JOURNAL OF VIROLOGY, Nov. 1988, p. 4185–4194
0022-538X/88/114185-10$02.00/0

Vol. 62, No. 11

A Herpesvirus Vector for Expression of Glycosylated Membrane Antigens: Fusion Proteins of Pseudorabies Virus gIII and Human Immunodeficiency Virus Type 1 Envelope Glycoproteins

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Received 26 April 1988/Accepted 5 August 1988

We describe experiments using the swine herpesvirus, pseudorabies virus (PRV), as a vector for expression of hybrid membrane protein genes. In particular, we present the construction and analysis of three infectious PRV mutants expressing chimeric viral membrane proteins composed of portions of the PRV envelope glycoprotein gIII and of the human retrovirus, human immunodeficiency virus type 1 (HIV-1), envelope glycoproteins gp120 and gp41. All of the chimeric genes contain the transcription control sequences and the first 157 codons of PRV gIII (known to contain signals sufficient for efficient export of the encoded peptide out of the cell) fused to different regions of the HIV-1 envelope. The mutant viruses express novel glycosylated fusion proteins that are immunoprecipitated by polyvalent sera specific for gIII, as well as acquired immunodeficiency syndrome patient sera. The levels of expression are lower than expected due primarily to instability or altered processing of the hybrid mRNA. We could not detect cleavage of chimeric proteins carrying the gp120-gp41 protease processing site. The use of localization signals contained within herpesvirus membrane proteins to direct chimeric proteins to desired cellular locations is discussed.

Large DNA animal viruses have recently become valuable tools for expression of foreign genes. Specifically, vaccinia virus (for a review, see reference 17) and herpesviruses (for a review, see reference 24) have been shown to be amenable to the genetic manipulation once reserved only for the small DNA viruses. Because of this newfound ease of in vitro manipulation, coupled with the importance of herpesviruses in disease and the characteristic ability to cause latent infections, we have been interested in determining the utility of herpesvirus vectors to carry foreign genes both for fundamental studies of gene function as well as for expression of novel gene products. The utility of such vectors would be not only in basic virology but also in new modes of disease intervention and novel vaccine design.

The swine herpesvirus, pseudorabies virus (PRV), is attractive in this regard because of its broad host range in culture, its lack of overt human pathogenicity, and its use as a live, killed, or subunit vaccine in the animal health industry (2, 8, 30). Many of the elegant methods for genetic engineering of large DNA viruses reviewed by Roizman and Jenkins (24) can be directly applied to PRV. For example, Thomsen et al. (30) have caused the expression of tissue plasminogen activator with a PRV vector. In general, these methods rely on the use of the thymidine kinase gene (*tk*) as the selective agent, although other techniques, including hybridization methods, can be used (6, 11, 20). The vectors usually contain a virus promoter fused to the foreign gene so that authentic foreign gene products are produced.

We have pursued another method for construction and identification of recombinant PRV mutants based on disruption of and fusion to gIII, a major virus envelope glycoprotein (13, 27). We have adapted well-characterized technology for immunostaining of plaques (the black-plaque test; 10, 28) to identify gIII-positive and -negative virus. This plaque color test has facilitated construction of a variety of infectious PRV gIII mutants and enabled initial assessment of the transcription, translation, and protein localization signals encoded in the gIII gene (23, 26).

This report describes our attempts to manipulate the gIII transcription and translation information to express foreign membrane proteins and to determine if the information contained in the gIII protein to direct it to particular membrane structures could be used to localize these foreign proteins by design.

We have used the human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins gp120 and gp41 as candidates for fusion to PRV gIII (7). Our intent was not only to explore the possibilities of producing glycosylated HIV-1 antigens for diagnostic purposes and for use as novel immunogens but also to study several features of the HIV-1 glycoproteins that have relevance to questions of protein localization and processing and virus envelopment in herpesvirus-infected cells. PRV and HIV-1 have contrasting sites of intracellular virus envelopment; PRV is thought to be enveloped at the inner nuclear membrane (2), while HIV-1 is enveloped at the plasma membrane (7). It is not known what signals, if any, direct PRV glycoproteins to the inner nuclear membrane. In addition, it is not known what signals, if any, direct PRV glycoproteins to the virus envelope. Specifically, we sought to determine if chimeric gIII-HIV envelope proteins could be incorporated into PRV virions. Another interesting characteristic of HIV-1 envelope proteins is the protease cleavage site in the gp160 envelope precursor that when cleaved by a cellular protease yields gp120 and gp41. Two PRV envelope glycoproteins, gX and gII, are also proteolytically processed (1, 19). PRV glycoprotein gII is found in the virus envelope as a disulfide-linked complex of the cleavage products (16). It is not known if a cell- or virus-encoded protease is involved. We therefore constructed gIII-HIV-1 fusion proteins containing the HIV-1 envelope processing site to determine if the hybrid proteins would be cleaved. It is of interest also to determine whether

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these hybrid proteins would be glycosylated and whether they would react with HIV-1 envelope-specific antisera.

We describe the construction and analysis of three infectious PRV mutants each expressing a novel gIII-HIV envelope fusion protein. These experiments demonstrate that certain gIII sequences facilitated the expression and localization of novel proteins to infected-cell membranes with concomitant core and complex glycosylation. The fusion proteins were reactive with a variety of HIV-1 envelope sera, including acquired immunodeficiency syndrome (AIDS) patient sera. Several unexpected findings emerged, including the less than expected levels of fusion protein expression due primarily to instability and unusual processing of the chimeric mRNA, the lack of proteolytic cleavage of gIII-gp120-gp41 fusion proteins, and the inability to localize fusion proteins to the PRV virus envelope.

(A preliminary report of these findings was presented at the 1986 Herpesvirus Workshop, Leeds, Great Britain, 21 through 26 July 1986.)

MATERIALS AND METHODS

Cells and virus. PK15 swine kidney fibroblast cells, the Becker strain of PRV (PRV-Be), and PRV2, a PRV-Be mutant expressing a truncated gIII protein, have been described previously (23).

Plasmid constructions. All *Escherichia coli* strains have been described previously (22), and plasmids were constructed by standard recombinant DNA techniques. The starting plasmid, pALM2, has been described previously (23). The HIV-1 sequences were all derived from lambda BH10 (21) and were provided by S. Petteway. For insertion into the *Sac*I site of pALM2, all fragments required the addition of synthetic adaptors. These were prepared on an Applied Biosystems DNA synthesizer and purified by reverse-phase high-performance liquid chromatography. Each linker was phosphorylated prior to ligation.

pALM24 was constructed to contain a 1,430-base-pair (bp) *Bgl*II fragment of HIV-1 that contains the 3′ 30% of gp120 and the entire coding region of gp41. The following *Sac*I-*Bgl*II adaptor was ligated to the *Bgl*II ends of the HIV-1 fragment:

5′-GAGCTCAATGAGTCCGA-3′
3′-CTCGAGTTACTCAGGCTCTAG-5′

After ligation, the fragment was purified, cut back with *Sac*I, and ligated with pALM2 previously digested with *Sac*I.

pALM33 contains an 850-bp fragment of HIV-1 that encodes the carboxy-terminal 30% of gp120 and amino-terminal 70% of gp41. pENV9 was digested with *Bgl*II, and the following *Sac*I-*Bgl*II adaptor was ligated to the *Bgl*II ends:

