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Encapsidation and Serial Passage of a Poliovirus Replicon Which Expresses an Inactive 2A Proteinase

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The multiple roles of the viral proteinase 2A in poliovirus replication have been difficult to assess because, to date, it has not been possible to isolate and characterize a viral genome with an inactive $2A^{pro}$. We have previously reported that a poliovirus replicon containing an inactive $2A^{pro}$ by virtue of a change at amino acid 109 from a cysteine to a serine (C109S) was replication competent when transfected into cells previously infected with vaccinia virus (R. Pal-Ghosh and C. D. Morrow, J. Virol. 67:4621–4629, 1993). To further develop this system, we have used a poliovirus replicon which contains the human immunodeficiency virus type 1 (HIV-1) *gag* gene positioned between nucleotides 1174 and 2470 of the poliovirus genome and have engineered a second mutation within this replicon to change the codon for amino acid 109 of the $2A^{pro}$ from cysteine to serine (2AC109S). Transfection of this replicon into cells previously infected with vaccinia virus results in the replication and expression of a protein with a molecular mass consistent with that of a P1–HIV-1 Gag–2A fusion protein. Using a recently described complementation system which relies on the capacity of a recombinant vaccinia virus (VV-P1) to provide the capsid precursor (P1) in *trans* (D. C. Ansardi, D. C. Porter, and C. D. Morrow, J. Virol. 67:3684–3690, 1993; and D. C. Porter, D. C. Ansardi, W. S. Choi, and C. D. Morrow, J. Virol. 67:3712–3719, 1993), we have encapsidated this replicon containing the 2AC109S mutation. By using reverse transcription PCR, we demonstrated that after 15 serial passages the encapsidated replicon still contained the 2AC109S mutation. Infection of cells with a stock of encapsidated replicon, either in the presence or in the absence of vaccinia virus, resulted in the expression of the P1–HIV-1 Gag–2A fusion protein. Expression of the P1–HIV-1 Gag fusion protein in cells infected with the encapsidated replicon containing the 2AC109S mutation was reduced compared with the expression of P1-HIV-1 Gag in those cells infected with a replicon containing a wild type 2A gene. The protein expression and replication of the replicon RNA in cells containing the 2AC109S mutation was maintained for a longer period of time than for the replicons containing the wild-type 2A gene, possibly because of a reduced cytopathic effect. Coinfection of cells with the encapsidated replicon containing the 2AC109S mutation and wild-type poliovirus resulted in the further processing of the P1–HIV-1 Gag–2A fusion protein to produce the P1–HIV-1 Gag fusion protein, demonstrating that the protease activity of 2A can be supplied in part in *trans*. This study is the first to describe a poliovirus genome (or replicon) that contains an enzymatically inactive P2 or P3 gene product. On the basis of this study, we conclude that the protease activity of 2A is not essential for viral RNA replication, although 2A is required for high-level replication and expression.

Poliovirus has a plus-stranded RNA virus genome approximately 7,500 bp in length (12). The primary translation product of the poliovirus genome is a single long polyprotein which is subsequently processed by virus-encoded proteases $2A^{pro}$ and $3C^{pro}$ (12). The long single polyprotein is functionally divided into structural (capsid) proteins, designated P1, and nonstructural proteins, designated P2 and P3 (13, 25). Initial cleavage of the polyprotein between the P1 and P2 regions is catalyzed by $2A^{pro}$ in what is believed to be a cotranslational reaction (27). Other subsequent proteolytic cleavages are catalyzed by $3C^{pro}$ or 3CD (10, 29, 30).

$2A^{pro}$ plays an important role in the life cycle of the virus. It is required for cleavage of the P1 capsid precursor from the long polyprotein (18, 19, 21, 27). The $2A^{pro}$ protein also indirectly induces cleavage of the 220-kDa component of the eukaryotic initiation factor eIF-4F, which results in the shutoff of host cell translation, leading to selective translation of the poliovirus RNA genome (6, 14, 15, 27). Finally, recent studies have demonstrated that 2A is directly involved in the process of cap-independent translation in a manner similar to that of a translational enhancer protein (7, 16).

The availability of an infectious poliovirus cDNA clone has allowed investigation of the role(s) of 2A in replication. Previous studies have clearly demonstrated that a mutation in the putative active site of the 2A proteinase, in which a cysteine amino acid at position 109 was changed to a serine (C109S), resulted in inactivation of the viral protease (8, 31, 32). Poliovirus cDNA clones containing this mutation were noninfectious when transfected into tissue culture cells (8). A drawback of these studies has been the necessity of generating an infectious virus. We have recently taken a different approach to study the role of 2A in viral replication. Our studies rely on the generation of poliovirus genomes in which a foreign gene sequence has been substituted into the P1 region of the poliovirus genome (replicons) (5, 22). Previous studies from this laboratory, as well as others, have demonstrated that RNA genomes with deletions in the P1 capsid region still have the capacity for replication when transfected into cells (5, 11, 22). We have used these chimeric replicons to begin to characterize features of viral replication that can be complemented in *trans*. In previous studies we found that the C109S mutation in $2A^{pro}$ resulted in a replicon which did not have the capacity to replicate when transfected into uninfected cells but was fully rep-

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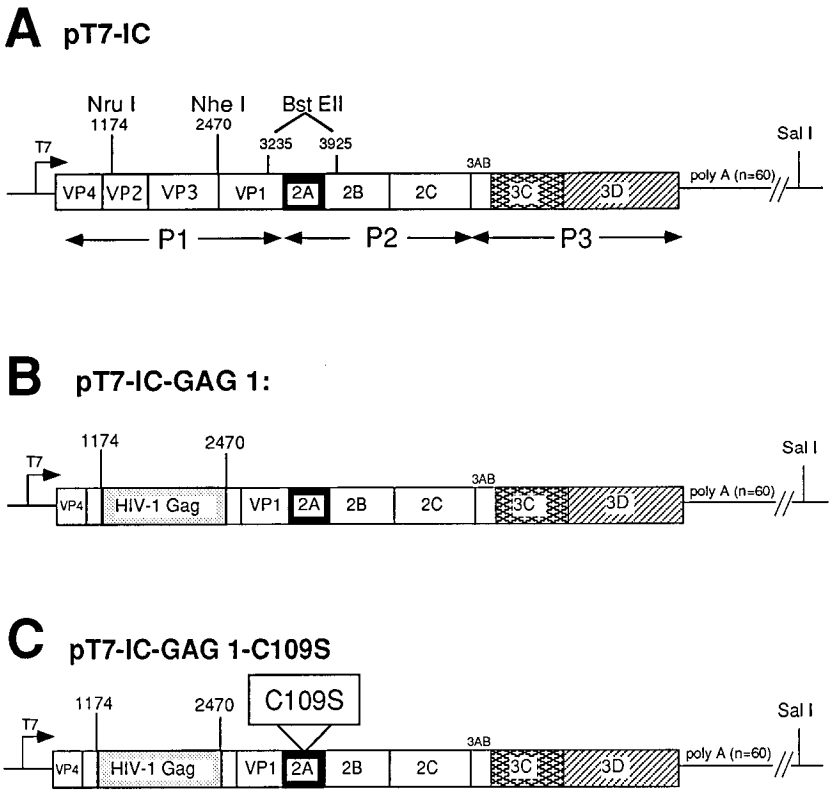

