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Encapsidation of Genetically Engineered Poliovirus Minireplicons Which Express Human Immunodeficiency Virus Type 1 Gag and Pol Proteins upon Infection

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The use of recombinant viruses for the expression of a wide array of foreign proteins has become commonplace during the last few years. Recently, we have described the construction and characterization of chimeric human immunodeficiency virus type 1 (HIV-1)–poliovirus genomes in which the *gag* and *pol* genes of HIV-1 have been substituted for the VP2 and VP3 capsid genes of the P1 capsid precursor region of poliovirus. Transfection of these RNAs into tissue culture cells results in replication of the RNA genome and expression of HIV-1–P1 fusion proteins (W. S. Choi, R. Pal-Ghosh, and C. D. Morrow, J. Virol. 65:2875–2883, 1991). Here we report on the encapsidation and amplification of the minireplicons to obtain sufficient quantities for biological characterization. To do this, HIV-1–poliovirus minireplicon genomes containing the *gag* or *pol* gene were transfected into cells previously infected with a recombinant vaccinia virus (VV-P1) which expresses the poliovirus capsid precursor protein, P1 (D. C. Ansardi, D. C. Porter, and C. D. Morrow, J. Virol. 65:2088–2092, 1991). The chimeric minireplicons replicated and expressed the appropriate HIV-1–P1 fusion proteins as determined by immunoprecipitation with HIV-1-specific antibodies. The encapsidated genomes were isolated by ultracentrifugation. Reinfection of cells with the encapsidated chimeric RNA genomes resulted in expression of the HIV-1–Gag–P1 or HIV-1–Pol–P1 fusion protein. Serial passaging of the encapsidated chimeric HIV-1–poliovirus genomes was accomplished by coinfecting cells with the encapsidated minireplicons and VV-P1, resulting in stocks of the encapsidated minireplicons. Northern (RNA) blot analysis of passaged material revealed that no detectable deletions of the chimeric genomes occurred during 14 serial passages. Infection of cells by the encapsidated minireplicons was blocked by antipoliovirus antibodies. Coinfection of cells with encapsidated minireplicons and type 1 Sabin poliovirus resulted in encapsidation of the chimeric genomes by wild-type poliovirus as measured by immunoprecipitation of the HIV-1–P1 fusion proteins with HIV-1-specific antibodies. The results of this study demonstrate the encapsidation of poliovirus minireplicons which express foreign proteins and point to the future use of this system as a potential vaccine vector.

The RNA genome of poliovirus is approximately 7,500 nucleotides in length (20, 24). Expression of the viral proteins occurs by translation of the entire viral genome, resulting in a long polyprotein which is subsequently processed by virus-encoded proteinases 2A and $3C^{pro}$ (14, 24, 34, 39). Initial proteolytic processing results in the release of a capsid precursor protein, P1, by proteinase 2A (14, 20, 22). Previous studies have demonstrated that this processing occurs immediately following translation of viral proteinase 2A (38). The subsequent processing of the nonstructural precursor proteins P2 and P3 is accomplished by proteinase 3C. Earlier studies have established that proteolytic processing of the P1 precursor polyprotein occurs in *trans* by the viral proteinase 3CD, which consists of a fusion protein between $3C^{pro}$ and $3D^{pol}$, the RNA-dependent RNA polymerase (40, 41). Recent studies have demonstrated that independent expression of the P1 polyprotein and 3CD polyprotein by using recombinant vaccinia viruses also results in processing of P1 protein and subsequent assembly of subviral intermediates in the coinfected cells (2).

The availability of an infectious poliovirus cDNA has provided an opportunity to use molecular genetics to investigate various aspects of viral replication (31, 36). Studies have demonstrated that deletion of the majority of the P1 coding region results in an RNA with the capacity to replicate when transfected into cells provided that the translational reading frame is maintained between the deleted regions (18). Recent studies have also described the construction of poliovirus replicons which contain the chloramphenicol acetyltransferase (CAT) gene inserted between nucleotides 743 and 1805 (in frame with the first AUG codon) of poliovirus (29). Replication and encapsidation of this chimeric replicon by helper virus (type 3 poliovirus) was demonstrated as measured by detection of CAT activity in infected cells. Our laboratory has previously demonstrated that foreign genes can be inserted into the poliovirus RNA genome, resulting in the generation of chimeric minireplicons which replicate and express the desired protein when transfected into cells (5). However, we were unable to demonstrate encapsidation of these minireplicons upon transfection of in vitro-transcribed RNA into type 1 Mahoney poliovirus-infected cells. To circumvent this problem, we have adopted a different strategy to encapsidate the chimeric genomes by using a recombinant vaccinia virus, VV-P1, which expresses the poliovirus capsid precursor protein upon infection (2). We report that transfection of human immunodeficiency virus type 1 (HIV-1)–poliovirus chimeric RNAs into cells previously infected with VV-P1 results in encapsidation of these chimeric RNA genomes and demonstrate serial passaging of the encapsidated chimeric genomes in the presence of VV-P1. Coinfection of cells with a stock of encapsidated minireplicons and type 1 Sabin poliovirus also resulted in encapsidation of the minireplicon