5′-GAGCTCAATGAGTCCGA-3′
3′-CTCGAGTTACTCAGGCT C TAG-5′

After verification of the presence of the adaptor, the plasmid was digested with *Bam*HI and the following adaptor was added:

5′-GAGCTCA -3′
3′-CTCGAGTCTAG-3′

The insertion of this adaptor results in the loss of the *Bam*HI site. Finally, the ligated DNA was digested with *Sac*I and ligated with *Sac*I-digested pALM2.

pALM37 was constructed by digesting pENV14 (a derivative of pENV9 with a synthetic *Bam*HI site at the gp120-gp41 junction; kindly provided by S. Petteway) with *Kpn*I and ligating with the following adaptor:

5′-GAGCTCGTAC-3′
3′-CTCGAG-5′

This linker does not recreate the *Kpn*I site. Next, the DNA was digested with *Bam*HI and a second adaptor was added:

5′-GATCCTAGGAGCTCG
3′-GATCCTCGAGCCTAG -5′

The insertion of this linker does not destroy the *Bam*HI site. This adaptor also inserts a translation termination codon (TAG) at the junction. The last step involved cleaving the DNA with *Sac*I and ligating it to *Sac*I-digested pALM2 DNA.

Plasmid constructions were confirmed by DNA sequence analysis across the gIII-HIV-1 envelope junctions.

Construction of recombinant viruses. The plasmids carrying the gIII-HIV envelope fusions were cotransfected with PRV-Be DNA into PK15 cells as described previously (23, 26). Selection, screening, and purification of gIII mutants have been described previously (13, 23, 26). All virus work was done at biosafety level 2 in accordance with the National Institutes of Health guidelines on recombinant DNA experiments.

Purification and analysis of viral DNA. The procedure for purification of viral DNA from PRV nucleocapsids and the subsequent analysis by Southern (29) blot hybridization are described by Robbins et al. (22).

Preparation of RNA from infected cells. For all RNA experiments, total cytoplasmic RNA was extracted from uninfected or PRV-infected PK15 cells at 12 h postinfection as described by Robbins et al. (22).

Slot blot and Northern (RNA) analysis of RNA. For slot blot analysis, RNA was prepared as follows. Twenty micrograms of each viral RNA to be analyzed was serially diluted twofold. The RNA was denatured at 65°C in 0.02 M MOPS (morpholinepropanesulfonic acid), 5 mM sodium acetate, 1 mM EDTA, 47% formamide, and 6% formaldehyde (3, 4). After denaturation, the RNA was diluted fivefold with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and transferred to nitrocellulose in a slot blot apparatus. The nitrocellulose was baked for 1 h at 80°C before hybridization at 42°C for 16 h with ^{32}P-labeled DNA probes. The slot blot hybridizations and Northern blots were carried out exactly as described by Robbins et al. (23). After autoradiography, blots were fixed and stained with methylene blue as described by Maniatis et al. (18). Densitometry analysis of Northern blot and slot blot films was done with an LKB Ultrascan XL Laser Densitometer interfaced with an AT&T microcomputer.

In vitro translation of cytoplasmic RNA. In vitro translation of total cytoplasmic RNA was done by using a rabbit reticulocyte lysate system obtained from Dupont NEN Research Products according to the specifications of the manufacturer.

Endoglycosidase H and F analyses. Endoglycosidase H (endo-H) and endoglycosidase F (endo-F) were purchased from Dupont NEN and were used according to the specifications of the manufacturer. Substrate for the enzymes was immunoprecipitated gIII from infected cells that had been steady state labeled with [^{3}H]glucosamine (60 μCi/ml; D-[1,6-^{3}H(N)]glucosamine hydrochloride, Dupont NEN).

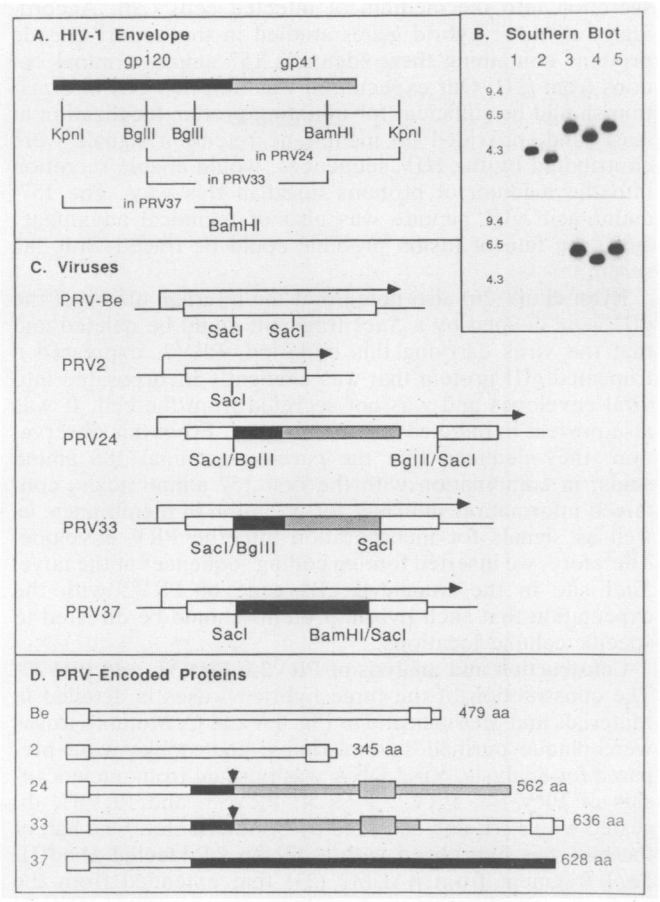

FIG. 1. Construction and analysis of PRV recombinants. (A) 2.7-kbp *Kpn*I fragment containing most of the HIV-1 envelope glycoprotein genes. The gp120 and gp41 coding regions are indicated in boxes above the line. Relevant restriction endonuclease sites and the fragments used to construct recombinant PRV strains are indicated. The *Bam*HI site indicated in the fragment inserted in PRV37 was created at the protease cleavage site between gp120 and gp41. The *Bam*HI linker inserted also contained a TAG termination codon in phase with gp120 (see Materials and Methods). The drawing is not to scale. (B) Southern blot analysis of nucleocapsid DNA extracted from PRV-Be (lane 1), PRV2 (lane 2), PRV24 (lane 3), PRV33 (lane 4), and PRV37 (lane 5). Virion DNA was digested with *Pst*I and the resulting fragments were resolved by electrophoresis in a 1% agarose gel and blotted onto nitrocellulose membranes as described in Materials and Methods. The top blot was hybridized with a 471-bp ^{32}P-labeled *Hin*dIII-*Sac*I fragment from pALM2 (23). The bottom blot was hybridized with a 2.5-kbp ^{32}P-labeled *Kpn*I fragment containing essentially all the HIV-1 envelope coding sequences (see panel A). The positions of molecular size standards (in kilodaltons) are indicated to the left of the blots. (C) Depiction of the wild-type and mutant gIII alleles present in various PRV strains. The gIII (▭), gp120 (■), and gp41 (▩) sequences are shown. Restriction endonuclease sites used for cloning are indicated. Note that the *Bam*HI-*Sac*I linker indicated in PRV37 contains a TAG termination codon in phase with gp120. The direction and extent of the expected gIII transcripts (→) are shown above the boxes. The drawings are not to scale. (D) Predicted gIII proteins encoded by the wild-type gIII gene and the mutant gIII alleles present in PRV strains. Abbreviations: Be, PRV-Be; 2, PRV2; 33, PRV33 (33); 37, PRV37. Symbols: ▭, gIII sequence; ■, gp120 sequence; ▩, gp41 sequence; □ at the left end of each protein, predicted 22-amino-acid signal peptide at the amino terminus of each protein; □ at the right end (carboxy terminus) of each protein (except for PRV37), putative transmembrane region of gIII; ▩, putative transmembrane region of gp41; ▼, protease cleavage site that defines the boundary between HIV-1 gp120 and gp41. The length of each polypeptide in amino acids (aa) is indicated at the carboxy terminus of each protein. Proteins are shown to approximate scale.