FIG. 1. HIV-1–poliovirus replicons. The initial plasmid used for the generation of the constructs described in this report was the complete infectious cDNA genome of poliovirus, positioned downstream from the phage T7 promoter (T7) (5). For in vitro transcription, the plasmid was first linearized at the unique *Sal*I site. Poliovirus genomes containing regions of the HIV-1 *gag* gene (nucleotides 718 to 1549 [23]) were substituted between nucleotides 1174 and 2470 of the poliovirus genome (22). A mutation in the 2A gene in which the codon for cysteine at amino acid 109 was changed to a serine was constructed (20). The fragment containing this mutation (contained in a *Bst*EII fragment) was subcloned into the plasmid pT7-IC-Gag1 to create the plasmid pT7-IC-Gag1-C109S. The replicon containing the C109S mutation in the 2A gene was otherwise isogenic with the replicon derived from in vitro transcription of pT7-IC-Gag1. Transfection of RNA transcribed in vitro from either pT7-IC-Gag 1 or pT7-IC-Gag1-C109S templates into cells previously infected with vaccinia virus resulted in expression of the poliovirus 3CD protein, a fusion protein between the $3C^{pro}$ and $3D^{pol}$ proteins, which has a molecular mass of 72 kDa. In addition, a VP4-HIV-1-Gag-VP1 fusion protein was expressed in cells transfected with RNA derived from pT7-IC-Gag1. Two fusion proteins consisting of VP4–HIV-1 Gag–VP1–2A and VP4–HIV-1 Gag–VP1–2A–2B–2C were expressed in vaccinia virus-infected cells transfected with RNA derived from pT7-IC-Gag1-C109S.

lication competent when transfected into cells previously infected with vaccinia virus (20). The results of these studies suggested that the proteinase function of 2A was not required for replication of the viral RNA and that the vaccinia virus provided some factor to allow replication of the viral genomes containing the inactive $2A^{pro}$.

To further investigate the role of $2A^{pro}$ in viral replication, we have made use of a complementation system, developed in this laboratory, to encapsidate replicons expressing foreign genes (1, 22). We have previously described the construction and characterization of a human immunodeficiency virus type 1 (HIV-1)-poliovirus replicon in which the *gag* gene was substituted for the VP2 and VP3 regions in the infectious cDNA of poliovirus (22) (Fig. 1B). In this plasmid construction, the HIV-1 *gag* gene (nucleotides 718 to 1549 of the HXB2 genome [23]) was positioned between nucleotides 1174 and 2470 of the poliovirus genome, substituting the majority of the VP2 and VP3 genes. The HIV-1 *gag* gene was inserted to maintain the translational reading frame between the poliovirus VP4 and VP1 genes. Transfection of in vitro transcribed RNA from this clone into cells results in the replication of the RNA and expression of the HIV-1 Gag–P1 fusion protein. In previous studies, a mutation in the 2A gene to change the codon for a cysteine to serine amino acid 109 (2AC109S) was found to result in an enzyme that had little or no proteolytic activity and did not induce the degradation of p220 (8, 31, 32). A poliovirus cDNA containing this mutation was noninfectious (8, 20). A chimeric HIV-2 Gag–poliovirus genome with the 2AC109S mutation was not capable of replication when transfected into uninfected cells, but it was capable of replication and protein expression when transfected into vaccinia virus-infected cells (20). We have reconstructed the 2AC109S mutation back into the replicon genome containing the HIV-1 *gag* gene. To do this we used a *Bst*EII restriction endonuclease site. The resulting plasmid, designated pT7-IC-Gag1-C109S, is isogenic to the parental plasmid pT7-IC-Gag1 except for the mutation encoding the cysteine-to-serine change at amino acid 109 of 2A (Fig. 1C).

In our first experiments, we analyzed the expression from the replicons transfected into cells previously infected with a recombinant vaccinia virus, VV-P1. In a previous study, we demonstrated that similar replicons, in which the HIV-2 *gag* gene was substituted in the P1 region, were able to replicate when transfected into cells previously infected with vaccinia virus (20). For the present study we used three independent clones (no. 1 through 3) as in vitro templates to generate replicon RNA (5). The RNA was transfected into cells previously infected with VV-P1. At 4 h posttransfection, the cells were metabolically labeled, and the proteins were subsequently immunoprecipitated with antibodies to $3D^{pol}$ (9) or HIV-1

