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An Amino-Terminal Deletion Mutation of Pseudorabies Virus Glycoprotein gIII Affects Protein Localization and RNA Accumulation

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We have constructed a pseudorabies virus mutant that contains virtually a complete deletion of the predicted signal sequence coding region for a nonessential envelope glycoprotein, gIII. No signal sequence mutants have been reported previously for a herpesvirus glycoprotein. Through endoglycosidase treatments and pulse-chase analysis, we have determined that the mutant gIII protein is not posttranslationally modified like the wild-type polypeptide, but rather is present as a single, stable species within the infected cell. The mutant polypeptide cannot be detected in the virus envelope, nor is it aberrantly localized to the tissue culture medium. Immunofluorescence studies have indicated that the mutant protein also is not localized to the surfaces of infected cells. In addition, Northern (RNA) and slot blot analyses, as well as in vitro translation experiments using infected-cell cytoplasmic RNA, have indicated that the mutant gIII allele is expressed at lower levels than the wild-type gene is. This is despite the fact that no alterations have been made upstream of the gIII coding sequence. From these results, it appears that the first 22 amino acids of the wild-type gIII protein define a necessary signal peptide that is responsible for at least the correct initiation of translocation and subsequent glycosylation of the gIII envelope glycoprotein within infected cells.

Many cellular and viral gene products are synthesized by membrane-bound ribosomes on the rough endoplasmic reticulum (RER) and then transported through the secretory pathway to the cell surface, where they are either released into the surrounding medium or incorporated into the plasma membrane of the cell. In general, the early events in this process are believed to adhere to the tenets of the signal hypothesis (4), whose proposed mechanisms for the initiation of protein export have been largely confirmed through in vitro experimentation (for a review, see reference 36). Briefly, most exported proteins are initially synthesized on free ribosomes with a 15- to 30-amino-acid extension, the signal sequence or peptide, at their amino-terminal end. As the nascent chain emerges from the ribosome, the polypeptide-ribosome complex interacts with a cytosolic ribonucleoprotein component of the cellular export machinery, the signal recognition particle (SRP) (35, 37). This interaction leads to a decrease in the rate, if not actual arrest, of translation. The arrested structure is then presented to the surface of the RER, where it associates specifically with at least one membrane component of the export machinery. This exchange between the SRP-polypeptide-ribosome complex and the SRP-receptor, or so-called docking protein (11, 20), displaces SRP from the arrested nascent chain and facilitates the translocation of the protein across the RER membrane. It is during the translocation event that the signal sequence interacts with at least two more membrane components: the signal sequence receptor (38) and signal peptidase, which cleaves the signal peptide after a consensus sequence of three amino acids (34). In addition, the mature portion of the protein may receive primary, N-linked glycosylation during its membrane crossing.

It is commonly held that herpesvirus glycoproteins can be localized to virtually all of the membranes of the infected cell, including those surrounding the nucleus. It is from the latter that the egressing nucleocapsid appears to take its viral envelope, budding through patches, where the only proteins present are virus-encoded glycoproteins (32). The initial events leading to a nuclear membrane localization of herpesvirus glycoproteins are believed to be signal sequence dependent, although this has not been demonstrated. The mechanisms by which the viral glycoproteins are specifically retained in the inner nuclear membrane after being localized are not understood. We report here the deletion of the signal sequence for a major component of a herpesvirus envelope and demonstrate its consequences, both for the mutant glycoprotein and for the resulting virus.

At least five envelope glycoproteins and one secreted glycoprotein are synthesized on pseudorabies virus (PRV) infection of cells in culture (13, 22). Investigators in our laboratory have cloned, mapped, and sequenced the gIII envelope glycoprotein of PRV (24, 25). We have demonstrated that gIII shares significant homology with the human herpes simplex virus type 1 and 2 gC glycoproteins (24) and, like these proteins, is not essential for growth of the virus in tissue culture (26). Glycoprotein gIII is predicted to be 479 amino acids in length, and it resides in the infected cell as two species: (i) a 74-kilodalton (kDa) pre-Golgi form bearing N-linked glycosylation of the high-mannose variety, and (ii) a 92-kDa mature protein, derived from the pre-Golgi form, whose polypeptide chain has been extensively modified; the 92-kDa protein also is found in mature virions (24, 28). The first 22 amino acids of the primary structure appear to meet all of the criteria for a functional signal peptide (see Fig. 1A). The first six residues constitute a hydrophilic domain and are immediately followed by a hydrophobic core of amino acids

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that are predicted to assume an α-helix (9). In addition, there are three consensus sequences for signal peptidase cleavage (Ala-X-Ala), but we favor the last site, because it is closely followed by a predicted β-turn, an important feature as noted by von Heijne (34). The mature portion of the protein contains eight consensus sites of N-linked glycosylation and a single hydrophobic stretch between amino acids 451 and 470 that probably functions as a membrane-spanning domain. We have demonstrated previously that the gIII glycoprotein is rapidly and efficiently exported in the infected cell (28). Moreover, an amber fragment representing only the first one-third of the full-length polypeptide is exported with wild-type kinetics, indicating that all information necessary for efficient export is present in the amino terminus of the protein (28).

In this report we present the construction of an additional gIII mutant to further define functional assignments of protein localization signals within the gIII gene. Specifically, we deleted amino acids 2 to 22 of the gIII protein in a bacterial expression vector. The mutant gene was then used to replace the wild-type gIII gene in the virus by cotransfection of plasmid and viral DNA. Analysis of the mutant gIII protein produced by the resulting virus has revealed that the polypeptide is present as one species and is not glycosylated or otherwise posttranslationally modified. Furthermore, the mutant protein apparently is found only in the cytosol and is not found in mature virions. Although no upstream sequences have been altered, the mutant allele is poorly expressed compared with the wild-type gene, with the mutant mRNA accumulating at reduced levels. This appears to be a novel consequence of the signal sequence deletion, and possible implications of this finding for herpesvirus gene expression are addressed.

