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Evolution of Poliovirus Defective Interfering Particles Expressing *Gaussia* Luciferase

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Polioviruses (PVs) carrying a reporter gene are useful tools for studies of virus replication, particularly if the viral chimeras contain the polyprotein that provides all of the proteins necessary for a complete replication cycle. Replication in HeLa cells of a previously constructed poliovirus expressing the gene for *Renilla* luciferase (RLuc) fused to the N terminus of the polyprotein H_2N-RLuc-P1-P2-P3-COOH (P1, structural domain; P2 and P3, nonstructural domains) led to the deletion of RLuc after only one passage. Here we describe a novel poliovirus chimera that expresses *Gaussia* luciferase (GLuc) inserted into the polyprotein between P1 and P2 (N_2H-P1-GLuc-P2-P3-COOH). This chimera, termed PV-GLuc, replicated to 10% of wild-type yield. The reporter signal was fully retained for three passages and then gradually lost. After six passages the signal was barely detectable. On further passages, however, the GLuc signal reappeared, and after eight passages it had reached the same levels observed with the original PV-GLuc at the first passage. We demonstrated that this surprising observation was due to coevolution of defective interfering (DI) particles that had lost part or all of the capsid coding sequence (ΔP1-GLuc-P2-P3) and wild-type-like viruses that had lost the GLuc sequence (P1-P2-P3). When used at low passage, PV-GLuc is an excellent tool for studying aspects of genome replication and morphogenesis. The GLuc protein was secreted from mammalian cells but, in agreement with published data, was not secreted from PV-GLuc-infected cells due to poliovirus-induced inhibition of cellular protein secretion. Published evidence indicates that individual expression of enterovirus polypeptide 3A, 2B, or 2BC in COS-1 cells strongly inhibits host protein secretion. In HeLa cells, however, expression of none of the poliovirus polypeptides, either singly or in pairs, inhibited GLuc secretion. Thus, inhibition of GLuc secretion in PV-infected HeLa cells is likely a result of the interaction between several viral and cellular proteins that are different from those in COS-1 cells.

Despite decades of genetic and biochemical studies, many details of poliovirus (PV) replication, including morphogenesis, remain unknown. Since *in vivo* poliovirus RNA replication is stringently dependent upon translation in *cis* (33, 49) and because encapsidation, in turn, requires RNA replication in *cis* (1, 33, 34, 45, 71), it is difficult to design reliable experiments that distinguish between defects in viral replication and morphogenesis.

Poliovirus belongs to the genus *Enterovirus* of the *Picornaviridae* and contains a 7.5-kb-long RNA genome encoding only one protein, the polyprotein. This polypeptide consists (N terminus to C terminus) of a structural domain (P1) and two nonstructural domains (P2 and P3), all of which are processed into functional precursor and mature polypeptides (70).

Recombinant enterovirus replicons containing a reporter gene have been used for several years in studies of protein translation and RNA replication. The most commonly used reporters have been light-emitting luciferases derived either from the firefly (firefly luciferase [FLuc]) (20, 38) or the sea pansy (*Renilla* luciferase [RLuc]) (40). To retain genetic stability, these reporter genes originally replaced the P1 capsid-encoding sequence (Luc-P2-P3) (4, 16, 73). These replicons, however, cannot be used in studies of virus morphogenesis unless the capsid proteins are supplied in *trans* (37, 54). Reporter viruses that contain a reporter gene fused to the N terminus of the polyprotein have also been developed (25). During infection of HeLa cells with the RLuc-P1-P2-P3 chimera, the reporter polypeptide is released from the viral polypeptide by the viral proteinase $3CD^{pro}$ (37). The advantage of this reporter system is that it allows one to distinguish between effects on RNA replication and those on encapsidation (37). However, because of the rigidity of the poliovirus capsid and the relatively large size of the parental genome (RLuc-P1-P2-P3), there is rapid selection of genomes from which the foreign gene has been spontaneously deleted during RNA synthesis. In the case of reporter viruses containing the RLuc gene (933 nucleotides [nt]), deletion commences during the first passage on HeLa cells (37). It should be noted that besides luciferase reporters, other marker proteins have also been engineered into the complete poliovirus polyprotein (59–61). These chimeras are expressing marker proteins in nonstructural proteins $2A^{pro}$ and 3A. They have been constructed predominantly with the aim of providing a method to visualize their subcellular localizations.

Here we describe the development and characterization of a novel recombinant reporter virus in which the smaller gene of the *Gaussia* luciferase (GLuc; 555 nt, encoding 185 amino acids) from the marine copepod *Gaussia princeps* was engineered into the viral polyprotein. GLuc is a remarkably stable polypeptide (57) that, in contrast to the larger luciferases, does not require ATP for activity (5). The humanized GLuc protein generates a much stronger (1,000-fold) signal intensity from cells in culture than RLuc or FLuc (57, 65). Notably, GLuc is secreted, a property that allows detection in the growth medium of cells transformed with appropriate expression vectors.

Experiments with PV-GLuc revealed that the luciferase gene

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and its intracellular expression were retained for three passages but were then gradually lost. On further passage, however, the GLuc signals reemerged and eventually reached the same level observed with the original PV-GLuc during the first passage. We found that this was due to the coevolution of two kinds of replicating genomes: (i) wild-type (wt)-like genomes (P1-P2-P3), the helper viruses, with most or all of the GLuc gene deleted and (ii) genomes lacking much of the P1 coding sequence but containing a complete GLuc gene. The latter evolved to form defective interfering (DI) particles that are only rarely formed in the life cycle of poliovirus in tissue culture (48, 70).

During enterovirus infection, host cells undergo metabolic and morphological alterations that are induced by diverse functions of viral nonstructural proteins. Most dramatic are the inhibition of cellular protein and cellular RNA synthesis, the rearrangement of existing cytoplasmic membranous structures, and the *de novo* synthesis of new vesicles (6, 9–11, 18, 55, 56, 58). In addition, the viruses induce a large number of changes in the host cell that are designed to favor viral replication and, at the same time, paralyze host cell defense mechanisms (1, 42, 56).

One of the profound changes induced by enterovirus infection is the inhibition of protein secretion (23, 24, 41). For example, infection of COS-1 cells with enteroviruses such as poliovirus or coxsackie B virus type 3 (CVB3) or the individual expression of enterovirus proteins in target cells (e.g., 3A or 2B/2BC) blocks endoplasmic reticulum (ER)-to-Golgi apparatus traffic and results in the inhibition of cellular protein secretion (23, 24, 64). Interestingly, it was shown that CVB3 3A inhibits transport by binding to cellular factor GBF-1 and inhibiting GBF-1 dependent COP-1 recruitment to membranes (43, 44). Polypeptides 3A and 2B and their precursors, 3AB and 2BC, contain hydrophobic transmembrane regions that play a role in their effect on secretion (2, 26, 62). In the case of foot-and-mouth disease virus (FMDV), a picornavirus of the genus *Aphthovirus*, the 2BC precursor polypeptide rather than 3A or 2B is responsible for the block in the secretory pathway (43, 44).

As expected, secretion of the GLuc protein from PV-GLuc-infected cells was inhibited. In experiments designed to pinpoint the PV proteins responsible for the block of GLuc secretion, we made the surprising observation that individually 3A, 2B, 2C, 2BC, and 3AB had either no or only a slight inhibitory effect on secretion in HeLa cells. This is different from reports of inhibition of protein secretion from COS-1 cells (23, 24). However, it was confirmed that GLuc is not secreted from COS-1 cells if coexpressed with the PV 3A polypeptide. Therefore, we suggest that the mechanism of GLuc secretion may be different in different cell types. In HeLa cells, inhibition appears to be the result of interaction between several viral and cellular proteins; thus, the biochemical properties of one or more of the HeLa proteins may be distinct from those of COS-1 or other tissue culture cells. Although GLuc does not have the advantage of being a secreted protein in PV GLuc-infected cells, this reporter virus promises to be a useful tool in studies of RNA replication and morphogenesis because of its increased genetic stability.

MATERIALS AND METHODS

Cell culture. HeLa R19 and COS-1 cell monolayers were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine calf serum (BCS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. All cells were grown at 37°C in a 5% CO_2 incubator.

Plasmids. (i) pCMV-GLuc. pCMV-GLuc, containing the GLuc coding sequence, was purchased from New England BioLabs (NEB).