Protein localization, black-plaque, and immunoprecipitation analyses. The expression of each gIII-HIV envelope fusion protein was analyzed by immunoprecipitation as follows. PK15 cells were infected at a multiplicity of infection of 5 with PRV-Be, PRV2, PRV24, PRV33, or PRV37. Infected cells were continuously labeled with [^{3}H]glucosamine (50 μCi/ml) until 16 h postinfection. Each plate was fractionated as descibed by Ryan et al. (26) into infected cells, released virions, and medium free of infected cells and released virions. Antisera used for immunoprecipitation are as follows. (i) Mouse monoclonal M3-specific antiserum was reactive with the PRV-processed glycoprotein gII (9) and used as the control. It was a kind gift from T. Ben-Porat and H. Hampl. (ii) Mouse monoclonal [Mab(2549.1.1.5); NEI9301 (Dupont NEN)]-specific serum was prepared against a peptide at the carboxy terminus of gp120 of HIV-1 and was reactive against denatured, but not native, gp120. It was a kind gift from P. Durda, Dupont Medical Products. (iii) Goat polyvalent 282 serum was prepared against a denatured *E. coli* Cro-gIII fusion protein and was reactive with native and denatured gIII antigen (26). (iv) Human AIDS patient serum was reactive with authentic HIV-1 envelope gp160, gp41, and gp120. It was a kind gift from S. Petteway, Dupont Medical Products. To control for efficiency of infection, each fraction was immunoprecipitated with the M3 monoclonal antibody that reacts with a unique, processed PRV glycoprotein designated gII (9).

Immunostaining of plaques using the black-plaque assay has been previously described (10, 28). Briefly, approximately 100 to 200 PFU of each virus to be tested was plated onto PK15 monolayers on 100-mm-diameter culture dishes and incubated at 37°C for 48 h. The medium was then removed, and the monolayer was covered with 282 antiserum. After 1 h, the 282 serum was removed through a series of washes and peroxidase-labeled anti-antibody was added, followed later by treatment with peroxide in the presence of 4-chloro-1-naphthol. Infected cells with gIII on the cell surface turn black, while cells not presenting gIII on the surface remain colorless.

Single-step growth curves. PRV single-step growth curves have been described by Ryan et al. (26).

RESULTS

Rationale. We constructed three unique PRV recombinant viruses predicted to express glycosylated gIII-HIV envelope fusion proteins localized (i) only in membranes of infected cells (PRV24), (ii) in membranes of infected cells and in PRV virions (PRV33), and (iii) secreted into the medium of infected cells (PRV37).

Ryan et al. (26) described a set of PRV gIII mutants whose phenotypes guided the construction of these PRV-HIV recombinants. They noted that a polypeptide composed of the first 157 amino acids of gIII was rapidly and efficiently exported from infected cells and was not found in virus envelopes. Significantly, the polypeptide expressed from the first 157 codons of gIII could be immunoprecipitated by the polyvalent gIII serum 282 in a variety of forms, including after in vitro translation, after high-mannose glycosylation, after endo-H digestion, and after Golgi modification and

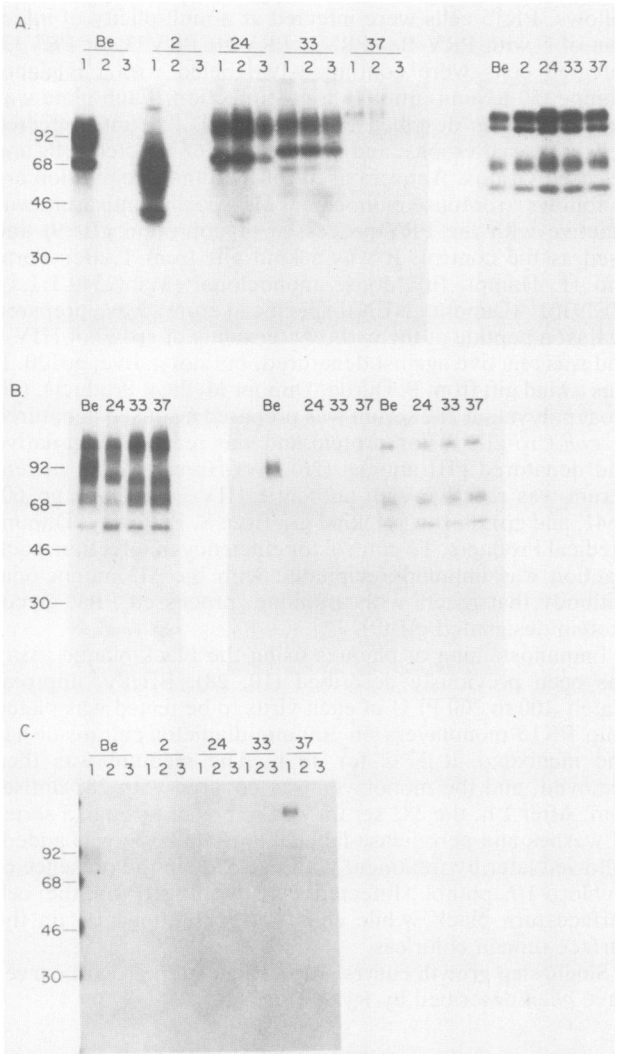

FIG. 2. Localization of wild-type and mutant forms of glycoprotein gIII. PK15 cells were infected at a multiplicity of infection of 5 and labeled continuously for 16 h with [^{3}H]glucosamine. At 16 h, the infected samples were fractionated into cell (A), virion (B), and medium (C) fractions and samples of each were used for immunoprecipitations. Proteins so precipitated were resolved on a 7 to 17% sodium dodecyl sulfate-polyacrylamide gradient gel, and fluorography was used for visualization of the ^{3}H-labeled polypeptides. The virus strain used for infection is indicated across the top of each panel. Be, PRV-Be; 2, PRV2; 24, PRV24; 33, PRV33; 37, PRV37. The molecular mass standards (in kilodaltons) are shown at the left of each panel. (A) Immunoprecipitations of infected cells. Antibodies used were gIII-specific polyvalent 282 serum (lane 1), AIDS patient sera (lane 2), and HIV-1 gp120-specific monoclonal antibody (lane 3). The five lanes on the gel to the right were immunoprecipitations with control gII-specific monoclonal antibody, M3. (B) Virions. The data are in sets of four lanes. The first four lanes are total virions, not immunoprecipitated. The second four lanes are immunoprecipitated virions with gIII-specific 282 serum. The final set of four lanes are immunoprecipitations with control PRV gII-specific antibody, M3. (C) Immunoprecipitation of medium fractions. Lane designations and antibodies are the same as in panel A.

secretion into the medium of infected cells (26). Accordingly, all three hybrid genes studied in this report encode proteins containing these identical 157 amino-terminal codons from gIII. Our expectation was that this gIII information should be sufficient for initiating protein localization at least, and, provided no membrane retention signals were contributed by the HIV sequences, would enable secretion into the medium of proteins fused in this way. The 157-amino-acid gIII peptide was also of technical advantage, since the fate of fusion proteins could be traced with 282 serum.