MATERIALS AND METHODS

Cells, virus, and DNA. The growth and properties of the Becker strain of PRV (PRV-Be) and the porcine kidney cell line PK15 have been described previously (25).

Escherichia coli NF1829 has been described (24). All plasmids were constructed by standard recombinant techniques. The structure of each plasmid was verified by restriction endonuclease analysis and, when applicable, by protein production or β-galactosidase assay. Plasmid pCL1405 has been described previously (17).

Mutagenesis of the gIII gene. Plasmid pCL1405 uses a tac promoter to express roughly one-third of the coding sequence of gIII fused to the *E. coli lacZ* gene and has unique *Nco*I and *Sal*I sites that flank the predicted gIII signal sequence coding region. Plasmid pCL1413 was created by digesting pCL1405 with *Nco*I and *Sal*I and inserting the following linker:

```
        5' C ATG GGC CCG      3'
        3'     CCG GGC AGC T 5'
gIII Codon:  1  Gly  23   24
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The addition of this linker also added a unique *Apa*I site (shown in the coding strand in bold type). To replace the tac promoter, PRV DNA homology upstream from the 5' end of the gIII gene was added in a two-step process, creating pCL1418. Downstream PRV homology was added to the 3' end of *lacZ* in the form of a 5-kilobase-pair (kbp) *Eco*RI-*Hin*dIII restriction fragment from pALM1 (28) that contains the remaining two-thirds of gIII lacking from pCL1405. The resulting plasmid, pCL1423, is depicted in Fig. 1B.

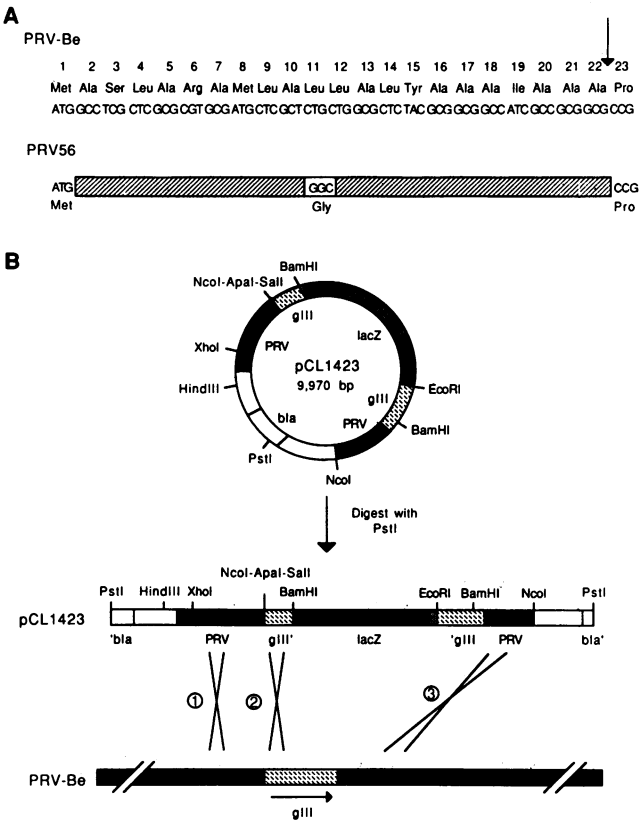

FIG. 1. Construction of PRV56. (A) The first 23 residues of the deduced amino acid sequence for wild-type gIII, including the entire predicted signal peptide and processing site (indicated by an arrow), are shown in the top portion. The corresponding nucleotide sequence of the sense strand also is given. In the bottom portion, the region deleted in the mutant allele is indicated (▨). Also depicted are the first three codons and encoded amino acids of the mutant. (B) Plasmid pCL1423 DNA was linearized by digestion with the restriction enzyme *Pst*I and used in a cotransfection experiment with PRV-Be genomic DNA. The three potential crossover sites for recombination between the plasmid and viral DNAs are indicated, as is the direction of gIII transcription in the virus genome. Relevant restriction enzyme sites also are shown. Symbols: ▭, plasmid vector sequences; ■, PRV sequences flanking gIII; ▨, gIII sequences; ▩, *lacZ* sequences.

Construction of PRV mutants. Recombinant viruses were obtained by using the calcium phosphate cotransfection and gene replacement techniques described previously (12, 26). Black-plaque assays (see below) were performed to identify mutant viruses. Viral plaques that did not react with the gIII-specific monoclonal antibody M1 in these assays were purified and analyzed further.

DNA and RNA analysis. Viral DNA preparation and Southern blot analysis of viral DNA, with a gIII-specific ^{32}P-labeled probe, were performed as described previously (26). For DNA sequencing, a 1.1-kbp *Xho*I-*Kpn*I fragment containing the 5' coding sequence of gIII was cloned from PRV genomic DNA into bacteriophage M13mp19. The dideoxy method (29) was used to sequence the gIII promoter region and signal sequence deletion endpoints by using a synthetic oligonucleotide primer that hybridized upstream of the gene.

The isolation of total cytoplasmic RNA from infected cells and subsequent Northern (RNA) blot analysis were conducted as previously described (26). For slot blot analysis,

RNA was prepared as follows. A 20-μg portion of each viral RNA to be analyzed was serially diluted twofold. The RNA was denatured at 65°C in 0.02 M MOPS (morpholinopropanesulfonic acid)–5 mM sodium acetate–1 mM EDTA–47% formamide–6% formaldehyde (2). The RNA was diluted ninefold with 20× SSC (1× SSC is 0.15 NaCl M plus 0.015 sodium citrate) and subsequently transferred to nitrocellulose in a slot blot apparatus. The nitrocellulose was baked for 1 h at 80°C and then hybridized at 42°C for 16 h. Autoradiograms resulting from slot blot analysis were scanned with an LKB densitometer interfaced with an AT&T microcomputer. After autoradiography, blots were fixed and stained with methylene blue as described by Maniatis et al. (18). For both the Northern and slot blot analyses, a 471-bp ^{32}P-labeled *Hin*dIII-*Sac*I fragment containing only the 5′ coding sequence of gIII was used.