(ii) pPV-GLuc. The pPV-GLuc plasmid was constructed from pT7PVM, which contains the cDNA of type 1 poliovirus [PV1(M)]. The GLuc coding sequence was inserted between the P1 and P2 domains of the PV polyprotein. The P1 domain was linked at its N terminus to a linker of 27 nt encoding an ENLTTY amino acid sequence. At its C terminus, the P1 domain was fused to a linker of 9 nt (GGA*GAATTC*; the EcoRI site is in italics) followed by the GLuc coding sequence. The C terminus of GLuc was fused to a modified FMDV 2A sequence (63 nt) terminated with the amino acid sequence ELTTY, which forms a PV $2A^{pro}$ cleavage site together with the first amino acid (G) of P2.

(iii) Dicistronic reporter plasmids. pCMV-T7-GFP/viral protein-HCV IRES/GLuc (HG) plasmids contain the cytomegalovirus (CMV) promoter and the T7 RNA polymerase promoter linked to any of the following protein coding sequences followed by a termination codon: PV 3A, PV mutant 3A (first 10 amino acids deleted), or enhanced green fluorescent protein (eGFP). The second cistron contains the GLuc and the FMDV 2A coding sequences and a termination codon. Between the two cistrons, the hepatitis C virus (HCV) internal ribosome entry site (IRES) was inserted. The nontranslated region and the poly(A) signal at the 3′ end were derived from plasmid pCMV-GLuc (NEB).

(iv) Tricistronic reporter plasmids. pCMV-3A-encephalomyocarditis virus (EMCV) IRES-GFP/viral protein-HG plasmids contain the CMV and T7 RNA polymerase promoters linked to PV 3A. The second cistrons contain any of the following protein coding sequences followed by a termination codon: 2B, 2C, 2BC, or eGFP. An EMCV IRES was inserted between PV 3A and the second cistron. The third cistron contains the GLuc sequence and a termination codon. Between the second and the third cistrons, the HCV IRES was inserted. The nontranslated region and the poly(A) signal at the 3′ end were derived from plasmid pCMV-GLuc.

Virus growth. Viruses were amplified by infection of HeLa R19 cell monolayers with ≥5 PFU per cell. Infected cells were incubated in DMEM (1% BCS) at 37°C until a complete cytopathic effect (CPE) was detected. After three rounds of freezing and thawing, the lysate was clarified of cell debris by a brief high-speed centrifugation and the supernatant containing the virus was used for further passaging or reinfection.

DNA transfection. Plasmid DNAs (1 μg per transfection) were introduced into HeLa or COS-1 cells growing in 12-well dishes using Lipofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. After 3 to 4 h at 37°C, DMEM was replaced with 1 ml fresh DMEM containing 10% BCS, and incubation was continued overnight. At different time points, samples of the growth medium and cell lysates were analyzed for luciferase activity. For quantitation of GFP expression, a fluorescence image was taken at 24 h posttransfection.

***In vitro* transcription and RNA transfection.** Templates for different viral RNAs were cut by EcoRI or DraI, followed by phenol-chloroform extraction and ethanol precipitation. About 1 μg of purified template was transcribed by T7 RNA polymerase (Stratagene), and the integrity of the RNAs was examined by agarose gel electrophoresis. Between 10 and 15 μl of fresh transcription reaction mixture containing between 1 and 10 μg of transcript RNA was used to transfect HeLa R19 cells on a 35-mm-diameter plate by the DEAE-dextran method as described previously (63). After a 30-min incubation at room temperature, the supernatant was removed and the cells were incubated at 37°C in 2 ml of DMEM containing 1% BCS until CPE appeared. Virus titers were determined by a standard plaque assay on HeLa R19 cell monolayers using a semisolid overlay of 0.6% tragacanth gum (Sigma-Aldrich) in minimal Eagle's medium. Plaques were visualized after 2 to 3 days incubation by staining cells with crystal violet.

RNA isolation from infected cells. Confluent HeLa R19 cell monolayers on 35-mm-diameter plates were infected with virus supernatants at 5 PFU per cell. When complete CPE was observed, the cells were subjected to three rounds of freezing and thawing and the supernatant was recovered by microcentrifugation. Total RNA was extracted from 200 μl of viral

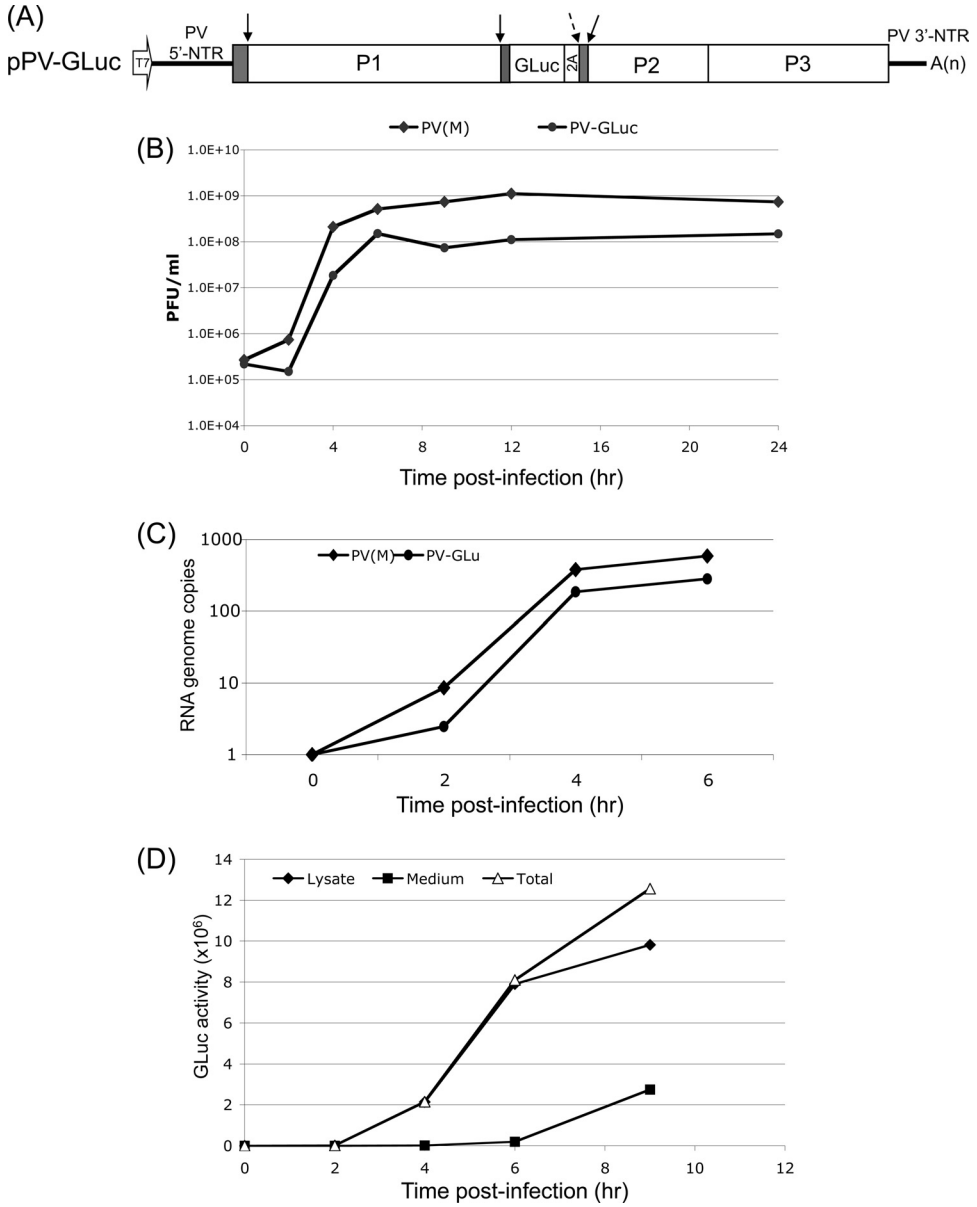

FIG 1 Characterization of novel monocistronic reporter construct pPV-GLuc. (A) Schematic representation of the GLuc-containing reporter construct. The polioviral reporter genome contains the coding sequence for GLuc inserted between capsid precursor P1 and the nonstructural P2 domain, followed by the FMDV 2A peptide flanked by two different in-frame clone linkers. Solid and dotted arrows, cleavage sites by PV 2A and FMDV 2A, respectively; shaded box followed by the 2A cleavage site at the N terminus of the polyprotein, a linker of 27 nt encoding the sequence ENLTTY. (B) One-step growth curves of wt PV1(M) and of the reporter virus PV-GLuc. HeLa cell monolayers were infected with viral supernatants of wt PV and of PV-GLuc (passage 1), and the virus yield was determined at the indicated time points by plaque assay (see Materials and Methods). (C) Number of RNA genome copies in PV1(M)- and PV GLuc-infected HeLa cells. The number of RNA genomes was measured at various times postinfection by quantitative RT-PCR (see Materials and Methods). (D) GLuc activity both in the growth medium and in the lysate was determined at the indicated time points postinfection.

supernatant with 800 μl TRIzol reagent (Invitrogen), according to the manufacturer's instructions.