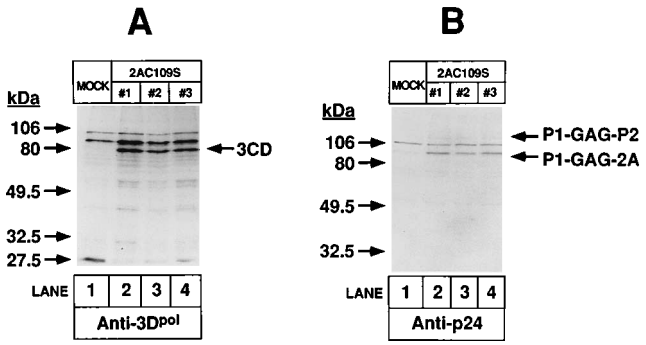

FIG. 2. Analysis of 3CD and P1-Gag fusion proteins in cells transfected with RNA derived from pT7-IC-Gag1-C109S. Cells were first infected with vaccinia virus P1 at a multiplicity of 10 PFU per cell. Cells were incubated for 2 h in complete medium, and then transfection was performed by using in vitro-transcribed RNA generated from three separate clones of pT7-IC-Gag 1-C109S (no. 1 through 3). Beginning at 4 h after transfection, the cells were metabolically labeled for 2 h, and then radiolabeled proteins were immunoprecipitated with anti-3D^{pol} (A) or anti-p24 (B) antibodies. The order of the samples for both panel A and panel B is as follows: lanes 1, VV-P1 infected, mock-transfected; lanes 2, VV-P1 infected, transfected with RNA derived from pT7-IC-Gag1-C109S (clone 1); lanes 3, VV-P1 infected, transfected with RNA derived from pT7-IC-Gag1-C109S (clone 2); lanes 4, VV-P1 infected, transfected with RNA derived from pT7-IC-Gag1-C109S (clone 3). The positions of the molecular mass standards are as indicated. The P1-Gag-P2 (P1-Gag-2A-2B-2C) and P1-Gag-2A fusion proteins are also indicated.

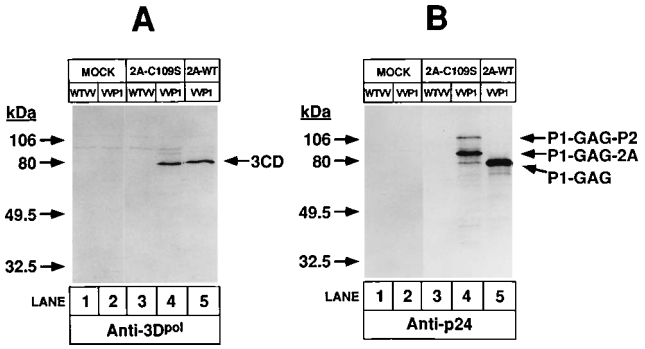

FIG. 3. Analysis of protein expression from cells infected with replicons that were encapsidated and serially passaged with capsid proteins provided by VV-P1. Pass 8 extracts were used to infect cells previously infected for 2 h with 20 PFU of VV-P1 per cell. After 6 h, the monolayers were metabolically labeled and radiolabeled proteins were immunoprecipitated with anti-3D^{pol} antibodies (A) or anti-Gag antibodies (anti-p24) (B). The samples originated with RNA genomes derived from pT7-IC-Gag1-C109S or pT7-IC-Gag1 that were transfected into VV-P1-infected cells. A mock-transfected extract was also included in this experiment. Upon removal of residual VV-P1 in the extracts from the transfected cells, the extracts were serially passaged eight times in the presence of either wild-type vaccinia virus (WTVV) or VV-P1. The lanes for both panel A and panel B contain samples generated as follows: lanes 1, mock-transfected sample, serially passaged with wild-type vaccinia virus; lanes 2, mock-transfected sample, serially passaged with VV-P1; lanes 3, sample derived from cells transfected with RNA transcribed from pT7-IC-Gag1-C109S, serially passaged with wild-type vaccinia virus; lanes 4, sample derived from cells transfected with RNA transcribed from pT7-IC-Gag1-C109S, serially passaged with VV-P1; lanes 5, sample derived from cells transfected with RNA transcribed from pT7-IC-Gag1, serially passaged with VV-P1. The migration of molecular mass markers is noted. For panel A, the migration of the 72-kDa 3CD protein is noted. For panel B, the migrations of the fusion proteins P1-Gag-P2 (P1-Gag-2A-2B-2C), P1-Gag-2A, and P1-Gag are also noted.

capsid (p24) protein. A protein with a molecular mass of 72 kDa, corresponding to the 3CD protein of poliovirus, was immunoprecipitated by anti-3D^{pol} antibodies from cells transfected with the replicon RNA but not by those from mock-transfected cells (Fig. 2A). We found that the 3CD protein was also immunoprecipitated from poliovirus-infected cells under the same conditions used for metabolic labeling (22). To determine if the P1-Gag fusion protein was also expressed, we immunoprecipitated the extracts with anti-p24 antibody. In this case, we detected two Gag-specific fusion proteins (Fig. 2B). One of these migrated with the molecular mass of a fusion protein consistent with that of P1-Gag-P2. A second, more predominant protein with a lower molecular mass, consistent with that of the P1-Gag-2A fusion protein, was also immunoprecipitated. A protein with a similar molecular mass was noted in our previous study (20). The results of these studies demonstrate that each of the RNAs derived from the three independent clones containing the 2AC109S mutation expressed 3CD as well as a P1-Gag-2A fusion protein upon transfection into VV-P1-infected cells. Consistent with our previous studies, we found that transfection of these RNA genomes into uninfected cells did not result in the expression of either 3CD or the P1-Gag fusion proteins (reference 20 and data not shown).

Previous studies from this laboratory have demonstrated that transfection of replicon RNAs containing the *gag* gene into cells previously infected with VV-P1 results in RNA encapsidation (22). To determine if this would be the case with the replicon RNAs containing the 2AC109S mutation, we transfected the RNA transcripts into cells previously infected with VV-P1. As a control, we also did a separate transfection using wild-type vaccinia virus. As a positive control for encapsidation, we transfected the RNA genome derived from pT7-IC-Gag-1, since in previous studies we have demonstrated the encapsidation of RNA from pT7-IC-Gag1 under these experimental conditions (22). At 16 h posttransfection, the cells and the medium were harvested by directly adding Triton X-100 to the medium to a final concentration of 1%. The medium-cell lysate was clarified in a microcentrifuge for 20 min at 14,000 × *g*. The clarified lysate was treated with 20 μg of RNase A per ml at 37°C for 15 min and then diluted to 4 ml with a buffer consisting of 30 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 1% Triton X-100 and overlaid on a 0.5-ml sucrose cushion (30% sucrose, 30 mM Tris-HCl [pH 8.0], 1 M NaCl, 0.1% bovine serum albumin [BSA]) in SW 55 tubes. The sucrose cushion was centrifuged at 45,000 rpm in an SW55 rotor (Beckman) for 2 h. Pelleted material was washed with phosphate-buffered saline–0.1% BSA and recentrifuged. The final pellet was resuspended in 0.6 ml of complete medium. BSC-40 cells were infected for 2 h with 20 PFU of VV-P1 per cell, and 0.25 ml of the 0.6 ml was used to infect cells infected with VV-P1; after 24 h, the cells and medium were harvested. After three freeze-thaw cycles, the sample was centrifuged at 13,000 × *g* to remove cellular debris and the supernatant was used to reinfect cells previously infected with either wild-type vaccinia virus or VV-P1. This procedure was repeated for eight serial passages to generate stocks of encapsidated replicons.