Ryan et al. (26) also noted that the internal 402 bp of the gIII gene defined by a *Sac*I fragment could be deleted and that the virus carrying this mutation, PRV2, expressed a truncated gIII protein that was efficiently incorporated into viral envelopes and was not secreted from the cell. It was also present in infected-cell membranes. From this observation, they deduced that the carboxy-terminal 188 amino acids, in combination with the first 157 amino acids, contained information sufficient for retention in membranes, as well as signals for incorporation into the PRV envelope. Therefore, we inserted foreign coding sequences at the novel *Sac*I site in the truncated gIII gene of PRV2 with the expectation that such hybrid proteins should be directed to specific cellular locations.

Construction and analysis of PRV24, PRV33, and PRV37. The construction of the three hybrid viruses is detailed in Materials and Methods and in Fig. 1A and C. Mutant viruses were plaque purified several times, and stocks were prepared for analysis. Viral DNA was purified from nucleocapsids of PRV-Be, PRV2, PRV24, PRV33, and PRV37, digested with *Pst*I, and analyzed by Southern blot. One half of the blot was hybridized with a 471-bp ^{32}P-labeled *Hin*dIII-*Sac*I fragment from pALM2 (23) that extended from the beginning of the gIII coding sequences to the gIII-HIV fusion junction. The second half of the blot was hybridized with a 2.7-kbp ^{32}P-labeled *Kpn*I fragment that contained essentially all the HIV-1 envelope coding sequences. The results of this analysis are shown in Fig. 1B. The upper panel shows the following results with the gIII probe. Lane 1 demonstrates that the normal gIII gene is located on a 4.3-kbp *Pst*I fragment. Lane 2 shows that PRV2 contains the expected 402-bp deletion. Lanes 3, 4, and 5 reveal the insertions within the gIII gene. PRV24 and PRV37 both contain an insertion of approximately 1.4 kbp, and PRV33 contains an insertion of 850 bp.

The second half of the blot was hybridized with the HIV-1 envelope probe and is shown in the lower panel (Fig. 1B); lanes 1 and 2 reveal no hybridization with either PRV-Be or PRV2. Lanes 3, 4, and 5 show the HIV-1 envelope-specific hybridization confined to the novel *Pst*I fragment that hybridized with the gIII-specific probe. Upon longer exposure, no other bands were observed, suggesting that the recombinant viruses have few, if any, unexpected rearrangements involving the gIII locus (data not shown).

Expression and localization of fusion proteins in infected PK15 cells. The three recombinant viruses express fusion proteins that react with both anti-gIII sera and HIV-1 antisera. We analyzed the expression and localization of each gIII-HIV envelope fusion protein by immunoprecipitation as described in Materials and Methods. Each plate was fractionated into infected cells, released virions, and medium free of cell debris and released virions as described by Ryan et al. (26). To control for efficiency of infection, each fraction was immunoprecipitated with the M3 monoclonal antibody that reacts with a unique, processed PRV glyco-

protein designated gII (9). The results of these immunoprecipitations are presented in Fig. 2.

Figure 2A shows the glucosamine-labeled proteins immunoprecipitated from the infected cells. Lane 1, under PRV-Be, revealed the characteristic steady-state labeling pattern for gIII in infected cells showing the heterogeneous 92-kilodalton (kDa) mature protein and the 74-kDa precursor protein (22, 26). A similar set of proteins was observed in lane 1 under PRV2; however, the proteins were truncated as expected, with apparent molecular masses of approximately 68 and 43 kDa. No immunoprecipitation was observed in either PRV-Be- or PRV2-infected cells with either HIV-1-specific antisera (lanes 2 and 3). Lane 2 gives results with AIDS patient sera, and lane 3 shows results with a monoclonal antibody directed against the HIV-1 exterior glycoprotein, gp120.

It is clear that all three gIII-HIV recombinant viruses produced glucosamine-labeled fusion proteins, since both the gIII-specific antibody 282 (lane 1) and the AIDS patient sera (lane 2) immunoprecipitated similar proteins. The gp120-specific monoclonal antibody recognized only the fusion proteins produced by PRV24 and PRV33 (lane 3), as expected.

The overall steady-state fusion protein profiles were similar to that for wild-type gIII. They all had a heterogeneous high-molecular-mass form of the protein and more homogeneous lower-molecular-mass species. The fusion proteins produced by PRV24 have an apparent molecular mass of 101 and 78 kDa, PRV33 fusion proteins were slightly larger at an apparent molecular mass of 104 and 86 kDa, and PRV37 fusion proteins had an apparent molecular mass of 121 and 111 kDa. It should be noted that approximately 10- to 20-fold-less gIII-HIV fusion proteins were produced. For the analysis shown in Fig. 2, approximately five times the amount of immunoprecipitated cell extract was loaded for each of the mutants. In contrast, for all virus infections, virtually identical amounts of the control PRV gII glycoprotein family were observed with no apparent change in its processing pattern (lanes Be, 2, 24, 33, and 37). This suggested that the low level of gIII-HIV fusion proteins was not due to a less than vigorous infection. A more likely reason for this lower than expected level of expression lies primarily at the level of chimeric gene mRNA stability and is considered below.

Both PRV24 and PRV33 should express proteins containing the protease cleavage site present in native HIV-1 gp160; however, we found little evidence for processing of either fusion protein in infected cells. We considered the possibility that PK15 cells may lack the proper protease and repeated these experiments in cells known to have the correct activity, including BSC-40 cells (12) and HeLa cells (5). The profiles were essentially the same as presented in Fig. 2 (data not shown). It remains a possibility that PRV infection shuts off the cellular protease responsible for cleavage of HIV-1 envelope proteins.

Further evidence for lack of processing of the fusion proteins is presented in the immunoprecipitation of the medium fractions (Fig. 2C). If the fusion proteins were processed at the gp120-gp41 junction, the amino-terminal fragment should be secreted into the medium. A control for this is the fusion protein expressed by PRV37. PRV37 contains a hybrid gene with a nonsense termination codon at the gp120-gp41 junction. The gIII-gp120 fusion protein produced by this virus should approximate the behavior of the predicted protease cleavage product of PRV24 and PRV33. Since neither gp120 nor the first 157 amino acids of gIII contain predicted transmembrane or anchoring sequences, the gIII-gp120 fusion protein should be excreted into the medium from PRV37-infected cells. It is clear that only PRV37 infection produced a protein that accumulated in the medium (Fig. 2C). Nothing was detected, even upon longer exposure, in the medium of PRV24- or PRV33-infected cells. The highly glycosylated, secreted form of the hybrid protein reacted only with gIII-specific antisera and not AIDS patient sera or HIV-1 envelope antisera.