RT-PCR, quantitative RT-PCR, and sequencing of virus variants. Single-strand cDNA synthesis was performed using either the SuperScript II or the SuperScript III first-strand synthesis system for reverse transcription-PCR (RT-PCR; Invitrogen) with primers specific to the appropriate poliovirus RNA sequences. From 2 to 4 μl of the RT-PCR mixture was used in the subsequent standard PCR. The PCR products were analyzed on a 1% agarose gel and purified for subcloning into a TOPO TA vector (Invitrogen). After transformation, single white colonies were picked and minicultured. Each target fragment was sequenced. Quantitative RT-PCR was performed, and the results were analyzed according to the protocols of the LightCycler RNA amplification kit SYBR green I (Roche). The regions amplified were from nt 2504 to 2603 for helper virus detection and nt 5144 to 5246 for total genomes.

Assay of luciferase activity. At the indicated time points, 10 to 20 μl of the culture medium was taken for the measurement of luciferase activity. To assay cell lysates, cells growing in 12-well plates were carefully washed twice with warm phosphate-buffered saline (PBS), followed by treatment with 120 μl 1× passive lysis buffer (PLB; Promega) by shaking for 15 min at room temperature. The supernatant was collected by centrifugation at 4°C and diluted with plain DMEM. Luciferase activity was determined in

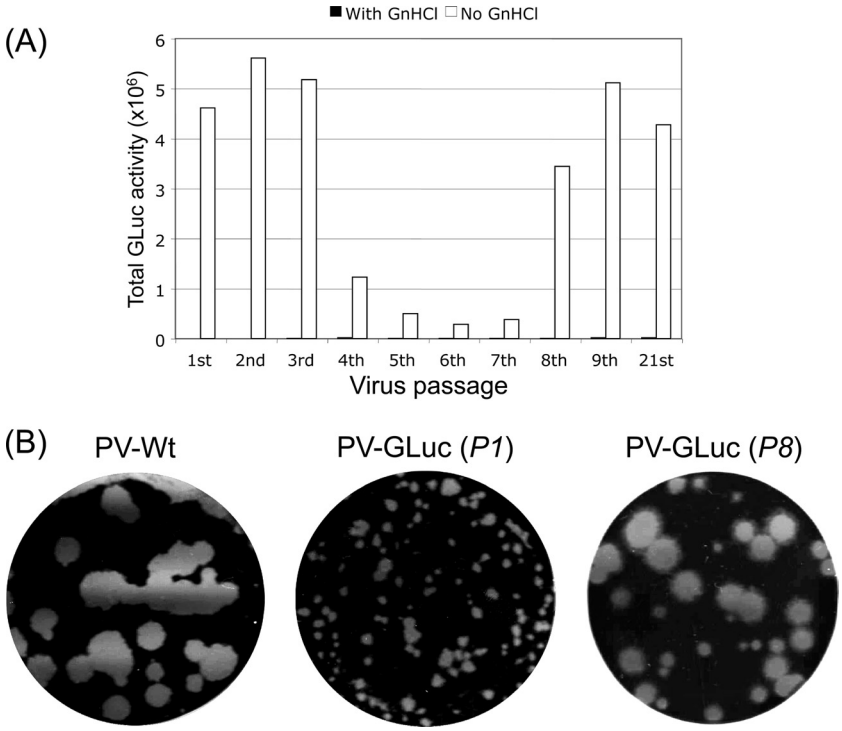

FIG 2 Growth properties of PV-GLuc. (A) Summary of total GLuc activities at different viral passages. All passages were carried out in the absence or presence of GnHCl (2 mM). GLuc measurements were made at between 18 and 20 h postinfection at the time when cytopathic effects were observed. (B) Plaque phenotypes of wt PV and of PV-GLuc at passage 1 (*P1*) and passage 8 (*P8*) (see Materials and Methods).

a luminometer (Optocomp I) by the addition of 5 to 10 μl diluted coelenterazine (NEB) to 10 to 20 μl of sample.

Western blot analysis. HeLa and COS-1 cells in 12-well culture plates were harvested 24 h after transfection and lysed with PLB. The cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14% or 17% acrylamide). The proteins were transferred to a nitrocellulose membrane (Roche) and were immunoblotted according to standard protocols. The efficiency of transfer was verified by using prestained molecular weight markers (Crystalgen). The resulting membranes were blocked for at least 60 min in a solution of 1% nonfat milk powder and 0.01% Tween 20 in Tris-buffered saline (TBS) at room temperature. After blocking, the blots were incubated with different antibodies (anti-2B, anti-2C, or anti-3A) overnight at 4°C. The blots were then washed five times for 5 to 15 min each in TBS containing 0.01% Tween 20 and incubated with antimouse horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution in TBS containing 0.01% Tween 20) for 1 h at room temperature. After five washes, the immune complexes with bound secondary antibodies were detected by enhanced chemiluminescence (ECL kit; Roche) according to the manufacturer's instructions.

RESULTS

Construction of a recombinant poliovirus expressing *Gaussia* luciferase. We previously analyzed the properties of a reporter poliovirus that contains the RLuc gene between the 5′ nontranslated region (NTR) and the capsid protein precursor P1 (RLuc-P1-P2-P3). This construct has proven useful for distinguishing between defects in RNA replication or encapsidation (37); however, the RLuc reporter gene was progressively deleted after the first passage. Numerous other studies have already shown that PV does not efficiently encapsidate RNAs containing inserts longer than 400 nt (3, 39, 41, 46, 72). Therefore, we assume that the RLuc gene, which is 933 nt long, was deleted because of its size.

Recently, a smaller luciferase protein-encoding gene, GLuc (555 nt, encoding 185 amino acids), was discovered in the marine copepod *Gaussia princeps*; the protein encoded by GLuc is naturally secreted (57). With the aim of constructing a more stable reporter virus, we have constructed PV genomes containing the GLuc gene. Fusion of GLuc to the N terminus of the polyprotein (GLuc-P1-P2-P3) yielded a nonviable virus (data not shown), which we believe was due to the peculiar and as yet unexplained observation that poliovirus polyproteins with an N-terminal signal sequence yield a dead phenotype (39). Although the GLuc protein contains a natural secretory signal that has been shown to be effective in directing secretion (35), we wondered whether this signal would be inhibitory within the polyprotein. Accordingly, we inserted a codon use-optimized gene of GLuc (57) between the P1 and P2 domains of the PV polyprotein (P1-GLuc-P2-P3). In this pPV-GLuc construct, the reporter sequence is flanked by two in-frame linkers: PV $2A^{pro}$ and FMDV $2A^{pro}$ protease cleavage sites at the N and C termini, respectively (Fig. 1A). GLuc produced by proteolytic processing of this fusion polyprotein is predicted to contain 3 additional amino acids at the N terminus and 21 additional residues at the C terminus compared to the wt protein. These extensions did not alter GLuc activity (data not shown).

RNA replication and growth phenotypes of PV-GLuc. Transfection of transcripts of pPV-GLuc into HeLa cells yielded a strong fluorescence signal in the cell medium at 16 h (the signal was weaker at 5 h). The signal was abolished when 2 mM guanidine hydrochloride (GnHCl), an inhibitor of PV RNA replication (53), was added posttransfection (data not shown). These results indicated that RNA translation and replication delayed release of GLuc into the medium, as would be expected if the nonstructural

proteins of PV-GLuc inhibited GLuc secretion and the discharge of GLuc occurred only at the onset of cell lysis (see below).