The in vitro translation of total cytoplasmic RNA obtained from PRV-infected cells has been described (26). Translated extracts were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis or used for the immunoprecipitation of gIII-specific proteins with 282 antiserum.

Fractionation and immunoprecipitation of PRV glycoproteins. Unless otherwise noted, in radiolabeling experiments PK15 cells were infected at a multiplicity of infection of 10 with either PRV-Be or PRV56 and grown from 7 to 16 h postinfection in medium containing 100 μCi of [^{35}S]cysteine per ml or grown throughout a 16-h infection in medium containing 100 μCi of [^{3}H]glucosamine per ml. Infected-cell, virion, and medium fractionation of PRV-infected PK15 cells was performed as previously described (1, 28). The preparation of infected cell extracts and the immunoprecipitation procedure have been described (25).

Antibody reagents. The antibodies used in these studies included mouse monoclonal antibodies M1, M7, and M16 (reactive only against the native form of gIII) (13); goat polyvalent sera 282 (28) and 286 (reactive with denatured gIII antigen); and mouse monoclonal antibodies M2 and M3 (reactive only against the native forms of the gII family of glycoproteins) (13).

Pulse-chase analysis and endoglycosidase treatment of PRV gIII proteins. The pulse-chase procedure used has been described previously (28). Briefly, PK15 cells were infected at a multiplicity of infection of 10 with either PRV-Be or PRV56. At 6 h postinfection a radioactive pulse with 100 μCi of [^{35}S]cysteine per ml was administered for 2 min, the radiolabel was removed, and the cells were incubated in the presence of excess nonradioactive cysteine for various times. At the desired chase times, the monolayers were harvested and the gIII species were immunoprecipitated with 282 serum.

Endoglycosidase H (endo H) and endoglycosidase F (endo F) were used as specified by the supplier (Du Pont, NEN Research Products). Substrate for the enzymes was wild-type or mutant gIII that had been steady-state radiolabeled from 7 to 16 h postinfection with 100 μCi of [^{35}S]cysteine per ml and immunoprecipitated with 282 serum.

Polyacrylamide gel analysis. All immunoprecipitates were loaded onto SDS–10% polyacrylamide slab gels. Electrophoresis was performed as described previously (25). Fluorography was conducted with sodium salicylate (5) and was followed by autoradiography.

Black-plaque assay. Black-plaque assays (14, 31) were performed to identify viral mutants and to determine the cell surface localization of viral glycoproteins. In the localization studies, PK15 cell monolayers were infected with PRV strains and incubated at 37°C until isolated plaques were visible. Individual culture dishes were exposed for 45 min at room temperature to one antibody out of a set of reagents: gIII monoclonal antibody M1, M7, or M16; gIII polyclonal serum 282 or 286; or the gII monoclonal antibody M2. After unbound primary antibodies had been removed with three washes of phosphate-buffered saline (PBS), peroxidase-labeled antibodies (goat anti-mouse immunoglobulin G or rabbit anti-goat immunoglobulin G [Kirkegaard & Perry Laboratories, Inc.]) were added at room temperature. After 45 min, any unbound antibodies were removed by three PBS washes. This was followed by treatment with peroxide in the presence of 4-chloro-1-naphthol. Reactive plaques turned black, while nonreactive plaques remained white.

Immunofluorescence. PRV glycoproteins were detected by immunofluorescent staining of fixed cells (to detect intracellular material) and of live, unfixed cells (to detect glycoproteins on the cell surface). In both cases, PK15 cells were grown on glass cover slips and infected at a multiplicity of infection of 4 with either PRV-Be or the mutant, PRV56. At 9 h postinfection live, unfixed cells were prepared by rinsing the glass cover slips in PBS. Alternatively, infected cells were fixed by immersion in 3.7% formaldehyde for 20 min, washed in PBS, and incubated in acetone at −70°C for 20 min. Goat polyvalent serum was diluted 1:500 in PBS and then added to the cover slips. After a 1-h incubation at 37°C, fluorescein-labeled rabbit anti-goat immunoglobulin G (Kirkegaard & Perry) was diluted 1:100 in PBS and added to the cover slips. The cells were treated for 1 h at 37°C and then observed and photographed with a Zeiss optical system.

RESULTS

Construction and isolation of a gIII glycoprotein signal sequence deletion mutant. The coding region for the predicted signal sequence of the gIII glycoprotein of PRV is fortuitously flanked by an *Nco*I site at the 5′ end and by a *Sal*I site at the 3′ end (24). To completely remove the signal sequence from gIII, we constructed an *Nco*I-*Sal*I deletion mutation in pCL1405, a plasmid containing a gIII-*lacZ* gene fusion (see Materials and Methods). After transformation of *E. coli* NF1829, potential mutants were identified by restriction enzyme analysis. The gIII-LacZ fusion protein produced by the resulting plasmid, pCL1413, had a single amino acid (glycine) substitution for the 21 amino acids that began at amino acid 2 and extended through the predicted signal sequence to amino acid 23 (Fig. 1A). The creation of this mutation in the framework of a *lacZ* gene fusion resulted in a convenient way to detect a phenotype for the deletion mutant in bacterial cells. We have found previously that fully induced expression of wild-type gIII is lethal in *E. coli* (24). However, under uninduced conditions, NF1829 cells containing plasmid pCL1413 were found to have 4.5-fold higher β-galactosidase activity than cells containing plasmid pCL1405 did (data not shown). By analogy to previously reported results for fusions of the *E. coli malE* gene to *lacZ* (21), we propose that the lower activity observed from cells harboring the parental plasmid pCL1405 may be the result of the gIII signal sequence directing the hybrid protein to the bacterial cell membrane, where it has difficulty forming an active tetramer.

