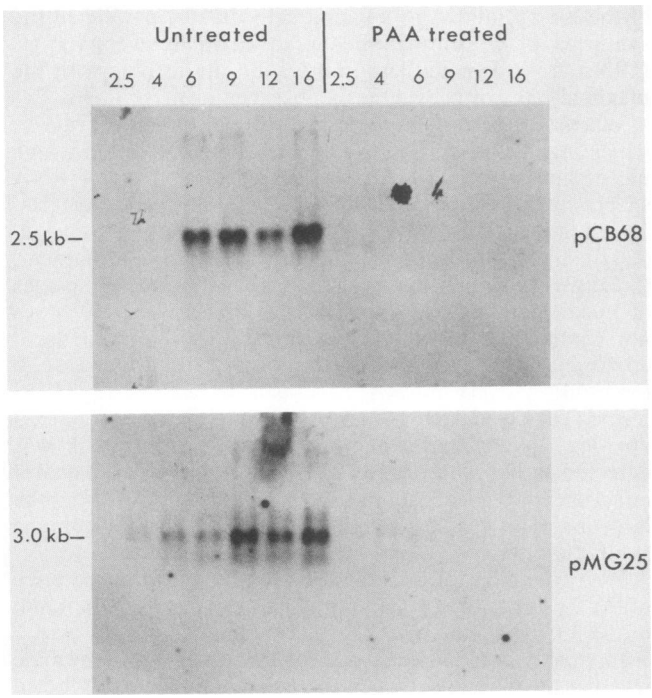

FIG. 3. Accumulation of gC and gD mRNAs at various times after infection with HSV-1 in the presence or absence of PAA (as described in the legend to Fig. 1). Cytoplasmic polyadenylated RNA was extracted from cells 2.5, 4, 6, 9, 12, and 16 h after infection of HEp-2 cells with HSV-1(HFEM) and electrophoresed on 1.5% agarose gels containing 2.2 M formaldehyde. RNAs were transferred to nitrocellulose and probed with either plasmid pCB68 (gC) or plasmid pMG25 (gD) (see Fig. 4). The quantity of RNA loaded in each lane was that extracted from 2×10^7 infected cells. Marker RNAs (18S and 28S rRNAs) were detected by staining the gel with ethidium bromide (22).

extracted at 6 or 8 h. This observation is in contrast to what was observed in vivo, where the rates of synthesis of gD decreased late in infection (Fig. 1 and 2).

DISCUSSION

We found that levels of translatable gD mRNA continued to increase at times after infection when the rate of gD polypeptide synthesis appeared to decline. Evidence has been presented elsewhere that there is no detectable difference in the rate of turnover (degradation) of pulse-labeled gD made at 5 or 14 h after infection and that both immature and mature forms of gD made throughout infection are almost quantitatively solubilized by the detergents used here (32). Therefore, unless an abnormal non-immunoprecipitable form of gD is made late, gD mRNA must be translated more efficiently early than late in the replicative cycle, suggesting translational control of gD synthesis. In contrast, the rate of gC synthesis at various times after infection appeared, to a first approximation at least, to reflect the amount of accumulated gC mRNA. The reduced rates of gD and gC synthesis resulting from inhibition of viral DNA replication appear to be the direct consequence of reduced accumulation of gD and gC mRNAs.

Regulation of gD mRNA translation might be achieved by one or more of several mechanisms. First, there could be structural features of the mRNA that differ early and late in infection and that influence rates of translation in vivo but not in vitro. Such differences in structure early and late in infection could result from differences in initiation or termination of transcription, splicing patterns, cap structure, or methylation patterns. Effects on translation of modifying the 5′-terminal cap structures of mRNAs have been well documented (reviewed in reference 2). Although 5′-terminal cap structures have been detected on HSV mRNAs (3, 25), no comparisons of cap structures on single mRNA species isolated at different times after infection have been reported. Even if there were differences in cap structure that could influence translation, differences in translation efficiency ought to be observed in vitro as well as in vivo unless the reticulocyte lysate contains capping or methyltransferase activities. Bartkoski and Roizman (4) noted, on analysis of bulk viral polyadenylated RNA, that methylation at internal positions was inhibited late in the replicative cycle. With regard to differential splicing patterns, Watson et al. (33) reported that gD mRNA is unspliced, and Ikura et al. (17) reported that it is spliced downstream of the coding sequence. The latter authors suggested that splicing patterns might change with time during the replicative cycle. We observed no significant changes in the sizes of gD mRNAs made at different times during the replicative cycle, as might be expected if gD mRNAs were spliced late but not early, for example. Clearly, comparisons of gD mRNAs made early