To compare the growth phenotype of virus expressing GLuc with that of wt PV, we transfected HeLa cells with transcript RNAs of both viral genomes. Cytopathic effects were apparent with wt PV transcripts at 18 to 20 h posttransfection but were barely detectable with PV-GLuc transcripts at 40 h posttransfection (data not shown). However, PV-GLuc isolated from passage 1 exhibited a small plaque phenotype (Fig. 2B) and, at a multiplicity of infection (MOI) of 5, replicated with a viral yield 1 log unit below that of the wt virus when tested in a one-step growth experiment (Fig. 1B). Quantitative RT-PCR analyses indicated that the primary reason for the lower yield of PV-GLuc than the wt virus is a reduction in the total number of RNA genomes synthesized (Fig. 1C). Intracellular synthesis of GLuc, as determined by analysis of cell lysates (Fig. 1D), paralleled the time course of virus production (Fig. 1B). The weak reporter signal in the growth medium during the first several hours after infection indicated potent inhibition of GLuc secretion (Fig. 1D). At 9 h postinfection, however, some GLuc appeared in the medium, most likely due to cell lysis (Fig. 1D).

Genetic stability of PV-GLuc. Although the GLuc coding sequence in PV-GLuc is relatively small, we expected that it would eventually be eliminated, as it presents no advantage to virus replication. We tested this hypothesis by assaying for GLuc activity after each of multiple successive passages on HeLa cells using an MOI of 5 (Fig. 2A). Although stable for the first three passages, the GLuc signal diminished at the fourth passage and was nearly undetectable by the sixth passage. These results suggest that the reporter gene was progressively eliminated from the genome, presumably by deletion. Remarkably, however, the GLuc signal returned to high levels by passage 9 and remained stable for more than 12 consecutive passages. This unexpected phenomenon was observed in two independent experiments (data not shown). The plaque size of the progeny virus was small after the first passage, most probably due to the large size of original reporter genome, but progeny virus developed into plaques with a mixture of sizes after passage 8, suggesting the presence of shortened genomes, nearly wt in size, that produced large plaques (Fig. 2B).

Characterization of defective interfering particles obtained during passaging of PV-GLuc on HeLa cells. To determine how PV-GLuc genomes were altered to lead to the fluctuation of the GLuc signal, we performed RT-PCR of total cellular/viral RNA with primers amplifying the entire coding sequence of P1 and that of the GLuc sequence. The sizes of the PCR fragments as analyzed by agarose gel electrophoresis indicated that the P1-GLuc fragment was intact for the first 2 to 3 passages (Fig. 3A; compare lanes 1 and 2). After passage 4, the amount of the full-length P1-GLuc segment was much reduced, and smaller fragments appeared (Fig. 3B, compare lane 2 with lanes 4 to 6).

The products obtained from passages 6, 9, and 21 were primarily of two sizes (Fig. 3B, lanes 4 to 6). The DNA fragments were subcloned into vectors, amplified, and subjected to sequence analysis. The larger fragment that was produced at passages 6, 9, and 21 revealed that essentially all of the GLuc coding sequence and some linker sequences were deleted. We concluded that these fragments represent the sequences of newly generated helper virus (wt-like PV) (Fig. 4; Table 1), which provides the capsid proteins necessary for encapsidation in the progeny mixture. Since the helper virus had lost the intact GLuc gene, no GLuc activity was associated with

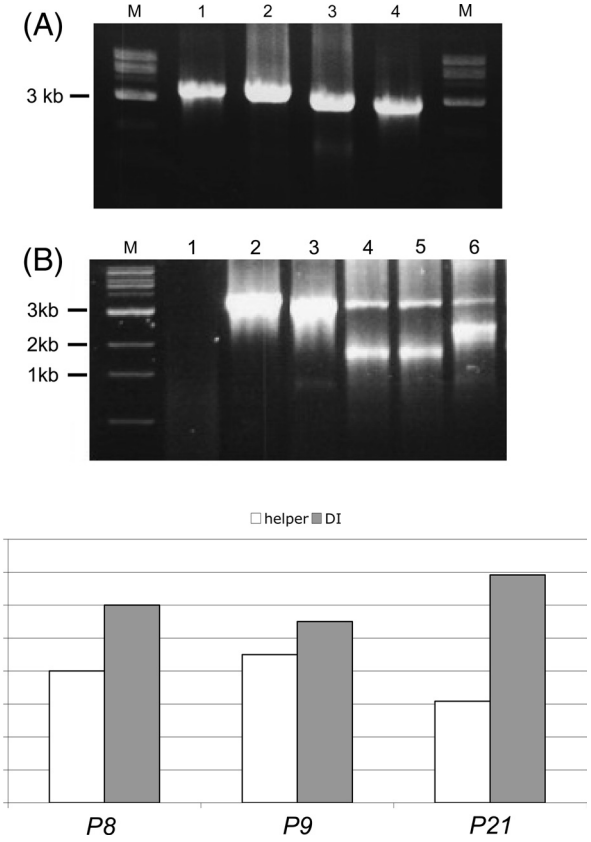

FIG 3 Genetic stability of PV-GLuc. (A) Genetic stability of PV-GLuc during passaging on HeLa cells. Viral RNA was reverse transcribed, followed by PCR, and the length of the cDNAs was analyzed on an agarose gel. Lanes M, DNA 1-kb marker (NEB); lane 1, PCR fragment derived from the entire P1 and GLuc coding sequences of PV-GLuc; lanes 2 and 3, PCR fragments derived from PV-GLuc RNA isolated after passages 3 and 4, respectively; lane 4, control PCR fragment derived from wt poliovirus RNA. (B) Identification of DI particles derived from PV-GLuc. Viral RNAs obtained at different passages were reverse transcribed, and following PCR, the DNA fragments obtained were analyzed on an agarose gel. Lanes M, DNA 1-kb marker (NEB); lane 1, negative control (no RNA); lane 2, PCR fragment derived from the entire P1 and GLuc coding sequences of pPV-GLuc; lane 3, PCR fragment derived from wt poliovirus RNA; lanes 4 to 6, PCR fragments derived from PV-GLuc RNA isolated at passages 6, 9, and 21, respectively. (C) Quantitation of helper virus and of DI genomes. The percentage of helper virus and of DI genomes in cell lysates obtained at passages 8, 9, and 21 was determined by quantitative RT-PCR (see Materials and Methods).

any of these infectious particles. The size of the smaller fragments (Fig. 3B) was consistent with genomes containing different deletions. Indeed, sequence analysis revealed a heterogeneous population of variants containing different deletions either in P1 (DI particles 5, 6, 8, and 9) or in both P1 and GLuc (DI genomes 1 to 4, 7, 10, and 11). Deletions in the P1 region ranged in size from 1,503 to 2,013 nt (Fig. 4; Table 1). These fragments that contained the entire GLuc coding domain originated from variants that produce the GLuc signal. Deletions in both P1 and GLuc ranged in size from 1,779 to 1,923 nt. DI variants containing such deletions likely lack GLuc activity. Quantitative RT-PCR analyses indicated that at passages 8, 9, and 21 the progeny mixture contained 60%, 55%, and 70% DI particles, respectively (Fig. 3C).

Interestingly, the small fragment isolated from passages 6 and 9 was about 1.6 kb in length, but by passage 21 the length that has