The addition of upstream and downstream PRV homology resulted in the formation of plasmid pCL1423 (Fig. 1B). This plasmid was capable of undergoing homologous recombination to replace the normal gIII gene with the mutant allele when cotransfected with PRV genomic DNA into PK15 cells, as described in Materials and Methods. By using this

plasmid, it was possible to obtain three distinct gIII mutants from a single cotransfection experiment. If the endpoints of the homologous recombination event were the result of the crossovers at positions 1 and 2, the resulting virus contained a gIII gene deleted for the putative signal sequence coding region (Fig. 1B). However, if the crossovers occurred at sites 1 and 3, the mutant virus encoded a gIII-LacZ fusion protein that is deleted for the predicted signal peptide. Lastly, if sites 2 and 3 were used, the resulting virus contained a gIII-*lacZ* gene fusion with a wild-type signal sequence coding region. We have reported previously on the construction of this third type of gIII mutant (17).

In the cotransfection experiments, viruses expressing an altered gIII protein were detected by the black-plaque assay (see Materials and Methods) through their nonreactivity with the gIII monoclonal antibody M1 (13, 14, 31). Although several nonreactive plaques were obtained, one such mutant was purified, characterized, and labeled PRV56.

Examination of PRV56 viral DNA by Southern blot analysis and DNA sequence analysis from a cloned *Xho*I-*Kpn*I fragment bearing the 5′ portion of gIII confirmed that the deletion constructed in pCL1413 had recombined onto the virus by using crossover sites 1 and 2 in Fig. 1B (data not shown). Consequently, the gIII allele found in PRV56 contained an intact gIII gene, with the exception of the substitution of 3 bases (GGC) for bases 4 through 66 of the wild-type coding sequence.

Characterization of glycoprotein gIII produced by PRV-Be and PRV56. We used immunoprecipitation and endoglycosidase digestion to analyze the gIII-specific proteins produced by PRV-Be and PRV56. PK15 cells were infected with either virus and incubated in medium containing [^{35}S]cysteine or [^{3}H]glucosamine as described in Materials and Methods. The results are shown in Fig. 2. As we have reported previously (24, 28), two forms of the gIII glycoprotein were found within cells infected with wild-type virus (Fig. 2, PRV-Be, lane 2). The polyclonal 282 serum immunoprecipitated a 74-kDa species and a more diffuse 92-kDa form. However, the monoclonal antibody M1 recognized only the 92-kDa species (lane 1). Ryan et al. (28) have demonstrated that the 74-kDa species contains primary N-linked glycosylation and is a pre-Golgi-localized, precursor form of the 92-kDa species that represents the mature post-Golgi-localized form. This relationship is shown in lanes 3 and 4, where the digestion products are resolved after endoglycosidase treatment of [^{35}S]cysteine-labeled, immunoprecipitated gIII. Endo H removes from the glycoprotein only the high-mannose form of N-linked glycosylation that is received as the polypeptide crosses the RER membrane (33). Endo F removes the Golgi-modified, complex variety of these carbohydrate side chains as well as the primary addition (8). After treatment with endo H (lane 3), the migration of the diffuse 92-kDa form of gIII remained unchanged, whereas treatment with either enzyme shifted the 74-kDa species to a faster-migrating position (lanes 3 and 4). Only after treatment with endo F did the mature form decrease in apparent molecular mass, indicating that its resistance to endo H was not due to the absence of N-linked glycosylation but rather was due to the complex modification of the side chains (lane 4). Additional evidence that the wild-type forms of gIII contains N-linked glycosylation can be seen in lane 5, where each form is readily detected by immunoprecipitation with 282 serum from infected-cell extracts radiolabeled with [^{3}H]glucosamine.

Sharply contrasting results were found for the gIII product encoded by PRV56. Since the PRV56 mutant was identified by its nonreactivity with M1 antibody in a black-plaque assay, we anticipated that this monoclonal antibody might not immunoprecipitate the mutant gIII protein from infected cells (Fig. 2, PRV56, lane 1). Conversely, the polyvalent 282 serum reacts with denatured and native gIII protein and has recognized all mutant forms of gIII that we have characterized to date (28; our unpublished observations). The 282 serum specifically immunoprecipitated a single, sharply defined protein with an apparent molecular mass of about 57 kDa, whose level of expression during the radiolabeling period seemed lower than that of the wild-type product (compare lane 2 in each set). This size was consistent with that predicted for the unmodified primary translation product of the mutant gIII allele. In contrast to the wild-type species, the migration of the 57-kDa protein was unaltered on treatment with either endo H or endo F (lanes 3 and 4). Moreover, no identifiable gIII form could be detected from PRV56-infected cells radiolabeled with [^{3}H]glucosamine (lane 5). Thus, the PRV56 gIII product appeared to be a single polypeptide species that did not receive N-linked glycosylation and accumulated in the infected cell to lower levels than those found for the wild-type protein.