To determine if RNA from the replicons containing the 2AC109S mutation was encapsidated under these experimental conditions, we used the pass 8 material to infect cells previously infected with VV-P1. At 6 h posttransfection, the cells were metabolically labeled for 2 h and immunoprecipitated with antibodies to either 3D^{pol} or the HIV-p24 protein. Poliovirus 3CD protein was immunoprecipitated from cells infected with the material derived from transfection of replicon RNA containing the wild-type 2A gene (positive control) (Fig. 3A). Surprisingly, 3CD protein was also immunoprecipitated from cells infected with the replicon containing the 2AC109S mutation that had been passaged in the presence of VV-P1. No 3CD was immunoprecipitated from cells infected with replicons that

had been passaged in the presence of wild-type vaccinia virus or from cells that were mock-transfected. These results indicated that the replicons containing the 2AC109S mutation were encapsidated when passaged in the presence of VV-P1. To test for the expression of the Gag fusion protein, we immunoprecipitated the extracts with anti-p24 antibody (Fig. 3B). The P1-Gag fusion protein was immunoprecipitated from cells infected with the encapsidated replicon containing the wild-type 2A protein (molecular mass, approximately 80 kDa). A minor protein with a lower molecular weight was also immunoprecipitated, possibly representing a breakdown product of nonspecific proteolysis. Analysis of the protein expressed in cells infected with the replicons containing the 2AC109S mutation revealed that the major protein immunoprecipitated had a molecular mass corresponding to that of the P1-Gag-2A fusion protein. A second protein, which had a molecular mass corresponding to that of the P1-Gag-P2 fusion protein, was also immunoprecipitated. We also immunoprecipitated a minor protein with a molecular mass corresponding to that of the P1-Gag fusion protein. At present, though, we do not know if this protein is the result of true processing of the P1-Gag-2A fusion protein by the 2A containing the C109S mutation or whether it is a nonspecific degradation, as occurs with the P1-Gag expressed from replicons containing the wild-type 2A gene.

To increase the yields of encapsidated replicons, the 2AC109S replicons were further passaged up to pass 15. No changes in protein expression were detected upon further passage with regard to the relative amounts of the P1-Gag-P2 and P1-Gag-2A fusion proteins produced upon infection of cells (data not shown). To investigate if reversions had occurred during the serial passage of the replicon containing the C109S mutation in 2A, we used reverse transcription PCR (RT-PCR) to amplify the region containing the mutation from the pass 15 encapsidated replicons. RT-PCR was carried out with RNA isolated from virions by the procedure of Rico-Hesse et al. (24). We followed the instructions for the Superscript Preamplification System for the RT-PCR of viral RNA. Negative control reactions were used for each assay to ensure that no contamination was present; samples with invalid negative controls were excluded from the analysis. The oligonucleotides used were designed to amplify the region in the 2A gene of poliovirus cDNA corresponding to nucleotides 3348 to 3872 as follows: 5′-GGA-GTG-GAA-TTC-AAG-GAT-GG-3′ (5′ oligonucleotide) and 5′-CTG-CTG-AGT-GGA-TCC-ACT-TCC-3′ (3′ oligonucleotide).

Conditions for PCR were as previously described (20): 94°C (1 min), 37°C (1 min), and 72°C (1 min). At the end of 30 cycles, a 72°C cycle for 7 min was included. The oligonucleotides were constructed to generate unique *Eco*RI (5′) and *Bam*HI (3′) restriction sites at the ends of the PCR-amplified product. The amplified product was extracted with phenol-chloroform and chloroform isoamyl alcohol and precipitated with ethanol at −70°C. After collection of the DNA by centrifugation, the DNA was dried and reconstituted with H_2O. The DNA was digested with restriction enzymes *Eco*RI and *Bam*HI, and then successive phenol-chloroform–chloroform isoamyl alcohol extractions and precipitation with ethanol under standard conditions were performed (17). The precipitates were collected by centrifugation, dried, and resuspended in H_2O. The digested PCR product was ligated into pUC119 digested with the same enzymes. Following ligation of the PCR-amplified product into pUC119, the plasmids were used to transform *Escherichia coli* DH5 α. Recombinants were identified by using the blue-white screen in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (28); restriction enzyme analysis was used to confirm that the recombinants contained the PCR-amplified gene product. Plasmids containing the DNA inserts were then used for DNA sequencing with an oligonucleotide complementary to nucleotides 3776 to 3762: CCA-ACC-CTT-CGC-CAC. DNA sequencing was carried out by the dideoxy chain termination method as previously described (26). The plasmids from 12 independent clones of pUC119 containing the 2A gene obtained from the RT-PCRs using RNA from encapsidated virions containing the 2AC109S mutation were analyzed. In every one of the clones sequenced, we found the 2AC109S mutation (Table 1). Thus, under our experimental conditions, reversion of the mutation in the 2A gene to the wild-type sequence did not occur, or if it did, it was below the level of detection by RT-PCR analysis.

TABLE 1. Analysis of the 2A gene sequence of encapsidated replicons from pass 15 containing the 2AC109S mutation

Sample[a]	2A sequence	Observed frequency (no. of clones with mutation/no. of clones tested)
WT[b]	GAT-TGT-GGT[c] D C G[d]	
2AC109S	GAT-AGC-GGT[c] D S G[d]	12/12[e]

[a] Samples were obtained from RT-PCR of replicons containing the wild-type sequence or the 2AC109S mutation.
[b] WT, wild type.
[c] Nucleic acid sequence.
[d] Amino acid sequence.
[e] Samples obtained from RT-PCR of replicons containing the 2AC109S mutation obtained after 15 serial passages. The numbers refer to DNA sequences of independent *E. coli* clones containing pUC119 with the PCR-amplified DNA insert corresponding to nucleotides 3348 to 3872 of the 2A gene.