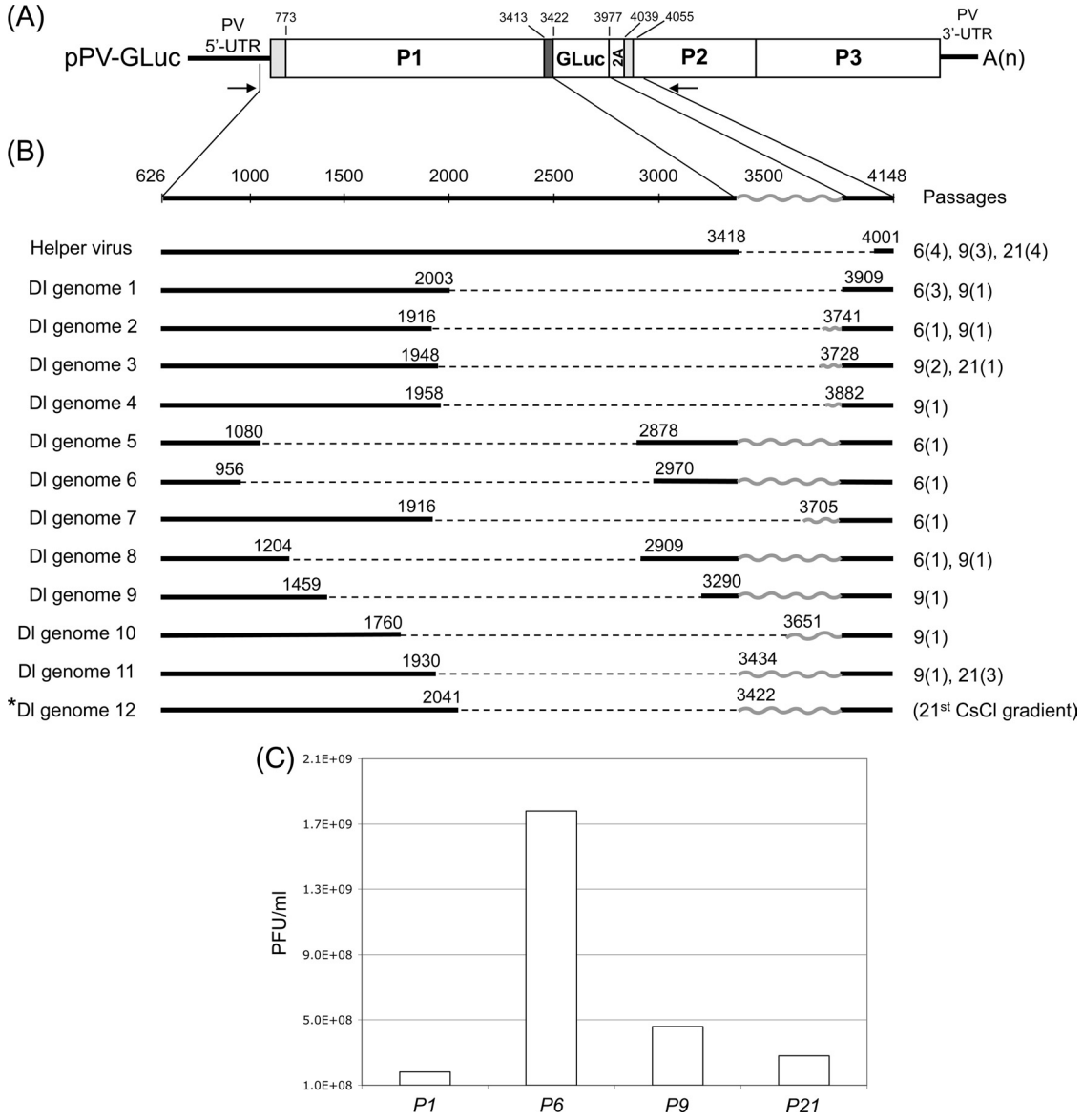

FIG 4 Genetic analysis of DI particles derived from PV-GLuc and interference with wt poliovirus growth by DI genomes. (A) Schematic diagram of PV-GLuc. The region from nt 626 to nt 4148 is enlarged in panel B. Arrows, location of primers used for RT-PCR. (B) Viral or DI RNAs were reverse transcribed, and following PCR amplification of the region from nt 626 to nt 4148, the DNA fragments were sequenced. Solid black lines, sequences retained from the parental PV-GLuc in the helper virus or the DI genomes; waved gray lines, GLuc coding sequences; dotted lines, deleted regions. Numbers above the lines indicate the position in the parental pPV-GLuc plasmid of the last upstream nucleotide before and the first downstream nucleotide after each deletion. On the right, the passage number from which the sample was derived is indicated. The total number of samples sequenced is indicated in parentheses after the passage number. DI genome 12, labeled with an asterisk, is derived from the CsCl gradient separation. (C) Virus titers at different passages (passages 1, 6, 9, and 21) of PV-GLuc on HeLa cells were determined by plaque assay (Materials and Methods).

evolved among the genotypes increased to 1.9 kb (Fig. 3B). Three out of four DI genomes examined from passage 21 contained the shortest deletion observed (1,503 nt; Table 1, small fragment 11). This suggests that after long-term passaging, the DI genome length that evolved was optimized for encapsidation in *trans* and RNA replication rate. Increase in replication rate, which is essential for DI particle survival, may be the reason for a DI genome nearly 1,000 nucleotides shorter than that of wt PV RNA. Sequences missing from the longest DI genome characterized were regions of P1, the C-terminal linker sequence, and the N-terminal 4 amino acids of the GLuc gene originally inserted into the PV polyprotein.

To confirm the genotypes of the stable DI genomes after passage 21, we separated the DI particles from the helper virus on a CsCl gradient (data not shown). The RNA of the DI particles isolated from the upper weak bands of the gradient was then subjected to RT-PCR. Sequencing of the PCR fragments indicated the presence of two types of DI particles, one containing the previously observed deletion of 1,503 nt (Fig. 4B; Table 1, DI genome 11) and the other containing a deletion of 1,380 nt that had not been detected in previous analyses (Fig. 4B; Table 1, DI genome 12). Both DI genomes should produce the GLuc signal. The identity of the helper virus isolated from the lower band of the gradient was also confirmed by sequencing of the N terminus of the P1

TABLE 1 Sequence analysis of RT-PCR fragments derived from helper virus and DI particles

Fragment size, fragment no.	Deleted nt range (no. of nt)	Final genome length (nt)
Big, 1	3419–4000 (582)	7,527
Small		
1	2004–3908 (1,905)	6,204
2	1917–3740 (1,824)	6,285
3	1949–3727 (1,779)	6,330
4	1959–3881 (1,923)	6,186
5	1081–2877 (1,797)	6,312
6	957–2969 (2,013)	6,096
7	1917–3704 (1,788)	6,321
8	1205–2908 (1,704)	6,405
9	1460–3289 (1,830)	6,279
10	1761–3650 (1,890)	6,219
11	1931–3433 (1,503)	6,606
12	2042–3421 (1,380)	6,729

domain that was fused to a linker containing heterologous sequences (27nt; Fig. 1A).

As expected from previous studies of PV DI particles, all of the DI RNA derived from our reporter virus contained in-frame deletions and, thus, intact reading frames for the synthesis of the nonstructural proteins (Table 1). Intact translation is a requirement for replication, and, thus, out-of-frame deletions are eliminated from the pool of competing genomes (48).

The ability of DI particles to interfere with the growth of wt poliovirus was first studied in detail by Cole and his colleagues (13, 14). Our experiments confirm this phenomenon. As shown in Fig. 4C, there is a nearly 8-fold reduction in virus titer between passages 6 and 21 of PV GLuc on HeLa cells, presumably as a result of the increasing number of DI particles produced. We obtained additional proof for interference by DI particles using cotransfection of wt PV and DI genome 11 transcript RNAs at ratios of 1:0 to 1:10 into HeLa cells and measurement of virus titers at the time of CPE. The results clearly indicate that virus titers decreased as a result of increasing the DI particle production, demonstrated by an increase in luciferase signal (Table 2).

Coexpression of PV polypeptide 3A with GLuc in HeLa cells does not inhibit secretion of luciferase. Previous studies have shown that infection of COS-1 cells with PV or expression of PV protein 3A inhibits the transport of secretory proteins in these cells (24). The N-terminal 10 amino acids of 3A (deleted in a mutant 3A) have been shown to be involved in this block of secretion (23). Since PV infection inhibited secretion of GLuc from HeLa cells (Fig. 1D), we assumed that PV 3A would also inhibit GLuc secretion in HeLa cells, as it does in COS-1 cells. This was not so. To test secretion of GLuc in the presence of 3A, we constructed a dicistronic expression vector (Fig. 5A) containing in the first cistron the coding sequences either of 3A, of mutant 3A (mut3A), or of eGFP. eGFP served as a control for intact secretion. DNA plasmids containing the dicistronic vector were transfected either into HeLa cells or into COS-1 cells, where they were transcribed under the control of a CMV promoter. In the resulting mRNAs, the first cistron was translated in a cap-dependent manner, whereas translation of the second cistron (GLuc open reading frame [ORF]) was controlled by the HCV IRES. Intracellular (based on analysis of cell lysates) and released (based on analysis of the growth medium) GLuc activities from HeLa cells (Fig. 5B) or from COS-1 cells (Fig. 5C) were measured at 24 h posttransfection. The expression of the 3A proteins was monitored by Western blot analysis (Fig. 5D), whereas the expression of eGFP was visualized by fluorescence microscopy (Fig. 5E). As shown in Fig. 5B, secretion of GLuc from HeLa cells was efficient in the presence of eGFP or mutant 3A (percentages in medium/lysate, 75/25 and 82/18, respectively), and there was hardly any inhibition of secretion with coexpressed 3A (70/30). In contrast, secretion of GLuc was significantly inhibited by 3A in COS-1 cells (percentages in medium/lysate, 20/80) but not by eGFP (82/18) or mutant 3A (81/19) (18). The ability of 3A to inhibit GLuc secretion is thus cell type dependent.