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genome which was stably maintained for 10 serial passages. The potential uses of the encapsidated minireplicons as an expression system and a means to examine the requirements for replication and encapsidation of poliovirus RNA are discussed.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co. Restriction enzymes were obtained from New England Biolabs. Tissue culture media was purchased from Gibco/BRL Co. ^{35}S Translabel (methionine-cystine) and methionine-cystine-free Dulbecco modified Eagle medium (DMEM) were purchased from ICN Biochemicals. T7 RNA polymerase was prepared in this laboratory by the method of Grodberg and Dunn (12).

Tissue culture cells and viruses. HeLa and BSC-40 cells were grown in DMEM supplemented with 5% A-γ newborn calf serum and 5% fetal calf serum (complete medium). The stock of the poliovirus type 1 Mahoney used in this study was derived from transfection of an infectious cDNA clone obtained from B. Semler, University of California at Irvine (36). The stock of type 1 Sabin poliovirus was obtained from the American Type Culture Collection. Wild-type vaccinia virus (wt VV) strain WR and the recombinant vaccinia virus VV-P1, which express the poliovirus P1 capsid precursor protein, have been previously described (2). Antisera to HIV-1 reverse transcriptase (RT) and HIV-1 p25/24 Gag (37) were obtained through the AIDS Research and Reference Reagent Program. Pooled AIDS patient sera was obtained from the Center for AIDS Research, University of Alabama at Birmingham.

In vitro transcription reaction. The in vitro transcription reactions were performed by using T7 RNA polymerase as described previously (5). Prior to in vitro transcription, DNA templates were linearized by restriction enzyme digestion, followed by successive phenol-chloroform (1:1) and chloroform extractions and ethanol precipitation. Reaction mixtures (100 μl) contained 1 to 5 μg of linearized DNA template, 5× transcription buffer (100 mM Tris [pH 7.7], 50 mM $MgCl_2$, 20 mM spermidine, 250 mM NaCl), 10 mM dithiothreitol, 2 mM each GTP, UTP, ATP, and CTP, 40 U of recombinant RNAsin (Promega), and approximately 5 μg of purified T7 RNA polymerase per reaction mixture. After 60 min at 37°C, 5% of the in vitro-synthesized RNA was analyzed by agarose gel electrophoresis.

Encapsidation and serial passage of minireplicons by VV-P1. HeLa cells were infected with 20 PFU of VV-P1 (a recombinant virus which expresses the poliovirus capsid precursor protein P1) or wt VV per cell. After 2 h of infection, the cells were transfected (by using DEAE-dextran [500,000 Da] as a facilitator) with RNA transcribed in vitro from the chimeric HIV-1–poliovirus genomes as previously described (5). The cultures were harvested at 24 h posttransfection. The cells were lysed with Triton X-100 at a concentration of 1%, treated with RNase A, and clarified by low-speed centrifugation at 14,000 × *g* for 20 min at 4°C as described previously (25). The supernatants were adjusted to 0.25% sodium dodecyl sulfate (SDS), overlaid on a 30% sucrose cushion (30% sucrose, 30 mM Tris [pH 8.0], 1% Triton X-100, 0.1 M NaCl), and centrifuged in a Beckman SW55Ti rotor at 45,000 rpm for 1.5 h. The pelleting procedure described above has been demonstrated to be effective for the removal of infectious vaccinia virus to below detectable levels (1). The supernatant was discarded, and the pellet was washed by recentrifugation for an additional 1.5 h in a low-salt buffer (30 mM Tris [pH 8.0], 0.1 M NaCl). The pellets were then resuspended in complete DMEM and designated passage 1 of the minireplicons encapsidated by VV-P1.

For serial passage of the encapsidated minireplicons, BSC-40 cells were infected with 20 PFU of VV-P1 per cell. At 2 h postinfection, the cells were infected with passage 1 of the encapsidated minireplicons. The cultures were harvested at 24 h postinfection by three successive freeze-thaws, sonicated, and clarified by centrifugation at 14,000 × *g* for 20 min. The supernatants were then stored at −70°C or used immediately for additional passages following the same procedure.