In preliminary experiments, we determined that transfection of RNA genomes containing the 2AC109S mutation into uninfected cells did not result in the expression of either 3CD or the Gag fusion protein (data not shown). From previous studies we know that the majority of the RNA transfected into uninfected cells is degraded (5). A small portion of this RNA is not degraded, and because this RNA can replicate, it is amplified in cells, resulting in the expression of viral proteins. Since previous studies have demonstrated that $2A^{pro}$ is involved in the inhibition of host cell translation via induction of an enzyme to degrade p220 (8, 14, 15, 31, 32), we wanted to determine if the encapsidated replicons containing the 2AC109S mutation would express proteins when transfected into cells in the absence of vaccinia virus. For these studies we compared the expression of the P1-Gag fusion protein in cells infected with replicons containing 2AC109S with and without coinfection with vaccinia virus over a 19-h period (Fig. 4). We also conducted a similar comparison in parallel with encapsidated replicons containing the wild-type 2A gene. Prior to these experiments, residual infectious vaccinia virus in the stocks of encapsidated replicons was removed by procedures described in previous studies (1, 22). To normalize the amounts of replicons used to infect cells, we estimated the titers of the stocks of encapsidated replicons. Since the encapsidated replicons have the capacity to infect a cell but lack capsid proteins, they cannot form plaques and therefore virus titers cannot be quantified by traditional assays. To overcome this problem, we used a method to estimate the titers of the encapsidated replicons by comparison with wild-type poliovirus of known titer. For these studies, RNA from a titered stock

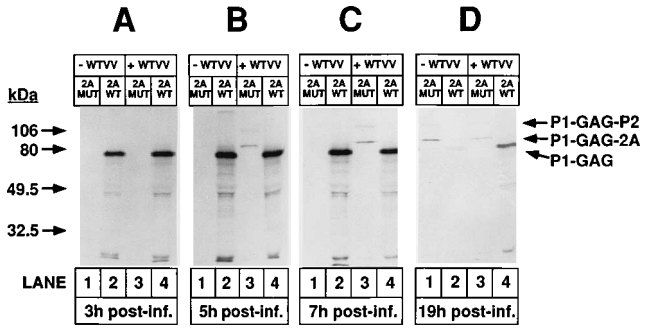

FIG. 4. Kinetics of expression of P1-Gag fusion proteins in cells infected with wild-type 2A replicons or 2AC109S replicons in the presence or absence of wild-type vaccinia virus. Cells were infected with wild-type vaccinia virus at a multiplicity of infection of 10 PFU per cell (+ WTVV) or were mock-infected (− WTVV). After 2 hrs, the cells were infected either with replicons containing the wild-type 2A gene (2AWT) or with replicons containing the 2AC109S mutation (2AMUT) at a multiplicity of infection of 3 IU per cell, as determined by the dot blot assay. The replicons were adsorbed for 1 h prior to the addition of complete medium (designated time 0). The infected cells were incubated in complete medium until 30 min prior to the times indicated in the figure. For this 30-min period, the cells were starved for methionine and cysteine, and then they were metabolically labeled for 1 h with [^{35}S]Translabel. After that time, the cells were lysed, and radiolabeled P1-Gag fusion proteins were immunoprecipitated from the lysates with anti-p24 antibodies. The times postinfection are labeled in each panel, and the samples were derived from cells infected as described for each lane: lanes 1, cells infected with the replicon containing the 2AC109S mutation alone; lanes 2, cells infected with replicon containing the wild-type 2A gene alone; lanes 3, cells infected with wild-type vaccinia virus and then with the replicon containing the 2AC109S mutation; lanes 4, cells infected with wild-type vaccinia virus and then with the replicon containing the wild-type 2A gene. The molecular mass markers are as indicated. The migrations of the P1-Gag-P2 (P1-Gag-2A-2B-2C), P1-Gag-2A, and P1-Gag fusion proteins are also indicated.

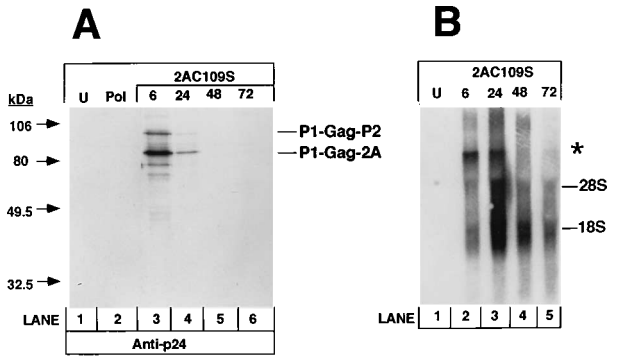

FIG. 5. Analysis of the expression of the P1-Gag fusion protein and levels of intracellular replicon RNA. Cells were infected with approximately 3 IU of the encapsidated replicon containing the 2AC109S mutation per cell. At 30 min prior to the times indicated below, the medium was removed and the cells were starved for methionine and cysteine (30 min); subsequently, they were labeled with [^{35}S]Translabel for 1 h. The cells were then analyzed for expression of P1-Gag fusion proteins by immunoprecipitation with anti-p24 antibodies. For analysis of viral RNA, cells were harvested at the times indicated below and total cellular RNA was isolated. Following electrophoresis and transfer of the RNA to nitrocellulose, the blots were probed with a radiolabeled riboprobe complementary to poliovirus nucleotides 671 to 1174 (5). The lanes for panel A contain proteins immunoprecipitated from cells treated as follows: lane 1, uninfected cells labeled at 6 h postinfection; lane 2, poliovirus-infected cells labeled at 6 h postinfection; lane 3, 2AC109S replicon-infected cells labeled at 6 h postinfection; lane 4, 2AC109S replicon-infected cells labeled at 24 h postinfection; lane 5, 2AC109S replicon-infected cells labeled at 48 h postinfection; lane 6, 2AC109S replicon-infected cells labeled at 72 h postinfection. The molecular mass markers and migrations of P1-Gag-P2 (P1-Gag-2A-2B-2C) and P1-Gag-2A fusion proteins are noted. The lanes for panel B contain the following RNA samples: lane 1, RNA from uninfected cells harvested at 6 h postinfection; lane 2, RNA from 2AC109S replicon-infected cells harvested at 6 h postinfection; lane 3, RNA from 2AC109S replicon-infected cells harvested at 24 h postinfection; lane 4, RNA from 2AC109S replicon-infected cells harvested at 48 h postinfection; lane 5, RNA from 2AC109S replicon-infected cells harvested at 72 h postinfection. The migration of the replicon RNA is indicated by an asterisk, and the migrations of 28S and 18S RNA are noted.