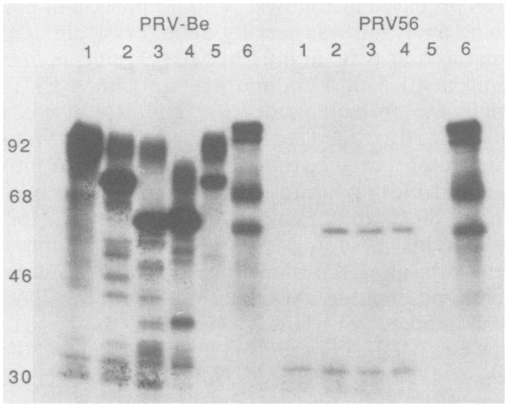

FIG. 2. Steady-state and endoglycosidase digestion profiles of immunoprecipitated proteins from PRV-infected cells. PK15 cells were infected with wild-type (PRV-Be) or mutant (PRV56) virus, and the viral proteins were radiolabeled, as described in Materials and Methods. Immunoprecipitations and endoglycosidase digestions were performed as described (25, 28), and the resulting samples were resolved on an SDS-10% polyacrylamide gel and then subjected to fluorography and autoradiography. The positions of molecular mass standards are indicated at the left in kilodaltons. For each set the lanes are as follows: 1, ^{35}S-labeled gIII immunoprecipitated with monoclonal antibody M1; 2, ^{35}S-labeled gIII immunoprecipitated with polyclonal antibody 282; 3, endo H treatment of 282-immunoprecipitated, ^{35}S-labeled gIII; 4, endo F treatment of 282-immunoprecipitated, ^{35}S-labeled gIII; 5, ^{3}H-labeled gIII immunoprecipitated with polyclonal antibody 282; 6, ^{35}S-labeled gII immunoprecipitated with monoclonal antibody M3.

For each infection in which the cells were radiolabeled with [^{35}S]cysteine, the gII family of glycoproteins (23) was immunoprecipitated from the infected cells. The patterns obtained were identical whether the infecting virus was PRV-Be or PRV56, indicating that each infection had progressed to an equivalent stage and that the gIII mutation had no effect on synthesis and posttranslational processing of an unrelated PRV glycoprotein.

Pulse-chase analysis of PRV-Be- and PRV56-encoded gIII. We wanted to examine the ability of the mutant gIII protein and to determine whether any transient, processed forms of the protein existed in the infected cell that might not be

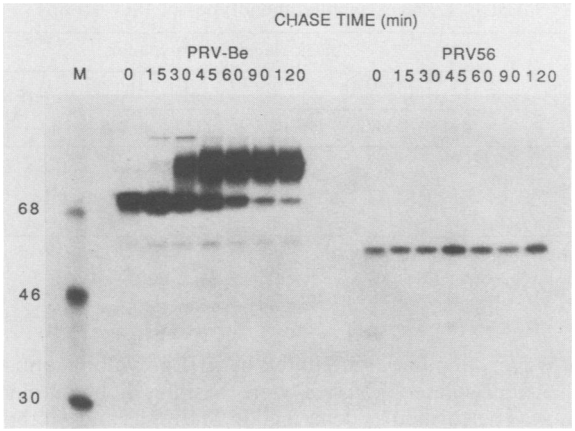

FIG. 3. Pulse-chase analysis of PRV-Be- and PRV56-encoded gIII. Cells infected with wild-type (PRV-Be) or mutant (PRV56) virus were used in a pulse-chase experiment as described in Materials and Methods. The gIII species were immunoprecipitated with 282 serum, resolved on an SDS-10% polyacrylamide gel, and subjected to fluorography and autoradiography. The length of each chase period (in minutes) is indicated at the top of each lane. Molecular mass standards (in kilodaltons) are shown in lane M.

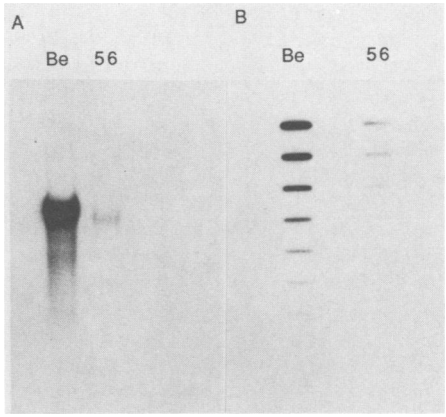

FIG. 4. Northern and slot blot analyses of gIII-specific RNA from PRV-infected cells. Total cytoplasmic RNA was harvested at 16 h postinfection from cells infected with wild-type (PRV-Be) or mutant (PRV-56) virus. (A) Autoradiogram of a Northern blot showing hybridization of a ^{32}P-labeled, gIII-specific probe to RNA that had been fractionated on an agarose-formaldehyde gel and transferred to nitrocellulose. (B) Autoradiogram of a slot blot showing hybridization of the same ^{32}P-labeled probe to RNA that had been serially diluted twofold. The highest concentration of RNA is in the top slot of each lane, and the lowest concentration is in the bottom slot of each lane. Equal amounts of total cytoplasmic RNA were used from each infection. See Materials and Methods for additional details.

detected under steady-state radiolabeling conditions. PK15 cells were infected with either PRV-Be or PRV56, and a pulse-chase experiment was performed as described in Materials and Methods. The results are shown in Fig. 3. As noted previously (28), export of wild-type gIII was rapid and efficient: the mature form appeared as early as 30 min after pulse-labeling, and only a small fraction of the precursor remained after 2 h. The pulse-chase profile obtained for the mutant form of gIII produced by PRV56 was strikingly different. The primary 57-kDa translation product was not processed to any other detectable form. Furthermore, this nonglycosylated species of gIII was stable, exhibiting a half-life of more than 120 min.

Analysis of viral RNA from infected cells. Because the gIII protein produced in PRV56-infected cells accumulated to lower levels than the wild-type protein did, and yet was stable in the cell, we analyzed the RNA from infected cells to determine whether this decrease in mutant gIII levels was due to a lower level of gene expression. Total cytoplasmic RNA from infected cells was purified and analyzed by the Northern blot technique as previously described (26). Cytoplasmic RNA extracted from PK15 cells infected with PRV-Be contained a discrete 1.55-kilobase gIII-specific transcript (Fig. 4A), as has been previously reported by Robbins et al. (26). A discrete 1.5-kilobase gIII-specific transcript was detected in RNA obtained from PRV56-infected cells. The size and appearance of the mRNA were therefore consistent with the introduction of a small deletion into the gIII-coding sequence and the use of the correct site for the initiation of transcription. However, this gIII-specific mRNA was significantly reduced in quantity, even though there was no apparent degradation of the transcripts. Slot blot experiments were performed to quantitate the reduction in total gIII mRNA that could be hybridized. When equal amounts of cytoplasmic RNA were probed, the amount of total gIII mRNA accumulated in PK15 cells infected with PRV56 was approximately 12-fold lower than that observed in a similar PRV-Be infection (Fig. 4B).