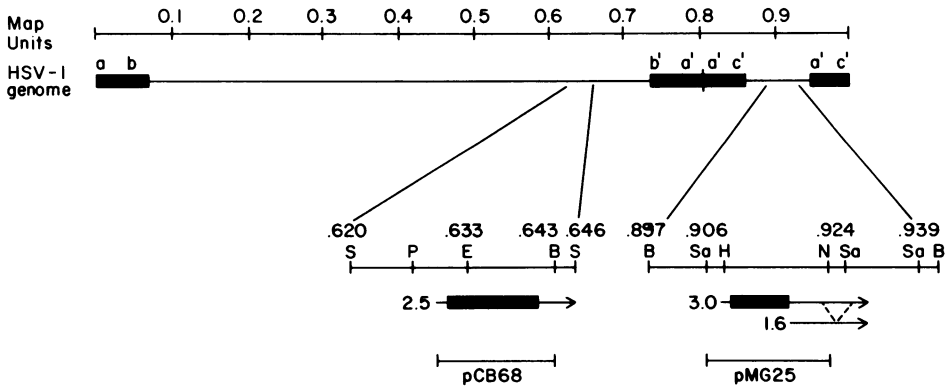

FIG. 4. Map positions of DNA sequences encoding the gC and gD mRNAs and of DNA sequences cloned in plasmids used as probes to detect these mRNAs. The 2.5-kb mRNA encoding gC (11) was detected with plasmid pCB68 (see text). The 3.0-kb mRNA encoding gD (33, 34) was detected with plasmid pMG25. Ikura et al. (17) have recently reported that the mRNA encoding gD is somewhat smaller than 3.0 kb (2.3 kb) and is spliced (indicated by dotted lines). The direction of transcription of the mRNAs is indicated, and the heavy black bars show the positions of the coding sequences. Restriction sites: *Sal*I (S), *Pst*I (P), *Eco*RI (E), *Bam*HI (B), *Sac*I (Sa), *Hin*dIII (H), and *Nru*I (N).

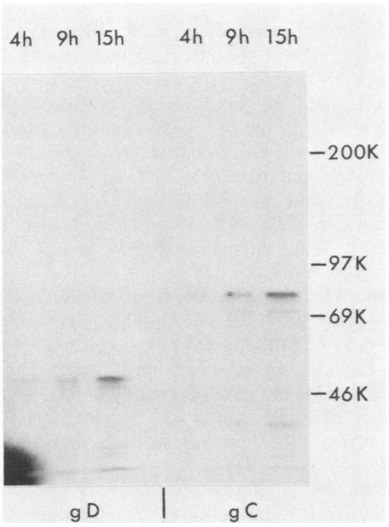

FIG. 5. In vitro translation of gC and gD from mRNAs extracted from cells at various times after infection of HEp-2 cells with HSV-1(HFEM). Cytoplasmic polyadenylated RNAs extracted 4, 9, or 15 h after infection were translated in vitro (each sample contained the amount of RNA extracted from 2×10^7 infected cells), and the translation products were immunoprecipitated with anti-gD monoclonal antibody II-436 or anti-gC antiserum R#24 for electrophoretic analysis. Molecular weight markers (in thousands [K]) were myosin (200), phosphorylase *b* (97), bovine serum albumin (69), and ovalbumin (46).

and late are necessary to investigate the possibility of more subtle differences in structure.

Second, it seems possible that ionic or metabolite imbalances in the infected cell or disruption of membrane systems brought about by viral structural components late in infection could have a selective inhibitory effect on the translation of certain viral mRNAs. For example, Carrasco and Smith (7) have proposed a model for the shutoff of host cell protein synthesis by picornaviruses, in which changes in cell membranes brought about by picornavirus structural proteins result in an influx of sodium ions. The change in sodium concentration in the infected cell cytoplasm results in the preferential translation of viral mRNAs.

Third, viral gene products present at particular times during the replicative cycle might interact with gD mRNA or the translation machinery to regulate the efficiency of translation. If this is the case, these putative products must discriminate between gD and gC mRNAs. Fusions between the gD and gC genes may permit mapping of the regions of gD mRNA that are essential for the translational regulation observed.

Future studies will reveal whether apparent regulation at the level of translation applies to other HSV polypeptides as well as to gD. It is somewhat puzzling that rates of gD synthesis decline before the period when demand for a structural protein ought to be greatest, during the interval (ca. 6 to 18 h after infection) when virions are produced. Nothing is known about the function of gD except that it can elicit the production of neutralizing antibodies (reviewed by P. G. Spear [*in* B. Roizman (ed.), *The Herpesviruses, Vol. 3*, in press]), and that anti-gD antibodies can block HSV-induced cell fusion (26).

The original classification of HSV genes into α, β, and γ regulatory groups was based on rates of polypeptide synthesis in the presence or absence of particular metabolic inhibitors (15). Later studies revealed that the regulatory groupings could be further subdivided (reviewed by E. K. Wagner [in press]). Quantitation of rates of production and turnover of mRNAs and polypeptides should permit even finer distinctions to be made so that genes subject to translational as well as transcriptional regulation can be identified and so that groups of genes possibly subject to coordinate regulation can be better identified.

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