of poliovirus was isolated according to previously described procedures (24). The virion RNA was precipitated with 2.5 volumes of ethanol by using 0.2 M lithium chloride. The encapsidated replicon RNA was extracted by the same procedure. Following precipitation, the RNAs were washed consecutively with 80% ethanol and 100% ethanol, dried, resuspended in diethyl pyrocarbonate-treated water, and serially diluted. The dilutions were then spotted onto a nitrocellulose paper with a dot blot apparatus (Bio-Rad). For detection of poliovirus-specific RNA, in vitro transcription with [α-^{32}P]UTP was used to generate a 503-bp riboprobe complementary to nucleotides 671 to 1174 of the poliovirus genome that could be used to detect the plus-strand RNA of both wild-type poliovirus and encapsidated replicon RNA genomes (5). To determine the relative titers of the encapsidated replicons, the radioactivity associated with each dilution was quantitated by using a Phosphorimager. A standard curve was generated by using wild-type poliovirus RNA for which the radioactive intensity was plotted versus the known PFU of the wild-type poliovirus genome. Extrapolation of the radioactivity of the encapsidated replicon samples to the curve gave an approximate estimation of the titers of the stocks of encapsidated replicons. The resulting titers were then expressed in infectious units (IU) of replicons, since the infection of cells with the replicons does not lead to plaque formation because of the absence of the P1 capsid genes. We have determined experimentally that the infectivity of equal amounts of IU of encapsidated replicons correlates with equal amounts of PFU of wild-type poliovirus (21a). Normalized amounts of replicons were then used to infect uninfected cells or those previously infected with wild-type vaccinia virus. At the designated times postinfection, the cultures were metabolically labeled for 1 h and then p24-specific proteins were immunoprecipitated. Expression of the P1-Gag fusion protein in cells infected with the replicon containing wild-type 2A protein was detected at 3 h postinfection in both the absence and the presence of wild-type vaccinia virus (Fig. 4A, lanes 2 and 4). Expression continued to increase at 5 and 7 h postinfection (Fig. 4B and C, lanes 2 and 4). At 19 h postinfection, expression from the replicons containing the wild-type 2A gene had started to decrease, although there was still detectable expression in cells that had been coinfected with wild-type vaccinia virus (Fig. 4D, lane 4). At this time, cells infected with replicons containing wild-type 2A, both uninfected and vaccinia virus-infected cells, were demonstrating a considerable cytopathic effect and detaching from the culture plates.

In contrast, cells infected with the replicon containing the 2AC109S mutation demonstrated a different pattern of expression of P1-Gag-2A fusion protein. The level of expression of the P1-Gag-2A fusion protein was very low at 3 h postinfection in cells coinfected with wild-type vaccinia virus (Fig. 4A, lane 3). Expression increased at 5 and 7 h posttransfection (Fig. 4B and C, lanes 3), but it was reduced by 19 h posttransfection. Surprisingly, we detected expression of the P1-Gag-2A fusion protein in cells infected with only the replicon containing the 2AC109S mutation at 19 h postinfection; expression was evident at early times postinfection, though, upon longer exposure of the autoradiogram (data not shown). These results demonstrate that expression of the P1-Gag fusion protein occurs at low levels in cells infected with the replicon containing the 2AC109S mutation. To further substantiate this result, we set up an experiment in which we extended the times for

analysis of the expression from 19 h to 3 days and used a Northern (RNA) blot analysis to correlate levels of protein expression with the presence of the replicon RNA genome (Fig. 5). Under these experimental conditions, we detected synthesis of the P1-Gag-2A fusion protein at 6 h postinfection, which was probably due to the higher IU/cell ratio used in these experiments. The levels of P1-Gag-2A expression had decreased by 24 h postinfection, but they were still detectable even as late as 72 h postinfection. At the same time points, we isolated total cellular RNA and analyzed it for the presence of replicon RNA. The levels of replicon RNA correlated with the levels of protein expression in that the highest levels were evident at 6 h, followed by a gradual decrease. Note that even at 72 h we could still detect replicon RNA in cells. In parallel experiments, we analyzed expression of the P1-Gag fusion protein in cells infected with replicons containing the wild-type 2A gene. In these studies we found that levels of expression and replicon RNA were greater than those found in cells infected with the replicon containing the 2AC109S mutation. Because of the high IU/cell ratio used in these experiments, infection of cells with replicons containing the wild-type 2A gene resulted in cell death by 24 h, thus precluding an analysis of protein expression at later time points (data not shown). Taken together, these results demonstrate that replicons containing the 2AC109S mutation have the capacity to infect and replicate in cells but that levels of replicon-specific protein expression as well as replication of RNA are lower than those for cells infected with replicons containing a wild-type 2A gene.

In a previous study, we determined that the proteolytic function of the 2AC109S mutation could be complemented by a wild-type $2A^{pro}$ provided in *trans* (20). For these studies we transfected the RNA genome into cells previously infected with wild-type vaccinia virus and poliovirus to determine if coinfection of cells with poliovirus and encapsidated replicons containing the 2AC109S mutation would also result in the complementation of the $2A^{pro}$ defect. For these studies, cells were infected with either poliovirus alone (5 PFU per cell) or the encapsidated replicon containing the 2AC109S mutation (approximately 3 IU per cell) alone, or they were coinfected with both poliovirus and the encapsidated replicon containing the C109S mutation. As a control, cells were also infected with the replicon containing the wild-type 2A gene to provide a marker protein for the P1-Gag fusion protein. At 4 h postinfection the cells were labeled for 1 h with methionine-cysteine, and then they were subjected to either lysis or chase in complete medium for an additional 30 or 90 min (chase). The lysates were then immunoprecipitated with anti-p24 antibody (Fig. 6). A protein with a molecular mass consistent with that of the P1-Gag-2A fusion protein was detected in cells infected with the replicon containing the 2AC109S mutation at both the 0- and 90-min chase times. In contrast, cells coinfected with the replicon containing the 2AC109S mutation and poliovirus demonstrated proteins with molecular masses consistent with that of the P1-Gag-2A fusion protein as well as that of the completely processed P1-Gag protein. There appeared to be no change in the levels of processing as the chase time was extended from 30 to 90 min. In addition, at time zero, we also detected a protein with a molecular mass consistent with that of the P1-Gag-P2 fusion protein. The results of these studies clearly demonstrate that the P1-Gag-2A fusion protein produced from infection of cells with the encapsidated replicon containing 2AC109S can be processed by the wild-type $2A^{pro}$ provided in *trans*. The processing appears to be inefficient, though, as not all of the synthesized P1-Gag-2A fusion proteins could be processed during the period examined (approximately 90 min).