In both the Northern blot and slot blot analyses, no gIII-specific hybridization was detected with cytoplasmic RNA obtained from mock-infected cells (data not shown).

In vitro translation of total cytoplasmic RNA extracted from PRV-Be- and PRV56-infected cells. To demonstrate that the bulk of the gIII-specific RNA detected by Northern blot and slot blot analyses could lead to the synthesis of protein, total cytoplasmic RNA from cells infected with PRV-Be or PRV56 was translated in vitro as described previously (26). In addition, gIII was immunoprecipitated with 282 serum from infected cells that had been incubated in medium containing [^{35}S]cysteine as described for Fig. 2. The gIII species then were either left untreated (Fig. 5, lanes C) or treated with endo H (lanes H). When immunoprecipitated with 282 serum, the in vitro-translated wild-type gIII polypeptide migrated slightly slower than the endo H-treated wild-type pre-Golgi form did (compare panel PRV-Be, lanes H and IVT). This difference in apparent molecular mass presumably reflected the presence of the uncleaved signal peptide on the in vitro-derived product. In contrast, the in vitro-translated gIII protein of PRV56 comigrated with the single form of gIII obtained from PRV56-infected cells (compare panel PRV56, lanes C, H, and IVT). Moreover, all of these polypeptides migrated more closely with the endo H-treated pre-Golgi form of wild-type gIII than with the wild-type in vitro-translated product. No gIII-specific proteins were immunoprecipitated from the translation of RNA obtained from mock-infected cells (panel PRV56, lane Mo).

We also examined whole profiles obtained from each in vitro translation reaction (Fig. 5, right panel). In general, the number and quantity of protein species observed in each profile were the same, indicating that an equal amount of RNA was used in each reaction and that, for PRV56, no other major alterations in RNA expression existed. A band migrating with the apparent molecular mass of the gIII in vitro-translated product could be detected in the wild-type profile (lane Be), but not in the mock- or PRV56- derived profiles (lanes Mo and 56, respectively). These results were consistent with the relative levels of gIII product observed

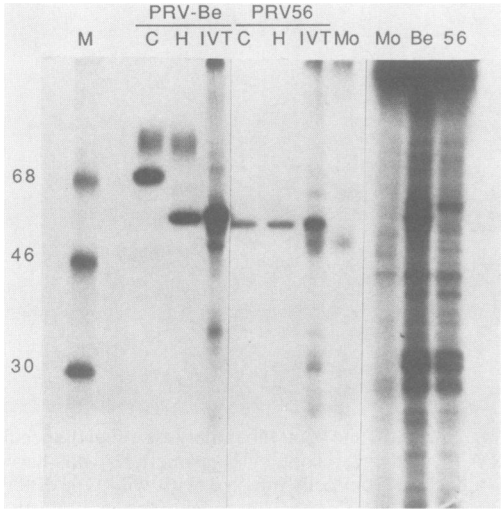

FIG. 5. In vitro translation of PRV-infected cell RNA. Total cytoplasmic RNA from mock-, PRV-Be-, or PRV56-infected cells was harvested at 16 h postinfection and incubated with [^{35}S]cysteine in an in vitro translation system as described (26). Samples of the total reaction mixtures and gIII immune precipitates from these mixtures were resolved on an SDS–10% polyacrylamide gel. Steady-state radiolabeled wild-type and mutant gIIIs that had been left untreated or treated with endo H were also fractionated on the gel. The gel then was subjected to fluorography and autoradiography. Four times more RNA was translated to obtain the PRV56-encoded gIII immune precipitate than for the wild type. The figure is a composite of different exposures of the same autoradiogram. For the left-hand eight lanes: M, molecular mass standards (in kilodaltons); C, 282-immunoprecipitated, ^{35}S-labeled gIII; H, endo H treatment of 282-immunoprecipitated, ^{35}S-labeled gIII; IVT, 282-immunoprecipitated, in vitro-translated gIII; Mo, 282-immunoprecipitated in vitro translation products from mock-infected cell RNA. For the three right-hand lanes: Mo, total in vitro translation profile for RNA from mock-infected cells; Be, total in vitro translation profile for RNA from PRV-Be-infected cells; 56, total in vitro translation profile for RNA from PRV56-infected cells.

through immunoprecipitation analysis and suggested that potential differences between the wild-type and mutant forms in their affinity for the polyclonal antibody played no significant role, if any, in the amount of gIII that was immunoprecipitated from in vitro translation extracts.

Localization of gIII produced by PRV56. Three methods were used to determine the localization of PRV56-encoded gIII in the context of a virus infection. In the first, infected cells, virions, and media that had been depleted of cells and virions were purified as described (1, 28). As demonstrated previously (28), in a wild-type infection both forms of gIII were found in infected cells, whereas only the mature form was localized to mature virions. The lone gIII species present in a PRV56 infection was found exclusively in infected cells, with no fraction detected in mature virions. In neither case was a form of gIII localized to culture media (data not shown).