TABLE 2 Interference with wt poliovirus growth by DI genome 11

Ratio of PV wt/DI RNA[a]	Titer (PFU/ml)	Total GLuc activity
1:0	8.9×10^8	698
1:1	6.4×10^8	22,539
1:2	2.8×10^8	47,657
1:5	1.6×10^8	182,558
1:10	1.5×10^8	340,106
0:1	0	23,929

[a] wt/DI RNA ratio, molar ratio of RNA used for transfection into HeLa cells.

PV proteins 2B, 2C, 2BC, and 3AB do not inhibit GLuc secretion in HeLa cells. The observation that expression of PV 3A by itself in HeLa cells does not inhibit GLuc secretion prompted us to determine whether one of the other membrane-binding viral nonstructural proteins (2B or 2C) of poliovirus or their precursors (2BC or 3AB) serve this function. In virus-infected cells, 2B, 2C, and 2BC are associated with membranes via their amphipathic helices, while 3A and 3AB are anchored through hydrophobic sequences (26, 51, 62, 64). Previous studies in Vero cells infected with FMDV have shown that 2BC (or coexpression of 2B and 2C), but not 3A, inhibits ER-to-Golgi apparatus protein transport (43). Therefore, we made dicistronic constructs in which the first cistron contained the coding sequence of 2B, 2C, 3AB, or 2BC (Fig. 6A). As a control, eGFP was also engineered into the first cistron. Plasmid DNAs were transfected into HeLa (Fig. 6B) and COS-1 (Fig. 6C) cells, and GLuc activity in cell lysates or in the medium was measured as described above. The expression of proteins was monitored by Western blot analysis (Fig. 6D). As shown in Fig. 6B, secretion of GLuc was as efficient in the presence of 2B, 2C, or 3AB as in the presence of eGFP. Expression of 2BC resulted in a slight inhibition of GLuc secretion. In comparison, in COS-1 cells (Fig. 6C), all of the proteins showed partial inhibition of GLuc secretion compared to expression of the control protein. In COS-1 cells, the strongest inhibitory effects (>50%) were seen with 2B and 2BC, although these were less striking than the effect that was observed with 3A (compare Fig. 5C and 6C).

Coexpression of 3A with another viral protein (2B, 2C, or 2BC) does not significantly increase the inhibition of GLuc secretion in HeLa cells. Since 3A, 2B, and 2BC expressed individually exhibited only a weak inhibitory effect on GLuc secretion in HeLa cells, we were interested to know whether a combination of two poliovirus nonstructural proteins (e.g., coexpression of 3A with 2B, 2C, or 2BC) would influence secretion of the luciferase protein. Therefore, we constructed tricistronic vectors, a strategy first described by Jang et al. (31). In our vectors, the first ORF

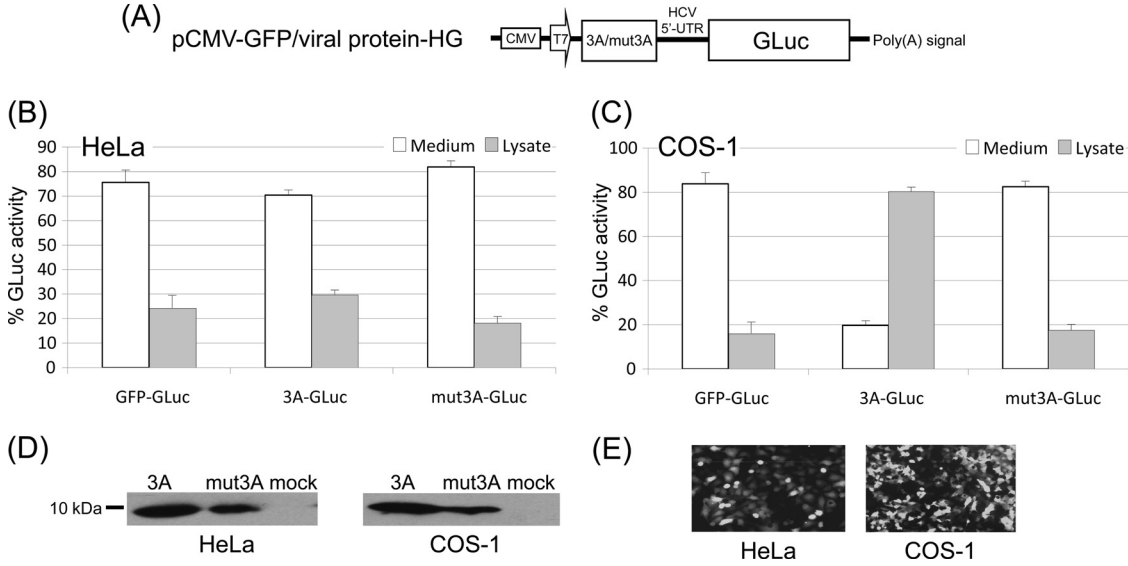

FIG 5 Effect of 3A expression on secretion of GLuc in HeLa and COS-1 cells. (A) Schematic representation of the dicistronic expression constructs used in this study. The first cistron contains the coding sequence either of 3A (wt or mutant 3A [mut3A]) or of a control protein (eGFP). The second cistron contains the GLuc coding sequence preceded by the HCV IRES. UTR, untranslated region. (B) Effect of 3A (wt or mutant 3A) or control protein expression on the secretion of GLuc in HeLa cells. HeLa cells were transfected with the dicistronic reporter DNA plasmids. GLuc activities, both secreted and intracellular, were measured at about 24 h posttransfection. The data are plotted as a percentage of the total GLuc activity. Error bars indicate the standard deviations of measurements from triplicate experiments. (C) Effect of 3A (wt or mutant 3A) or control protein expression on the secretion of GLuc in COS-1 cells. The experiment was the same as described for panel B, except that COS-1 cells were used. (D) Expression of wt 3A or mutant 3A proteins in HeLa and COS-1 cells. The expression of 3A proteins was monitored by Western blot analysis of samples collected at 24 h posttransfection. (E) Expression of eGFP in HeLa and COS-1 cells at 24 h posttransfection visualized by fluorescence microscopy. The efficiency of transfection was determined by flow cytometry: 51% of HeLa cells and 61% of COS-1 cells were transfected.

encoded 3A, the second contained either a viral protein (2B, 2C, or 2BC) or the control (eGFP), and the third encoded GLuc (Fig. 7A). The translation of 3A was cap dependent. Translation of the second and third cistrons was controlled by the EMCV IRES and HCV IRES, respectively. The plasmid DNAs were transfected into HeLa or COS-1 cells, and the GLuc signal in both the growth medium and the lysate was monitored at 24 h posttransfection. As shown in Fig. 7B, there was no significant difference in the secretion of GLuc in HeLa cells when 3A was coexpressed with 2B, 2C, 2BC, or the control, eGFP. However, the results were different when COS-1 cells were used in the experiment (Fig. 7C). Whereas 3A-eGFP and 3A-2B produced strong inhibition of secretion, as expected, coexpression of 3A with 2C or 2BC mitigated the inhibitory effect (compare Fig. 5C and 7C). The expression of proteins was monitored by Western blot analysis to ensure that the amounts of proteins produced were comparable (Fig. 7D).

Secretion of GLuc from HeLa cells occurs through the conventional ER-to-Golgi apparatus pathway. To test whether GLuc was transported and secreted in HeLa cells through the conventional ER-to-Golgi apparatus secretory pathway, we tested the effect of brefeldin A (BFA), a drug that inhibits anterograde ER export to the Golgi apparatus. HeLa cells were transfected with plasmid for expression of GLuc from the CMV promoter (Fig. 8A). At 6 h posttransfection, BFA was added over a range of concentrations, and the secretion of GLuc was assayed in the medium after 16 h. A striking reduction (>95%) of GLuc activity was observed in the presence of 1 μg/ml of BFA (Fig. 8B). These results confirm that the secretion of GLuc occurs by the conventional ER-to-Golgi apparatus pathway (5, 52).