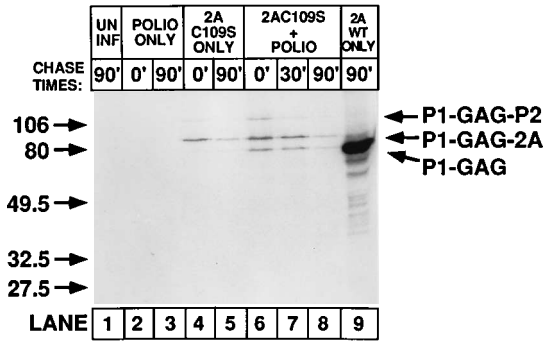

FIG. 6. Processing of P1-Gag-2A fusion protein by 2A provided in *trans*. Cells were infected with poliovirus alone (5 PFU per cell) or the replicon containing the 2AC109S mutation alone (2.5 IU per cell), or they were coinfected with poliovirus and the replicon containing the 2AC109S mutation. Cells were infected by adsorbing the viruses mentioned above for 1.5 h. Complete medium was then added, and the time of addition was designated 0 h postinfection. At 4 h postinfection, the cells were starved for 40 min in methionine-cysteine-free medium and then metabolically labeled with [^{35}S]Translabel for 70 min. The cells were washed twice in complete medium and either lysed immediately (0 h postchase) or further incubated for 30 or 90 min in complete medium prior to lysis and analysis for P1-Gag fusion protein expression by immunoprecipitation with anti-p24 antiserum. The samples in each lane were derived from cells treated as follows: lane 1, uninfected cells labeled and then chased for 90 min; lane 2, poliovirus-infected cells labeled with no chase; lane 3, poliovirus-infected cells labeled and then chased for 90 min; lane 4, cells infected with the replicon containing the 2AC109S mutation and labeled, with no chase; lane 5, cells infected with the replicon containing the 2AC109S mutation, labeled, and then chased for 90 min; lane 6, cells coinfected with poliovirus and the replicon containing the 2AC109S mutation and labeled, with no chase; lane 7, cells coinfected with poliovirus and the replicon containing the 2AC109S mutation, labeled, and then chased for 30 min; lane 8, cells coinfected with poliovirus and the replicon containing the 2AC109S mutation, labeled, and then chased for 90 min; lane 9, cells infected with 2.5 IU of the replicon containing the wild-type 2A (2AWT) gene per cell, labeled, and then chased for 90 min. The migrations of the molecular mass standards are as noted. The migrations of the fusion proteins P1-Gag-P2 (P1-Gag-2A-2B-2C), P1-Gag-2A, and P1-Gag are also indicated.

In conclusion, we have described the characterization of a poliovirus replicon with the *gag* gene of HIV-1 in the P1 region containing a second mutation to change the codon at amino acid 109 of the 2A gene from a cysteine to a serine. Transfection of RNA containing this genome into cells previously infected with vaccinia virus resulted in expression of a P1-Gag-2A fusion protein. In the presence of a recombinant vaccinia virus, VV-P1, that expresses poliovirus capsid proteins upon infection, we encapsidated and serially passaged the replicon containing the 2AC109S mutation. We found that the levels of protein expression as well as the levels of RNA were reduced in cells infected with the replicon containing the 2AC109S mutation compared with those in cells infected with the replicon containing the wild-type 2A gene. The P1-Gag-2A fusion protein expressed from the replicon containing the 2AC109S mutation could be processed in *trans* by $2A^{pro}$ expressed from a coinfecting wild-type poliovirus.

The results of this study are important because it is the first report of the generation of an infectious poliovirus, or replicon RNA, that contains a stable mutation in a P2 or P3 region gene that results in inactivation of a specific gene product. Previous studies have described the construction of mutations engineered into the P2 and P3 regions of the poliovirus genome (2–4, 9). The hallmark of these studies, including two which described mutations in 2A (2, 3), was the fact that the viruses that arose from the transfection of RNA containing these mutations would revert to the wild type after replication. No mutants with an inactive P2 or P3 region gene have been isolated, making it difficult to assess the roles of individual P2

or P3 region proteins in viral replication. We were, in fact, somewhat surprised that the 2AC109S mutation was stable in the replicon after 15 serial passages. A possible explanation for this stability is that the vaccinia virus (VV-P1) used to encapsidate and propagate this replicon relieves selective pressure for reversion of the replicon to wild-type 2A. One of the functions of $2A^{pro}$ in the poliovirus life cycle is to catalyze the initial proteolytic cleavage of the capsid precursor away from the primary translation product of the viral genome, which allows the process of capsid maturation to occur. Because VV-P1 provides the P1 capsid precursor in the absence of $2A^{pro}$ activity, the requirement for one of the enzymatic functions of $2A^{pro}$ has been eliminated. We do not believe that the stability of the 2AC109S mutation occurs because of selective pressure against reversion induced by the recombinant vaccinia virus used to provide the poliovirus capsid precursor. We have previously demonstrated that VV-P1 and replicons containing wild-type 2A genes are compatible in the complementation system described here for serial passage and encapsidation of the mutant replicon (1, 22). Therefore, we do not believe that maintenance of the 2AC109S mutation in serial passage of the mutant replicon is artifactual on the basis of an incompatibility between replicons containing wild-type 2A genes and VV-P1 in this complementation system. It is also possible that reversions of the mutations engineered into replicons do not occur with the same frequency as that observed for mutations engineered in the wild-type poliovirus genome.