Second, to test for the presence of gIII on the surfaces of infected cells, we tested PRV plaques for their reactivity with various antibodies via a peroxidase-linked immunoassay, the black-plaque assay (14, 31). Virus stocks of PRV-Be, PRV2, or PRV56 were diluted and used to infect PK15 monolayers. PRV2 produces a truncated form of gIII that is localized to the infected-cell surface but has lost the epitopes recognized by our monoclonal antibodies; the truncated gIII is, however, immunoprecipitated by gIII polyclonal antisera (28). After isolated plaques were visible, a black-plaque assay was performed as described in Materials and Methods, and the results are summarized in Table 1. PRV-Be plaques reacted (turned black) with all of the antibodies tested, whereas PRV2 plaques reacted with all of the antibodies except the gIII monoclonal species. PRV56 plaques reacted only with the gII monoclonal antibody. We did not expect PRV2 or PRV56 plaques to react with monoclonal antibodies directed against native, wild-type gIII. However, if either of the mutant gIII proteins was present on the infected-cell surface, we would have expected black plaques when the polyclonal sera were used, since these sera immunoprecipitated both of the mutant gIIIs from lysed infected cells. These results agreed with our previous findings for cell surface localization of wild-type and PRV2-encoded gIII and indicated that PRV56-encoded gIII either was not found on the surfaces of infected cells or was present at levels too low to detect in this assay.

TABLE 1. Black-plaque phenotypes of PRV strains

Strain	Black-plaque phenotype for:					
	gIII MAb[a]			gIII PAb[b]		gII MAb
	M1	M7	M16	282	286	M3
PRV-Be	+[c]	+	+	+	+	+
PRV2	−[c]	−	−	+	+	+
PRV56	−	−	−	−	−	+

[a] MAb, Monoclonal antibody.
[b] PAb, Polyclonal antibody.
[c] +, Black plaque; −, white plaque.

In a third method, PRV-Be- or PRV56-infected cells were reacted with 282 antiserum and tagged with a fluorescein-conjugated second antibody as an additional way to determine whether the mutant protein could be found on the cell surface (Fig. 6). Fixed cells from both infections exhibited substantial intracellular fluorescence (panels C and D). In contrast, nonpermeabilized PK15 cells infected with PRV56 exhibited only trace surface fluorescence (panel B) under conditions in which wild-type-infected cells clearly fluoresced (panel A).

DISCUSSION

We have described the construction of a defined 63-bp deletion within the PRV gIII glycoprotein gene that results in the removal of 21 of the first 22 amino-terminal residues of the primary translation product. The effect of this deletion on protein processing provides experimental support for the conclusion that the first 22 amino acids of the gIII protein define a functional signal sequence that acts as a sorting signal for the correct translocation and processing of the gIII glycoprotein. The alteration presumably eliminated the initial translocation of the PRV56-encoded gIII polypeptide across the RER membrane, resulting in a stable, nonglycosylated species of 57 kDa that appeared to be slightly smaller than the in vitro-translated wild-type product. Localization studies indicated that the mutant gIII was not found on the infected cell surface or in the mature virus envelope, but rather resided exclusively in the cytosol.

Few viral glycoprotein signal sequence mutants have been described, and, when characterized, these export-defective proteins often have been found to be unstable (10, 39). The stable gIII signal sequence deletion mutant reported here is the first example of a herpesvirus glycoprotein signal se-

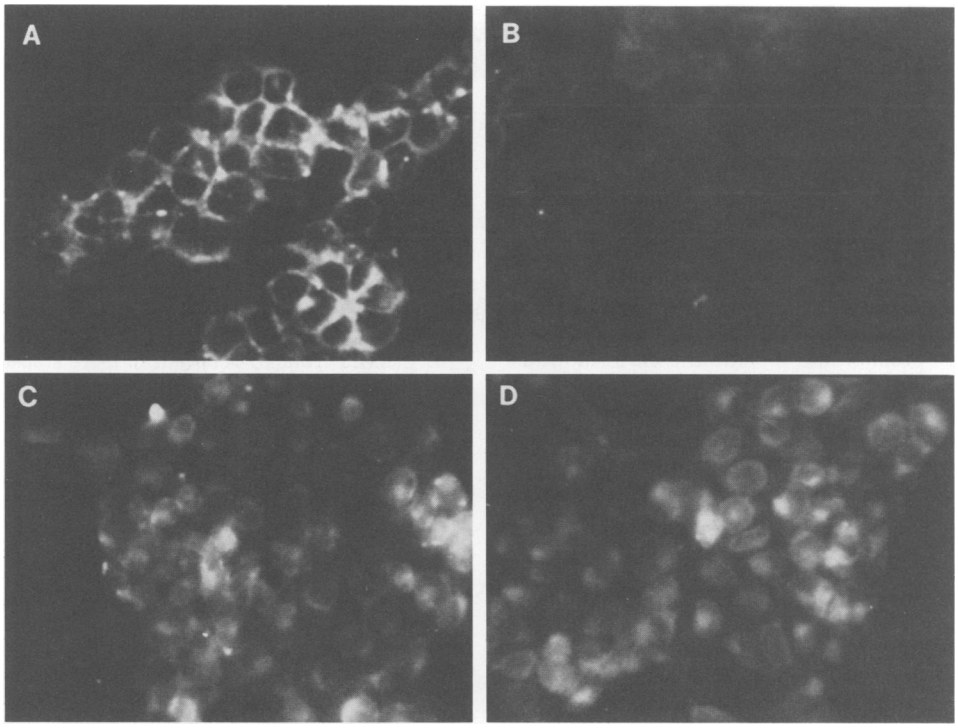

FIG. 6. Immunofluorescence of PRV-infected cells. PK15 cells were infected with PRV-Be or PRV56 at a multiplicity of infection of 4 and prepared for immunofluorescence studies at 9 h postinfection as described in Materials and Methods. (A) Live, unfixed cells from a PRV-Be infection. (B) Live, unfixed cells from a PRV56 infection. (C) Formaldehyde-fixed cells from a PRV-Be infection. (D) Formaldehyde-fixed cells from a PRV56 infection.