DISCUSSION

Characterization of a PV-GLuc and derived DI particles. The aim of our study was to develop an additional tool for the identification of PV variants defective in encapsidation. We selected GLuc (555 nt, the smallest luciferase gene known) as the reporter, which in the context of the PV genome was expected to be retained longer than the larger RLuc (933 nt). Originally, we fused the GLuc sequence to the N terminus of the PV polyprotein. The corresponding genome, however, was nonviable. In retrospect, we should have expected this result since we have shown previously that a signal sequence directing polypeptides to the rough endoplasmic reticulum, when placed at the N terminus of the poliovirus polyprotein, renders the construct nonviable for unknown reasons (39). It should be noted that inactivating the signal sequence of GLuc by deletion allowed its expression at the N terminus of the PV polyprotein and rendered the engineered genome viable (data not shown). However, because of reports that modifying the signal sequence of GLuc influenced its expression (57), we have not pursued these experiments further.

We then inserted the GLuc ORF into the polyprotein between domains P1 and P2 (Fig. 1A). PV-GLuc replicated to high titers, albeit 1 log unit below that of wt PV. The GLuc polypeptide is released from the polyprotein by two distinct proteolytic activities, PV $2A^{pro}$ and the autocatalytic 2A activity of FMDV. The released luciferase has additional amino acid residues at its N and C termini, which did not inhibit production of a strong fluorescence signal. PV-GLuc retained luciferase activity for three passages on HeLa cells; thus, the PV-GLuc genome was significantly more stable than that of our original PV-RLuc construct (37).

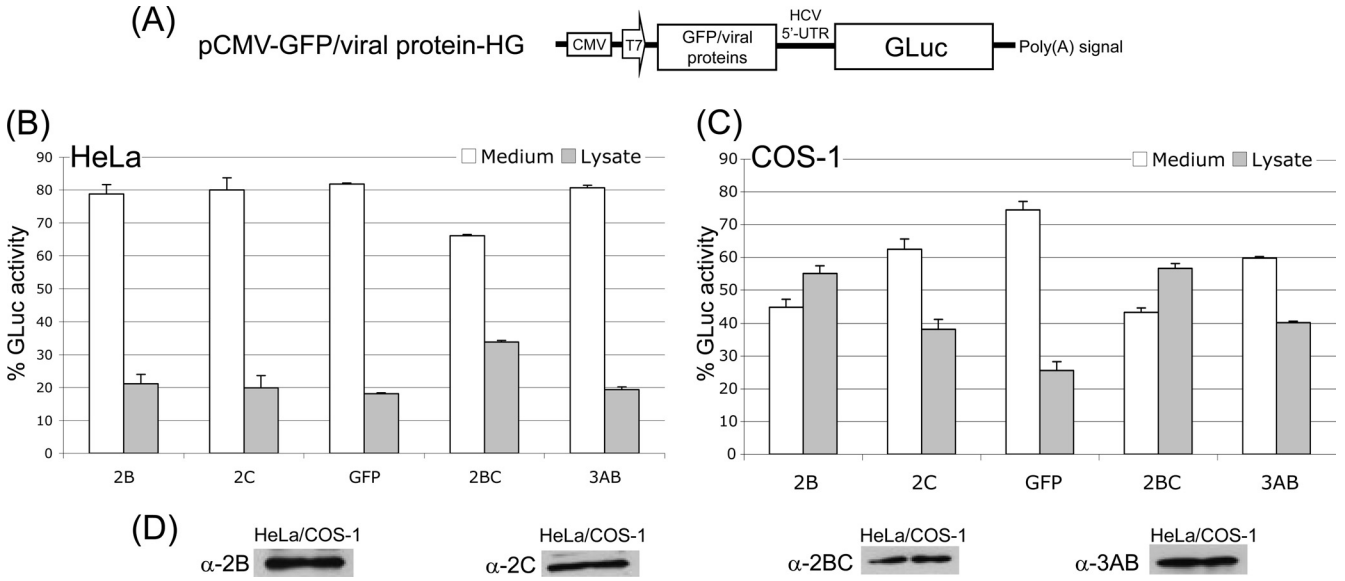

FIG 6 Effect of expression of viral proteins 2B, 2C, 2BC, and 3AB individually on the secretion of GLuc in HeLa and COS-1 cells. (A) Schematic representation of the dicistronic constructs used in the study. The first cistron contained either the viral or the control (eGFP) protein, while the second cistron contained the GLuc coding sequences. (B) Effect of viral or control protein expression on the secretion of GLuc in HeLa cells. HeLa cells were transfected with the dicistronic reporter DNA plasmids. GLuc activities, both secreted and intracellular, were measured at about 24 h posttransfection. The data are plotted as a percentage of the total GLuc activity. Error bars indicate the standard deviations of measurements from duplicate experiments. (C) Effect of viral or control protein expression on the secretion of GLuc in COS-1 cells. The experiment was the same as described for panel B, except that COS-1 cells were used. (D) Expression of viral proteins in HeLa and COS-1 cells was monitored by Western blot analysis of samples collected at 24 h posttransfection.

Therefore, PV-GLuc is expected to be a useful tool in studies of enterovirus replication and encapsidation.

Continued passaging of PV-GLuc beyond passage 3 led to the near complete loss of the GLuc signal by passage 6. The signal was, however, completely recovered by passage 8. This unusual phenomenon resulted from the coevolution of virus variants of two different genotype groups: one with a genotype resembling that of wt-like viruses and the other with a genotype resembling that of DI particles. After 21 passages the predominant DI genomes were shorter than the original PV-GLuc genome by 1,380 nt or 1,503 nt, but they retained, surprisingly, almost the complete coding sequence of GLuc. The interpretation of our data does not exclude the possibility that numerous other genotypes exist throughout passaging and even at passage 21 escape detection because of low abundance. The genome sizes of 6,600 to 6,700 nt (DI genomes 11 and 12) may be optimal for efficient encapsidation and virion stability, a result consistent with data previously reported by Kuge and colleagues (36). We propose that the genotypes of these passage 21 genomes provide the DI particles with the properties essential for their survival: a significantly higher rate of genome replication compared with that of the wt-like helper virus and maximal efficiency of encapsidation with capsid polypeptides "stolen" from wt-like virus. This is the reason for interference with the growth of the wt virus by the DI particles, a phenomenon which we have confirmed with one of our DI particles derived from PV-GLuc (Fig. 4C; Table 2).

DI particles were discovered more than 60 years ago (28, 66), but their role in the pathogenesis of viruses is poorly understood (30). The genomes of these particles always contain deletions that spontaneously arise during virus replication, preferentially in infections at high multiplicity. The DI variants competed with the wt-like variants for capsid proteins, thereby interfering with the replication of the latter. The deletions render the DI genomes noninfectious, but the defects are complemented in *trans* at the expense of homologous wild-type helper viruses. Poliovirus DI particles were first observed as contaminants in laboratory stocks (14), but they are not readily generated in cell culture (70). Because poliovirus DI genomes replicate efficiently, it was assumed that the deletions map to the P1 region and not to the nonstructural proteins. This was proven correct by heteroduplex mapping and biochemical procedures (48).

Whereas deletions in DI genomes of PV type 1 Sabin, selected in tissue culture experiments, spanned between 4% and 16% of the wt genome (32), viable genetically engineered deletions of PV type 1 (M) can be much larger, spanning nearly the entire P1 region (70). There is evidence that a 20% reduction in genome size may represent the largest deletion compatible with DI virion stability, and Nomoto and his colleagues (27, 36) made the important observation that deletions in the P1 region must be in frame with the initiation codon at nt 743 in order for DI genome replication to occur (15). This has led to the discovery of the secondary proofreading mechanism in picornavirus proliferation: correct translation and processing of the polyprotein are required for RNA replication, which, in turn, is required for encapsidation (45, 49, 50, 71).