The analysis of purified, glucosamine-labeled virions is shown in Fig. 2B. The whole virion profile (not immunoprecipitated) for PRV-Be, PRV24, PRV33, and PRV37 is presented in the left-hand portion. The three recombinants had a noticeable lack of gIII, a diffuse 92-kDa glycoprotein that was present in PRV-Be. The three gIII-HIV recombinants did not show any obvious compensatory increase in other labeled glycoproteins. When these same virion samples were immunoprecipitated with gIII-specific 282 antiserum (shown in the center four lanes of panel B), no gIII-HIV fusion proteins were detected, even though six times the amount of virus was used for each of these immunoprecipitations. The lane labeled Be in this set shows the mature gIII protein in PRV-Be virions. The final set of four lanes in panel B showed that essentially identical quantities of processed gII proteins were immunoprecipitated from all virions.

gIII-specific RNA in infected cells. The low level of fusion protein expression by each recombinant virus can be accounted for by mRNA instability and improper processing. The evidence for this conclusion is described below. Total cytoplasmic RNA present at 12 h after infection from wild-type and chimeric gIII genes was analyzed in slot blot and Northern blot assays, as described in Materials and Methods (Fig. 3A, B, and C). The left half of Fig. 3 contains the slot blots, and the right half contains the Northern blots. The gIII and HIV-1 envelope probes used in the Northern blots were the same as those used in the Southern blot analysis. In the slot blot analysis, three probes were prepared: one that hybridized to the 5′ gIII mRNA up to the fusion junction (Fig. 3A), one that hybridized to the 3′ gIII mRNA sequences downstream of the fusion junction (Fig. 3B), and one that hybridized only to the HIV-1 envelope sequences (Fig. 3C).

Uninfected-cell RNA showed no hybridization with any of the probes in slot blot and Northern blot assays (Fig. 3A, B, and C, lane M). Densitometer analysis of the slot blots demonstrated that in comparison to PRV-Be or PRV2, the hybrid viruses PRV24, PRV33, and PRV37 produced significantly less gIII-specific message. When a probe that is 5′ to the fusion junction was used, the level of gIII-specific message produced by these viruses decreased threefold (Fig. 3A). The differences in relative amounts of specific RNA between these viruses was more apparent with the 3′ probe when PRV24 decreased sixfold, PRV33 decreased fivefold, and PRV37 decreased ninefold (Fig. 3B). With the HIV-1 envelope-specific probe (Fig. 3C), no hybridization was seen to control, PRV-Be, or PRV2 RNA. Significant and similar levels of HIV-specific RNA were produced by the recombinants.

Northern blot analysis of these same RNA fractions using identical probes indicated that not only was the absolute level of chimeric RNA reduced but that little full-length RNA was produced (Fig. 3, right-hand panels). The gIII-specific transcript of PRV-Be was 1.55 kilobases (Fig. 3, top right panel, lane Be) which was replaced by a 1.15-kilobase transcript in PRV2 (lane 2). PRV33 (lane 33) produced barely detectable gIII-specific transcripts of approximately the pre-

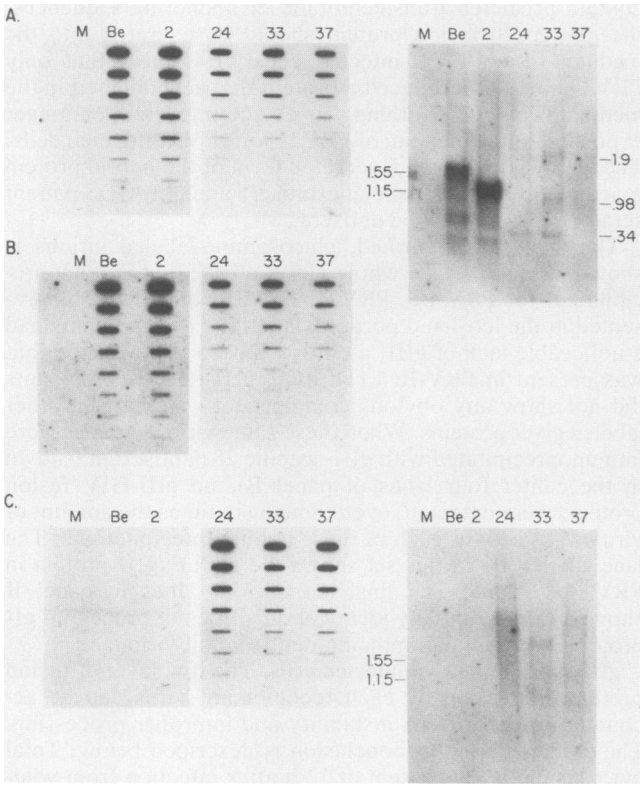

FIG. 3. Northern and slot blot analysis of RNA extracted from uninfected, wild-type and mutant PRV-infected PK15 cells. The slot blots are on the left in each panel, and the Northern blots are on the right. Total cytoplasmic RNA was extracted at 12 h postinfection. For slot blots, 20 μg of each RNA was serially diluted twofold, denatured, and subsequently transferred to nitrocellulose in a slot blot apparatus. For Northern blots, 5 μg of each RNA was denatured and fractionated on an agarose-formaldehyde gel and transferred to nitrocellulose for subsequent hybridization. The probes used were as follows: (A) A 471-bp *Hin*dIII-*Sac*I fragment from pALM2 (23) that hybridized only to the 5′ gIII mRNA up to the fusion junction, (B) a 504-bp *Sac*I-*Bam*HI fragment of pALM2 (23) that hybridized only to the 3′ gIII mRNA downstream of the fusion junction, and (C) a 2.7-kbp *Kpn*I fragment that hybridized only to HIV-1 envelope sequences. In the Northern blots shown on the right, the wild-type band specific for gIII and the PRV2-derived, truncated band specific for gIII are indicated on the left (in kilobases). The novel RNA species produced by PRV24, PRV33, and PRV37 are indicated. The upper blot was hybridized with the probe used in panel A, and the lower blot was hybridized with the probe used in panel C. Lanes: M, mock infected; Be, PRV-Be; 2, PRV2; 24, PRV24; 33, PRV33; 37, PRV37.

dicted size (1.9 kilobases), as well as two smaller transcripts of approximately 980 and 340 bases. No full-length transcript were detected from either PRV24 or PRV37 (lanes 24 and 37, respectively). Only a 340-base transcript was visible in PRV24-infected-cell RNA. Hybridization using the HIV-1 probe (Fig. 3, lower right panel) revealed only a smear of hybridizing RNA and no distinct transcripts. As expected, the HIV-1 probe did not hybridize to any messages produced by PRV-Be or PRV2.

The Northern and slot blots together demonstrated that at 12 h postinfection, cells infected with the three gIII-HIV recombinants contained less gIII-specific RNA compared with cells infected with PRV-Be or PRV2. Moreover, little or no full-length message was detectable with these viruses.

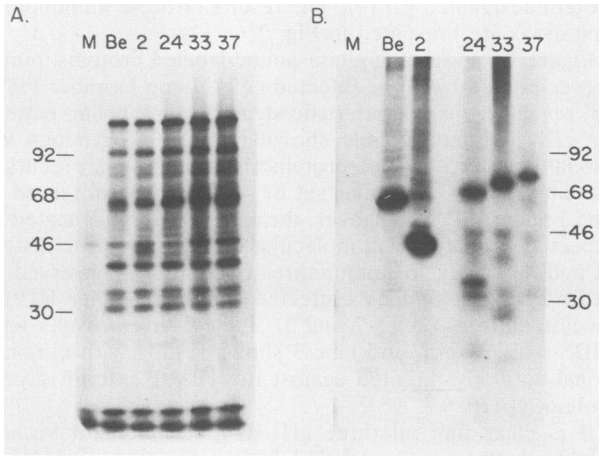

FIG. 4. In vitro translation of RNA extracted from uninfected cells and from cells infected with wild-type and mutant PRV virus. Total cytoplasmic RNA was extracted at 12 h postinfection and translated in a rabbit reticulocyte system with [^{35}S]methionine. (A) Fluorogram of total translation products fractionated on a 7 to 17% sodium dodecyl sulfate-polyacrylamide gradient gel. (B) Fluorogram of in vitro-translated polypeptides immunoprecipitated with gIII-specific 282 serum fractionated on a 7 to 17% sodium dodecyl sulfate-polyacrylamide gradient gel. Note that the data in Fig. 4B was obtained by loading four times the amount of immunoprecipitated protein for PRV24, PRV33, and PRV37 in comparison to the amount loaded for PRV-Be and PRV2. Molecular mass markers (in kilodaltons) are indicated on either side. Lanes: M, mock infected; Be, PRV-Be; 2, PRV2; 24, PRV24; 33, PRV33; 37, PRV37.