quence mutant. However, the existence of cleavable signal peptides for the herpes simplex virus glycoproteins gB and gD has been inferred by comparing the amino termini of the intracellular forms of these proteins, as determined by amino acid sequencing, with the amino acid sequences deduced from DNA sequence analyses (6, 7). For both gB and gD, the polypeptides found in infected cells specifically lacked the signal sequences that were predicted by DNA sequencing. Moreover, the addition of gB mRNA to an in vitro reticulocyte system has shown that glycoprotein gB is cotranslationally translocated across membranes (6). Thus, genetic and biochemical approaches are demonstrating a role for functional signal sequences in the targeting of herpesvirus glycoproteins to their correct cellular destination, including their presumed localization to the inner nuclear membrane.

For gIII, the requirement for a functional signal peptide appears to be an absolute one, since no form of the mutant gIII was detected in the export pathway or in mature virus envelopes. This is in contrast to results obtained for certain exported proteins of *Saccharomyces cerevisiae*. More than 10% of the polypeptides of pulse-radiolabeled yeast carboxypeptidase Y were posttranslationally glycosylated in vivo, despite the complete deletion of the wild-type signal sequence from the mutant allele (3). In a separate study, a complete deletion of the signal sequence of *S. cerevisiae* acid phosphatase still allowed up to 30% of the mutant polypeptides to be translocated, core glycosylated, and localized to the cell wall (30). These results suggest that stricter requirements for cotranslational translocation of exported proteins may exist in higher eucaryotes as compared with *S. cerevisiae*.

If an altered signal peptide was efficiently recognized by SRP but subsequently did not interact correctly with docking protein, a predicted phenotype might be a reduced synthesis of the mutant polypeptide, owing to an SRP-induced translational arrest. Although we observed smaller amounts of PRV56-encoded gIII than wild-type protein in the infected cell, the decreased gIII expression appeared to result from decreased RNA accumulation rather than defects in translation. We demonstrated a 12-fold reduction in the accumulation of PRV56 gIII-specific mRNA. The mRNAs were apparently transcribed with the correct initiation and termination sites and were not detectably degraded. These findings suggest an additional consideration for the regulation of herpesvirus glycoprotein genes.

Herpesvirus genes adhere to cascade regulation, in which the transcription products fall into three major classes: immediate-early, early, and late (reviewed by Roizman and Batterson [27]). A number of *cis*-acting domains have been identified as being necessary for the efficient expression of herpesvirus genes, with the immediate-early class requiring enhancerlike elements, Sp1-binding sites, and CAAT and TATA box structures (19). Many of these sequences serve, or are believed to serve, as sites for DNA-binding proteins. The early and late genes need fewer of these domains for regulated and efficient expression but exhibit an additional requirement for certain immediate-early gene products (16). Recently it was concluded for the herpes simplex virus type 1 gC gene, a gIII homolog, that a specific, *cis*-acting 15-bp sequence was sufficient for efficient and fully regulated expression (15). This sequence lay upstream of the transcribed region, and its only recognizable promoter element was a TATA box. The determination was made through a deletion analysis of sequences upstream and in the 5′ portion of the gC gene. The only indication that sequences other than a TATA box might be involved was the finding that a

large deletion removing most of the untranslated mRNA leader sequence reduced the efficiency of gC expression. It was suggested that this deficiency indicated a length requirement for the leader sequence to facilitate efficient transcription in perhaps a nonspecific manner (15).

Considering these results, it is worth noting that several inverted repeats lie in the DNA sequences that correspond to the 5′ noncoding and coding regions of gIII mRNA. These inverted repeats may lead to DNA-mediated control of gene expression, perhaps by acting as binding sites for regulatory proteins. An alternative consideration is the possible consequence for mRNA secondary structure. The 5′ sequence of gIII mRNA has the potential to fold into two stem-loop structures. The first stem would include the entire untranslated leader region and the codons 1 through 9 of the gIII signal sequence, whereas the second stem would encompass codons 13 through 23 of gIII. The deletion contained within PRV56 would eliminate both of these structures without affecting the length of the leader sequence, and its consequences may suggest a role for the stable formation of stem-loop structures in the efficient expression of gIII. It is also possible that such structures play a role in RNA stability. However, we cannot at present distinguish this possibility from several others, including one in which the signal sequence deletion has introduced a new stem-loop that interferes with expression. Still, it appears that more than simply an intact TATA box or full-length leader sequence is required for efficient gIII expression. We are presently examining the potential role of mRNA secondary structure in gIII transcription.

Finally, we would like to discuss our use of the black-plaque assay as a method for demonstrating cell surface localization of a protein species. Previously, we used this technique to identify gIII mutants that did not express glycoprotein gIII at all or whose altered gIII product as expressed and correctly localized to the cell surface but had lost the epitope recognized by the M1 monoclonal antibody (17, 26, 28). In each of these last cases, the mutant viruses did react (i.e., formed black plaques) with polyclonal antibodies specific for gIII. Although it is true that the PRV56-encoded gIII had lost the M1 epitope, most probably owing to a conformational change resulting from the absence of carbohydrate modification, plaques of this virus differ from those previously described (e.g., PRV2) in that they did not react with any gIII-specific polyclonal antibodies. This is despite the fact that the PRV56-encoded gIII was efficiently immunoprecipitated with these antibodies from lysed infected-cell extracts. Thus, the lack of antigen-antibody reactivity was due to an inaccessibility of antigen (gIII) to the antibody at the infected-cell surface. On the basis of these observations, we have used PRV56 and the black-plaque assay to construct gIII signal sequence mutants that are altered for specific amino acids (J. P. Ryan et al., manuscript in preparation). Moreover, preliminary results obtained with these mutants have indicated that perhaps as little as 10% of the wild-type levels of gIII need be present on the cell surface to elicit a black-plaque phenotype. We therefore view the black-plaque assay as a potentially important and sensitive assay for cell surface localization in future studies.

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