The results of these studies highlight some of the multiple functions of the 2A protein in poliovirus replication. The 2A protein has been found to be a critical component in the shutoff of the host cell translation that is characteristic of poliovirus-infected cells (6, 8, 15). Previous studies have suggested that the 2A protein induces a cellular protease that results in the degradation of the 220-kDa component of the cap binding protein complex (14, 15). The activity of the $2A^{pro}$ is required for this function, since mutations in or around the putative active site of $2A^{pro}$ have resulted in an enzyme which does not have the capacity to induce the cleavage of p220 (8, 31, 32). A cysteine-to-serine change in amino acid 109 of 2A (2AC109S) results in a protein that is incapable of inducing the cleavage of p220 (8, 31, 32). If this mutation is re-engineered back into the infectious poliovirus cDNA, the resulting cDNA is noninfectious upon transfection into uninfected cells (8). These results led to the suggestion that the enzymatic activity of the $2A^{pro}$ was required for viral replication. Recent studies from this laboratory have demonstrated that transfection of replicon RNAs containing the 2AC109S mutation into vaccinia virus-infected cells produced replicons that were replication competent (20). Since these RNAs were not replication competent in uninfected cells, we proposed that vaccinia virus provides a factor or intracellular milieu conducive to the translation and replication of the replicon RNA. A drawback of our previous study was the fact that in order to assess the replication competency of the RNA containing the 2AC109S mutation the RNA had to be transfected into cells. Since we, as well as others, have demonstrated that the vast majority of the transfected RNA is degraded and that the in vitro transcribed RNA has a lower level of infectivity than virion RNA as evidenced by a lower ratio of PFU to micrograms of RNA, this experimental approach is insensitive. To approach this problem from a new perspective, we have used a system in which we can encapsidate replicon RNA by transfection into cells previously infected with a recombinant vaccinia virus that expresses the capsid precursor, VV-P1 (22). Surprisingly, infection of cells with this encapsidated replicon resulted in the replication of the RNA and expression of the P1-Gag-2A fusion protein regardless of whether the cells had been previously infected with vaccinia virus. The levels of RNA replication and protein expression, though, were lower than those for a similar replicon RNA containing the wild-type 2A gene. From these studies we can conclude that enzymatically active $2A^{pro}$, while not absolutely required for replication of the viral RNA, is required for high-level expression. This interpretation is consistent with the fact that $2A^{pro}$ is known to induce the degradation of p220, leading to host cell protein shutoff, which is required for the increased production of viral proteins found in poliovirus-infected cells. Without this host cell protein shutoff, the RNA still replicates, but at a reduced level compared with genomes with an enzymatically active $2A^{pro}$. The development of strategies for generating encapsidated replicons containing mutations that otherwise would appear to be lethal in the context of a full-length poliovirus genome will permit a more thorough analysis of the role of $2A^{pro}$ in replication.

Previous studies have suggested that the $2A^{pro}$ might act to increase the translation of poliovirus RNA before a complete shutoff of host cell synthesis is evident (7, 16). The results of the experiments described in this paper lend support to the idea that the translational enhancement capacity of 2A is not dependent upon the enzymatic activity. The observed replication and expression of the replicon containing the 2AC109S mutation might be due to the fact that the mutant 2A protein could still act as a translational enhancer to allow the expression of the genome. The mechanism for this enhancement of translation by 2A has not been determined. Two possibilities are that the 2A directly binds to the viral RNA, possibly the 5′ nontranslated region, to facilitate translation or that the 2A modifies a host cell protein, resulting in translational enhancement (7). Our results might favor the first hypothesis. Since the mutation 2AC109S results in an inactivation of the $2A^{pro}$, the replicons containing the 2AC109S mutation would not induce the cellular p220 cleavage found with wild-type 2A. Recent studies have mapped the regions of the 2A that might be involved in translational enhancement. Most of these mutations appear to be at or near the surface of the $2A^{pro}$ and not close to the putative active site (amino acid 109). On the basis of these observations, we speculate that the translational enhancement observed for 2A is probably not a function of the enzymatic activity of $2A^{pro}$. Furthermore, if the 2A protein containing the C109S mutation is acting as a translational enhancer in the system described here, this enhancement activity most likely can still occur when $2A^{pro}$ is linked to other viral polypeptides in the form of a polyprotein, since the 2AC109S mutant protein is present predominantly in a polyprotein form in the experiments described in this report (P1-Gag-2A or P1-Gag-P2). Further experiments to substantiate this claim are ongoing.

The proteolytic activity of 2A is essential for the cleavage of the P1 precursor from the growing poliovirus polyprotein. In a recent study, we provided evidence that the $2A^{pro}$ activity could be complemented in *trans* by transfection of replicons into cells previously infected with vaccinia virus and poliovirus (20). In this paper, we further substantiate that result by demonstrating that coinfection of cells with poliovirus and the encapsidated replicon containing the 2AC109S mutation resulted in a complementation of the proteolytic activity of the 2A protein. Cleavage of the P1-Gag-2A fusion protein to produce a P1-Gag fusion protein was observed in cells coinfected with poliovirus and the encapsidated replicon containing 2AC109S. These results further confirm that $2A^{pro}$ can act in *trans* to process the poliovirus proteins. The fact that the complementation was not complete could be due to several reasons. It is possible that in a coinfected cell, diffusion of the

wild-type 2A protein and/or the P1-Gag-2A fusion protein is restricted. A compartmentalization of the replication process in coinfected cells may occur, such that mixing of the viral products from wild-type poliovirus and replicons containing 2AC109S does not occur with great frequency. An alternative interpretation of the inability of $2A^{pro}$ provided in *trans* to completely process all of the P1-Gag-2A fusion protein is that only some of the P1-Gag-2A fusion proteins are in an appropriate conformation for proteolytic processing. This seems unlikely, since the P1-Gag-2A is a fusion protein between poliovirus and HIV proteins and it is doubtful that any protein conformation required for $2A^{pro}$ cleavage is maintained. Further support for restricted diffusion of $2A^{pro}$ or the P1-Gag-2A fusion protein comes from preliminary experiments in which we have engineered a synthetic $2A^{pro}$ cleavage site into foreign genes expressed from a replicon at a site distant from 2A (i.e., at the VP4-VP2 junction). Upon transfection of these replicon RNAs into cells, the complete processing of the synthetic 2A cleavage site was observed. Thus, the expression of the $2A^{pro}$ and fusion proteins in the same intracellular proximity resulted in complete processing by $2A^{pro}$ in *trans*.

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