Given that these chimeric genes had 471 bp of normal gIII sequences on their 5′ and 3′ ends, one explanation consistent with these observations is that the chimeric RNAs are degraded or abnormally processed.

In vitro translation. The slot blot and Northern blot experiments addressed the question of RNA quantity and size, and the following in vitro translation experiments addressed the ability of the isolated RNA to be translated.

Total cytoplasmic RNA from uninfected and infected cells was prepared as described in Materials and Methods. After in vitro translation of equal amounts of RNA, a portion was immunoprecipitated with the gIII-specific antibody 282 and run with total translation products on a 7 to 17% sodium dodecyl sulfate-polyacrylamide gel (Fig. 4).

The lanes containing the total translation products (Fig. 4A) revealed several points. Most notable was that, other than the specific gIII protein produced, there were no major differences in the translation products. This corroborated our earlier conclusion from steady-state labeling of glycoproteins that the gIII-HIV recombinants established a normal infection at high multiplicity. Panel B shows the immunoprecipitated translation products. As predicted, the PRV-Be gIII primary translation product had an apparent molecular mass of 57 kDa (predicted to be 51 kDa from primary DNA sequence) and the truncated gIII gene of PRV2 gave a primary translation product when an apparent molecular mass of 44 kDa (predicted to be 37 kDa from primary DNA sequence). The discrepancy in apparent molecular mass versus predicted molecular mass has been noted by Robbins et al. (22) and is due presumably to the proline-rich primary amino acid sequence of gIII.

The predicted molecular mass of the gIII-HIV fusion protein expressed by PRV24 should be 60 kDa. Immunoprecipitation revealed a gIII-specific protein of 67 kDa, as well

as two smaller proteins of approximately 34 and 30 kDa. PRV33 is predicted to encode a fusion protein of 68 kDa. A gIII-specific protein of 70 kDa and two smaller proteins of approximately 41 and 23 kDa are immunoprecipitated. The translation of PRV37 RNA resulted in one major gIII-specific protein of 71 kDa. The predicted molecular mass of the PRV37 fusion protein was 69 kDa. For all three constructions, the major gIII-specific protein detected by in vitro translation corresponded well to that predicted from the DNA sequence. The smaller polypeptides are not easily explained without further analysis. There were no obvious gIII-specific messages in the Northern blots that could result in the 34- or 30-kDa proteins of PRV24 or the 23-kDa protein of PRV33. The 41-kDa protein, however, could result from the 980-base transcript of PRV33.

The quantity of immunoprecipitated protein for the three recombinant virus translations was substantially reduced compared with the amount recovered for PRV-Be and PRV2. The data in Fig. 4B was obtained by loading four times the amount of immunoprecipitated protein for PRV24, PRV33, and PRV37 compared with the amount loaded for PRV-Be and PRV2. This would indicate that at 12 h postinfection, the amount of functional chimeric RNA in the recombinants is reduced by approximately 5- to 10-fold compared with PRV-Be or PRV2 infection. This corresponded reasonably well with the conclusions from in vivo RNA and steady-state protein level experiments. We conclude that a major reason for the reduced expression of fusion protein is a lower level of functional chimeric RNA due to instability or abnormal processing.

Endoglycosidase analyses. Ryan et al. (26) have demonstrated by endoglycosidase analysis that the two forms of wild-type gIII seen by immunoprecipitation result from different states of glycosylation. The 74-kDa, less-diffuse species is a precursor with core glycosylation only and is completely sensitive to endo-H. The diffuse 92-kDa species represents highly processed gIII where, in the Golgi organelle, the high-mannose core glycosylated residues have been converted to complex carbohydrates and further significant modification, perhaps O-linked glycosylation, has occurred. This conclusion was based on the resistance of the 92-kDa species to endo-H treatment and at least partial sensitivity to endo-F (26).

Endo-H analysis of the two forms of fusion protein labeled by glucosamine is shown in Fig. 5. Infected cells were labeled with [^{3}H]glucosamine continuously until 16 h postinfection. The infected cells were harvested, gIII was immunoprecipitated with anti-gIII 282 serum, and the precipitate was digested with endo-H as described previously (26). Lanes labeled C are control, untreated samples, and lanes labeled H are endo-H treated. Treatment with endo-H removed core glycosylation from the 74-kDa form of wild-type gIII, giving rise to a 58-kDa product (Fig. 5B, lane H). Each glucosamine-labeled fusion protein (Fig. 5A, lanes 24, 33, 37, and H) reacted similarly to endo-H digestion, in that the lower-molecular-mass species was sensitive, resulting in a shift in apparent molecular mass of 13 kDa for PRV24, 10 kDa for PRV33, and 28 kDa for PRV37. The higher, more-diffuse molecular mass forms of PRV24 and PRV33 fusion proteins remained completely resistant to endo-H. In contrast, the corresponding form of PRV37 (121 kDa) was partially sensitive, shifting approximately 11 kDa, indicating the presence of high mannose or core glycosylation on this apparently mature species.

This data is complemented by similar experiments with endo-F (data not shown). Endo-F has been shown to remove not only the high-mannose core glycosylation but also the complex forms of N-linked side chains. Treatment with endo-F had a similar effect as endo-H on the migration of the lower-molecular-mass forms of the various gIII-specific glycoproteins. Moreover, treatment with this enzyme converted the higher-molecular-mass species into a faster-migrating but still-diffuse form, indicating that other modifications, perhaps O-linked glycosylation, had occurred. In the case of PRV37, the diffuse endo-F-cleaved species had a lower molecular mass than the endo-H-cleaved species, which suggests partial conversion from core glycosylation to complex carbohydrate side chains had occurred.

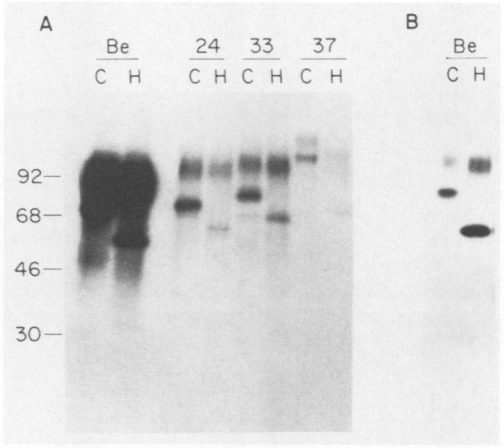

FIG. 5. Endo-H treatment of wild-type and mutant forms of glycoprotein gIII. PK15 cells were infected with PRV strains at a multiplicity of infection of 5 and radiolabeled with [^{3}H]glucosamine continuously for 16 h postinfection. At this time, monolayers were harvested and gIII species were immunoprecipitated with 282 serum. Prior to fractionation on a 7 to 17% sodium dodecyl sulfate-polyacrylamide gel, the immunoprecipitated polypeptides were either not treated (control lanes [labeled C]) or treated with endo-H (lanes labeled H). Polypeptides were visualized by fluorography. The molecular mass standards (in kilodaltons) are indicated on the left. The infecting virus is indicated across the top. Panel B is a shorter exposure of the PRV-Be lanes in panel A.