GLuc secretion is inhibited from PV-GLuc-infected HeLa cells but not by the expression of individual PV nonstructural proteins. Kirkegaard and her colleagues observed inhibition of cellular protein transport in and secretion from COS-1 cells after infection with enterovirus PV or CVB3, an effect caused by the viral protein 3A or 2B (or precursor 2BC), presumably by different mechanisms (23, 24). It was subsequently concluded that restricting protein secretion does not impact intracellular viral replication *per se* but rather inhibits host responses, thereby stimulating

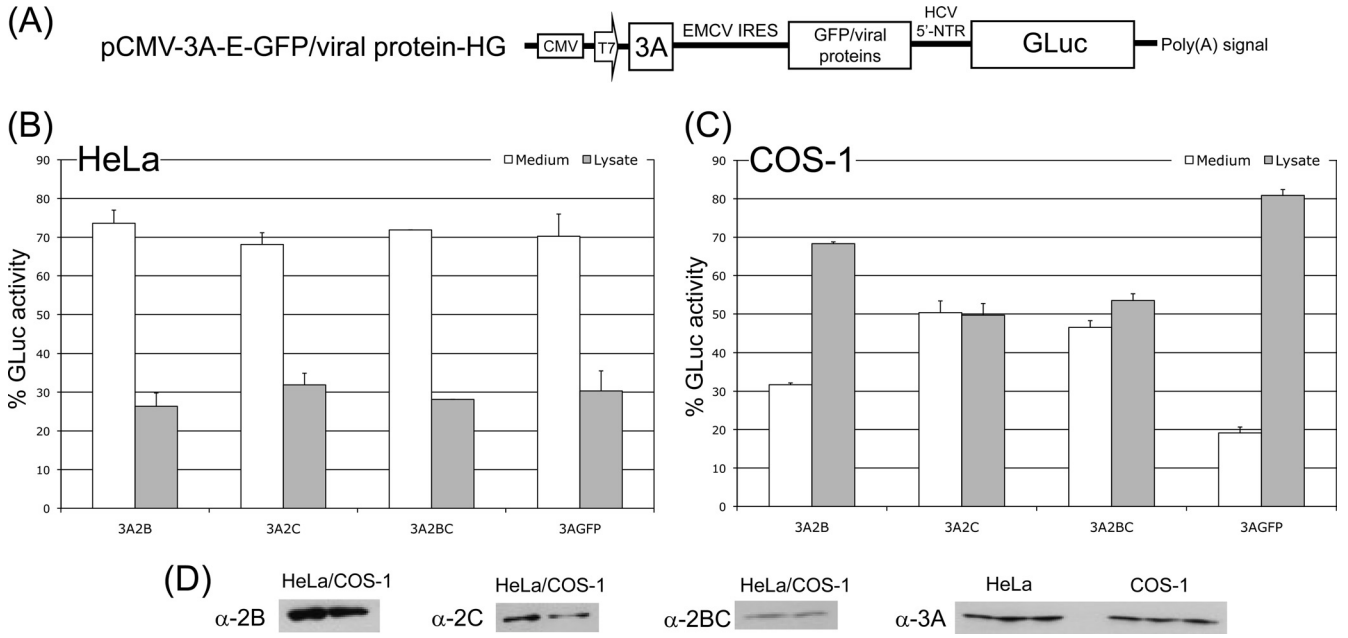

FIG 7 Effect of coexpression of 3A with another viral protein (2B, 2C, or 2BC) on the secretion of GLuc in HeLa and COS-1 cells. (A) Schematic representation of the tricistronic constructs used in the study. In the first cistron, translation of PV 3A is initiated by a cap-dependent mechanism. The second cistron contained either a viral protein (2B, 2C, or 2BC) or the control (eGFP) protein, while the third cistron contained the GLuc coding sequences. (B) Effect of viral or control protein coexpression on the secretion of GLuc in HeLa cells. HeLa cells were transfected with the tricistronic reporter DNA plasmids. GLuc activities, both secreted and intracellular, were measured at about 24 h posttransfection. The data are plotted as a percentage of the total GLuc activity. Error bars indicate the standard deviations of measurements from duplicate experiments. (C) Effect of viral or control protein coexpression on the secretion of GLuc in COS-1 cells. The experiment was the same as described for panel B, except that COS-1 cells were used. (D) Expression of viral proteins in HeLa and COS-1 cells was monitored by Western blot analysis of samples collected at 24 h posttransfection.

viral infection (17, 19, 21, 64, 68, 69). This is supported by the observation by van Kuppeveld and his colleagues that a mutant of the CVB3 3A protein defective in inhibiting ER-to-Golgi apparatus traffic has attenuated infectivity in mice (68). Enterovirus infection or the expression of individual viral protein 3A or 2B (or precursor 2BC) results in the recruitment of GBF1 and Arf1, which enhances the binding of phophatidylinositol-4-kinase IIIβ (PI4KIIIβ) to secretory organelle membranes (29). This induces the disintegration of cellular organelles and the assembly of special

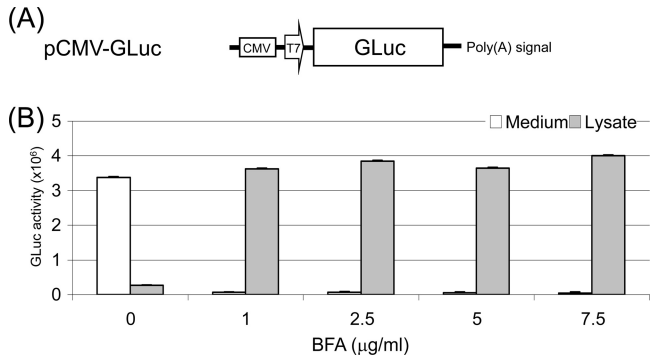

FIG 8 Effect of brefeldin A on GLuc secretion. (A) Schematic representation of the GLuc-expressing reporter construct pCMV-GLuc. (B) Inhibition of GLuc transport in the presence of the indicated concentrations of BFA. HeLa cells were transfected with a GLuc-expressing vector. At 6 h posttransfection, cells were treated with different amounts of BFA. Samples taken from growth medium and lysates were assayed for GLuc activity at 16 h after addition of BFA.

vesicles that are rich in phosphatidylinositol-4-phosphate (PI4P) lipids, where RNA replication takes place. Specifically, these processes lead to an inhibition of Arf1 activation because 3A interferes with Arf-dependent COP-1 recruitment to membranes by binding and inhibiting the function of GBF-1 (7, 67).

A failure to block protein transport by 3A or other small enterovirus proteins corresponding to those of PV has been reported for many picornaviruses, such as hepatitis A virus, enterovirus 71, Theiler's virus, and rhinovirus 14 (1, 12). In FMDV replication, the 2BC polypeptide, rather than 3A or 2B, is responsible for the blockage of ER-to-Golgi apparatus traffic (43, 44). In view of the observed heterogeneity in protein structure of these small picornavirus proteins, it has been concluded that the mechanisms by which they exert their inhibition of protein secretion must be different (1, 12). On the basis of several studies reporting inhibition of protein secretion by picornaviruses or picornavirus nonstructural proteins (8, 22, 23, 47, 67), we expected that GLuc would not be released from PV-GLuc-infected cells. This assumption was supported by the data shown in Fig. 1D. However, extending these experiments to expression of proteins 3A, 2B, 3AB, and 2BC in HeLa cells individually or as pairs (3A-2B, 3A-2C, or 3A-2BC), we did not observe inhibition of secretion of GLuc. Indeed, GLuc was secreted from HeLa cells when coexpressed with 3A, a 3A mutant (which does not inhibit secretion), or eGFP (Fig. 5B). In contrast, in COS-1 cells, our results were consistent with previously published data since 3A robustly inhibited secretion, whereas the 3A mutant or eGFP did not (Fig. 5C). Similar results were obtained when 2B, 2C, 2BC, or 3AB was expressed individually with GLuc or when GLuc was coexpressed with pairs of viral

proteins, 3A-2B, 3A-2C, or 3A-2BC (Fig. 7). These results suggest that the effect of 3A or 2B expression on GLuc secretion is possibly cell type dependent.

At present we cannot explain the difference between the effect of PV infection and expression of individual polypeptides on secretion of GLuc from HeLa cells. It is possible that the entire complement of PV proteins encoded in the polyprotein is necessary for the inhibition of secretion of GLuc from HeLa cells and that this mechanism involves a set of cellular proteins in addition to the set interacting with 3A, 2B, 2BC, or 3AB. Moreover, the sequence and/or binding even of very similar cellular proteins to the viral proteins may differ between HeLa cells, on the one hand, and COS-1, BGM, and mouse 3T3 cells, on the other hand. Irrespective of the mechanism, PV-GLuc should be a useful tool in studies of enterovirus replication and encapsidation.

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