Cell-surface localization of fusion proteins. Infected PK15 cells were used for localization of gIII-specific antigens using the black-plaque assay, as described in Materials and Methods (Fig. 6). This assay identified those cells within a plaque expressing cell-surface gIII molecules capable of reacting with gIII-specific 282 serum; internal gIII proteins were not available to react with the antiserum. The data in Fig. 6 are photographs of plaques of PRV-Be, PRV24, PRV33, and PRV37, prepared for the black-plaque assay. Individual infected cells were clearly visible in each plaque. PRV-Be was the wild-type virus and gave rise to black plaques as expected. All infected cells are stained more or less uniformly (Fig. 6A). The PRV24 plaques were clearly reactive, but staining was not as dark as PRV-Be (Fig. 6B). PRV33 plaques were less reactive than PRV24 plaques but were clearly reactive (Fig. 6C). PRV37 plaques were nonreactive (Fig. 6D) and indistinguishable in color from a gIII null mutation (data not shown). These results were corroborated by standard indirect immunofluorescence techniques using unfixed cells, 282 serum, and rhodamine-conjugated second antibody (data not shown). We conclude that PRV24 and PRV33, and not PRV37, expressed fusion proteins that are presented on the cell surface.

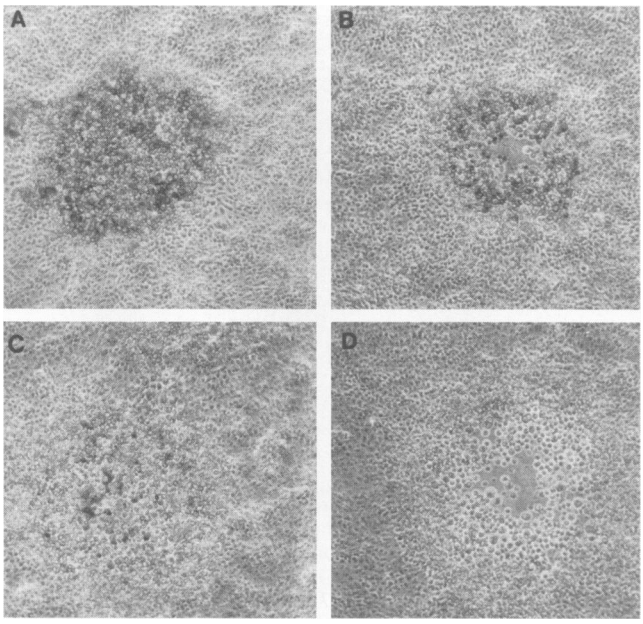

FIG. 6. Black-plaque analysis of PRV-Be and mutant viruses. Virus stocks were diluted, and approximately 100 PFU were plated on PK15 cells in 100-mm-diameter dishes. After plaques were visible, the medium was removed and the monolayer was covered with 282 antiserum. After washing away the 282 serum, peroxidase-labeled anti-goat antibody was added and followed later by treatment with peroxide in the presence of 4-chloro-1-naphthol. Infected cells with gIII accessible to 282 serum turn black, while cells not presenting gIII remain colorless. Pictured are micrographs of single representative plaques from wild-type PRV-Be (A), PRV24 (B), PRV33 (C), and PRV37 (D). Magnification, ×56.

Single-step growth curves. As has been shown previously, PRV virions lacking wild-type gIII in their envelopes grow to somewhat lower titers in PK15 cells (13, 26, 31). We have suggested that one reason may be defective release of gIII-deficient virions from the cell. It was of interest to see if this phenotype was expressed by viruses with hybrid gIII genes and if the unusual envelope fusion proteins would have other effects on virus yield. Single-step growth curves of the three gIII-HIV recombinants measuring intracellular and extracellular infectious virus are given in Fig. 7. PRV2 (truncated gIII) and the three recombinants reproducibly gave reduced numbers of total PFU throughout the experiment, yet their appearance inside the infected cell paralleled that for PRV-Be. PRV24, PRV33, and PRV37 exhibited a significantly lower number of infectious particles released into the medium. PRV33 and PRV37 also had a significant lag in infectious virus release from infected cells. The 6-h lag exhibited by PRV33 is particularly striking. By 24 h in a normal PRV-Be infection, more infectious particles were found in the medium than associated with the cell. This is to be contrasted with PRV2, PRV24, PRV33, and PRV37 when more cell-associated infectious particles than released particles were found.

DISCUSSION

These experiments demonstrated the construction of infectious PRV recombinants expressing novel hybrid envelope glycoprotein genes composed of the nonessential PRV gIII gene and the HIV-1 envelope genes. Such hybrid proteins were glycosylated and recognized by gIII-specific antisera as well as HIV-1 envelope antisera, including AIDS patient sera. The expression of these hybrid genes was lower than expected, primarily due to instability or aberrant processing of the chimeric RNA.

The gIII signal sequence functioned in these fusion proteins to initiate entrance to the membrane protein export pathway. PRV24 expressed a fusion protein with glycosylation modifications consistent with it reaching a Golgi or post-Golgi compartment; however, the protease cleavage site defining the junction of gp120 and gp41 present in the primary sequence of this hybrid gene was apparently not recognized. Similarly, the fusion protein expressed by PRV33 contained gIII sequences that were sufficient to direct the protein to the cell surface as well as the PRV virus envelope; however, it was poorly detected on the cell surface and not detected in the virus envelope.

The low, but detectable presentation of PRV24 and PRV33 gIII-HIV fusion proteins on the cell surface is consistent with their lower intracellular level of expression. It is also possible that some fraction of these fusion proteins reached the cell surface but was rapidly endocytosed and degraded as was the case for a vesicular stomatitis virus G-herpes simplex virus gC hybrid protein (25).

PRV37 expressed a gIII-HIV fusion protein lacking transmembrane or anchoring functions of both gIII and gp41. This protein was expressed at a low level and was secreted into the medium. Both the intracellular and extracellular proteins were highly glycosylated, reflecting the large number of potential N-linked glycosylation sites encoded in the HIV-1 gp120 sequences. Not predicted however, was our observation that the highly glycosylated, secreted form of the hybrid protein reacted only with gIII-specific antisera and not AIDS patient sera or HIV-1 envelope antisera. The size of the secreted species is not detectably different from the reactive species from infected cells, so loss of reactivity is not due to massive proteolysis. Further work must be done to determine how the HIV-1 sequences were masked.

We did not expect to find the fusion proteins of PRV24 or PRV37 in the virus envelope, since both lacked the carboxy-terminal amino acids of gIII thought to contain the transmembrane, anchoring sequences or envelope localization sequences. We did, however, expect to find the PRV33 fusion protein in the virus envelope, since it is simply an insertion into the truncated gIII gene of PRV2 whose product is efficiently inserted into viral envelopes (26). The protein is produced and receives modifications characteristic of passage through the Golgi yet is presented poorly on the cell surface and is not detectable in virus envelopes. Since the PRV capsids are thought to be enveloped initially at the inner nuclear membrane, apparently this fusion protein either cannot be localized or retained there because of signals in the HIV-1 envelope residues, or the protein is folded improperly and never reaches the site of PRV envelopement. One possible explanation may be that the protein has not only the predicted gIII transmembrane but also a portion of HIV-1 gp41 transmembrane sequence. This new transmembrane segment could cause improper insertion in membranes so that the fusion protein cannot be localized or retained in the inner nuclear membrane for initial envelopment of PRV capsids. To test these ideas, a number of experiments come to mind, including selective removal of segments of the HIV-1 envelope residues in the hybrid gene. These experiments are in progress.

We did not find the inappropriate localization of herpesvirus signal sequence-HIV-1 fusion proteins that was noted

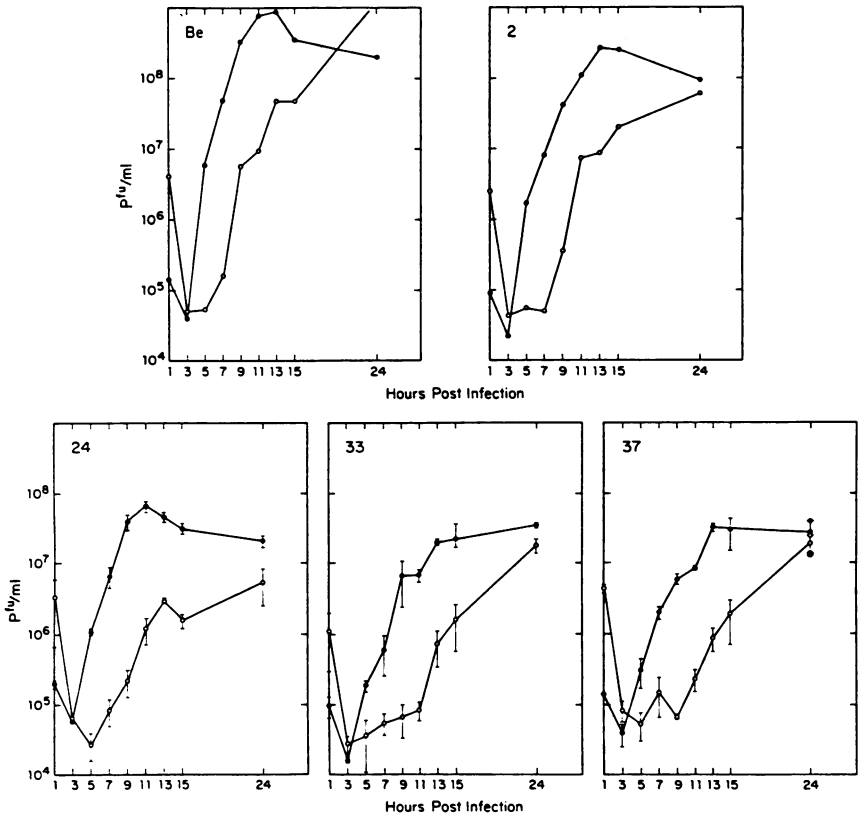

FIG. 7. Single-step growth of PRV-Be and mutant viruses. PK15 cells were infected with PRV strains at a multiplicity of infection of 5 and incubated at 37°C. At 1, 3, 5, 7, 9, 11, 13, 15, and 24 h postinfection, plates were harvested, and the virus titers of the cell and medium fractions were determined separately and plotted. The infecting virus strain is indicated in the upper left corner of each plot. Abbreviations: Be, PRV-Be; 2, PRV2; 24, PRV24; 33, PRV33; 37, PRV37. Symbols: ●, cell-associated virus; ○, virus found in the medium. With the exception of the PRV-Be and PRV2 growth curves, the results are the average of two independent experiments. The error bars represent the range of the two values.

previously in engineered cell lines. Laskey et al. (15) constructed mammalian cell lines expressing an HIV-1 envelope fusion protein using the first 50 amino acids of herpes simplex virus type 1 gD to furnish a functional signal sequence replacing the amino terminus of the HIV-1 gp160 envelope precursor. The removal of the HIV-1 gp160 amino-terminal sequences was essential for efficient expression. Subsequently, expression of such full-length gD-HIV envelope fusion proteins resulted in the production of a highly glycosylated protein that was retained in intracellular compartments with no evidence of proteolytic processing or export to the cell surface (15). When essentially all of gp41 was deleted, the desired truncated protein was secreted into the medium.

Our results with infectious PRV recombinant viruses are somewhat reminiscent of these findings in cell lines. One hypothesis is that the hybrid proteins fold improperly, blocking correct modification and cellular localization. This aberrant folding is not too severe, since the PRV-expressed proteins apparently leave the endoplasmic reticulum, pass into the Golgi apparatus (deduced by carbohydrate modifications), and appear on the cell surface or in the medium as predicted.

The defect in accumulation of functional hybrid mRNA is striking. The expression level of HIV-1 envelope sequences is highly regulated, involving transcriptional and translational control, both in HIV-1 infections or in transfection experiments with plasmids (7, 14). Since the HIV-1 *tat* or *trs/art* functions known to be required for efficient envelope gene expression are not present in PRV infection of PK15 cells, it is possible that their absence leads to the mRNA defect. We think this is unlikely, since HIV-1 envelope gene expression in other heterologous systems (e.g., vaccinia virus and simian virus 40) did not require *tat* or *trs/art* (5, 12, 14). In addition, preliminary experiments involving infection of cell lines expressing the HIV-1 regulatory proteins with the PRV recombinants gave essentially the same results as infection of PK15 cells (unpublished observations). Furthermore, the RNA defect may be idiosyncratic of hybrid gIII genes, since we have observed similar reduced expression phenotypes with gIII hybrids made with a variety of foreign proteins, including *E. coli* LacZ (13) and herpes simplex virus type 1 gC (unpublished observations). We have not ruled out the possibility that PRV infection itself may result in this general hybrid RNA phenotype.

We have established that PRV gIII mutants are somewhat defective in virus release in PK15 cells (26, 31). It is therefore interesting that these three gIII-HIV recombinants have a more pronounced release defect than a complete gIII deletion (PRV10 [26, 31]). For expression of such a phenotype, these hybrid proteins must interfere with normal PRV egress, perhaps either as a consequence of being brought to virus assembly points by gIII signals or by simply blocking PRV egress as a consequence of improper folding or aggregation. It will be informative to see if these proteins exert a dominant effect in coinfection with wild-type virus.

In conclusion, our experiments show that PRV can be used to express foreign membrane fusion proteins and that some, but not all, of the localization information thought to be part of the gIII glycoprotein can be exploited. The expression levels are significant, although not yet optimized, and the proteins produced are glycosylated and recognized by appropriate antibodies. Given the broad host range of PRV (2, 8), the foreign proteins can be produced in a wide variety of cell types. These results and those of Thomsen et al. (30) demonstrate that PRV represents a useful herpesvirus vector for production of foreign proteins. Potential applications of this approach are in the production of novel diagnostic antigens and immunogens, as well as the creation of safe live-virus vaccines for animals.

ACKNOWLEDGMENTS

S. Petteway and the members of his group (Applied Biotechnology Group, Dupont Medical Products) provided essential materials and information on HIV-1. Paul Durda (Dupont Medical Products) provided antisera and instruction on their use. We thank Pat Ryan, Nels Pederson, and the members of the Central Research and Development Molecular Genetics group for suggestions and comments on the work.

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