Metabolic labeling and immunoprecipitation of viral proteins. To metabolically label viral proteins from infected-transfected or infected cells, the cultures were starved for methionine-cystine at 6 h postinfection by incubation in DMEM minus methionine-cystine for 30 min. At the end of this time, ^{35}S Translabel was added for an additional hour. Cultures were then processed for immunoprecipitation of viral proteins by lysing the cells with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 10 mM Tris [pH 7.8], 1% Triton X-100, 1% sodium deoxycholate, 0.2% SDS). Following centrifugation at 14,000 × *g* for 10 min to pellet any debris, designated antibodies were added to the supernatants, which were incubated at 4°C rocking for 24 h. The immunoprecipitates were collected by addition of 100 μl of protein A-Sepharose (10% [wt/vol] in RIPA buffer). After 1 h of rocking at room temperature, the protein A-Sepharose beads were collected by brief centrifugation and washed three times with RIPA buffer. The bound material was eluted by boiling for 5 min in gel sample buffer (50 mM Tris [pH 6.8], 5% SDS, 10% glycerol, 0.01% bromophenol blue, 10% β-mercaptoethanol). The proteins were analyzed by SDS-polyacrylamide gel electrophoresis, and radiolabeled proteins were visualized by fluorography.

Nucleic acid hybridization. RNA from a stock of minireplicons encapsidated by VV-P1 was analyzed by Northern (RNA) blotting. Stocks of encapsidated minireplicons at passage 14 and a high-titer stock of type 1 Mahoney poliovirus were subjected to RNase A treatment and overlaid on a 30% sucrose cushion (30% sucrose, 30 mM Tris [pH 8.0], 1% Triton X-100, 0.1 M NaCl). The samples were centrifuged in a Beckman SW55Ti rotor at 45,000 rpm for 1.5 h. Pelleted virions were resuspended in TSE buffer (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA) and adjusted to 1% SDS and 1% β-mercaptoethanol as previously described (33). The resuspended virions were disrupted by extraction three times with phenol-chloroform equilibrated to acidic buffer and one time with chloroform. The extracted RNA was precipitated with 0.2 M $LiCl_2$ and 2.5 volumes 100% ethanol. The RNA was denatured and separated on a formaldehyde-agarose gel. The RNA was then transferred from the gel to a nitrocellulose filter by capillary elution (35) and cross-linked by using a UV Stratalinker (Stratagene). The conditions used for prehybridization, hybridization, and washing of RNA immobilized on filters were previously described (35). Briefly, the blot was prehybridized in hybridization buffer (50% deionized formamide, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% SDS, 0.1% Tween 20, 100 μg of yeast tRNA per ml). The blot was then incubated in hybridization buffer containing 10^6 cpm of a [^{32}P]UTP-labeled riboprobe complementary to nucleotides 671 to 1174 of the poliovirus genome (5) per ml. After hybridization, the blot was washed two times with 0.1× SSC–0.1% SDS at room temperature and one time at 65°C.

The blot was then exposed to X-ray film with an intensifying screen.

Neutralization of the minireplicons encapsidated by VV-P1, using antipoliovirus antibodies. For antibody neutralization, encapsidated minireplicons at passage 9 were pelleted by ultracentrifugation and resuspended in 250 μl of phosphate-buffered saline (pH 7.0)–0.1% bovine serum albumin. Samples were preincubated with 25 μl of either rabbit anti-poliovirus type 1 Mahoney antisera or preimmune sera per sample at 37°C for 2 h. Neutralization experiments were conducted on the basis of the results of preliminary experiments analyzing the capacity of antipoliovirus antisera to prevent infection of cells by 10^6 total PFU of poliovirus under the experimental conditions (1). The preincubated samples were then analyzed for protein expression by infection of BSC-40 cells which were metabolically labeled at 6 h postinfection followed by immunoprecipitation of viral proteins.

Encapsidation of the minireplicons by type 1 Sabin poliovirus. BSC-40 cells were coinfected with 10 PFU of type 1 Sabin poliovirus and a stock of encapsidated minireplicons (passage 14) per cell. The infected cells were harvested at 24 h postinfection by three successive freeze-thaws, sonicated, and clarified by centrifugation at 14,000 × *g* for 20 min as described previously (25). Approximately one-half of the supernatant was used for serial passaging by reinfection of BSC-40 cells. After 24 h, the cultures were harvested as described above, and the procedure was repeated for an additional 10 serial passages.

RESULTS

Expression of chimeric HIV-1–poliovirus genomes in VV-P1-infected cells. We have previously described the construction and characterization of chimeric HIV-1–poliovirus minireplicons in which the *gag* or *pol* gene was substituted for VP2 and VP3 regions of the poliovirus P1 protein in the infectious cDNA of poliovirus (Fig. 1). In vitro transcription from these plasmids generated an RNA molecule which, upon transfection into HeLa cells, resulted in the replication of the RNA and expression of the HIV-1 proteins as fusion proteins with the poliovirus P1 protein (5). In previous studies, we determined that transfection of these chimeric RNA genomes into type 1 Mahoney poliovirus-infected cells did not result in encapsidation of these RNA genomes (4). Under the experimental conditions used, it was possible that the minireplicons did not efficiently compete with wild-type RNA genomes for capsid proteins. To circumvent this problem, we used a recombinant vaccinia virus (VV-P1) which expresses the poliovirus capsid precursor protein P1 upon infection, since recent studies have shown that in cells coinfected with VV-P1 and poliovirus, P1 protein expressed from VV-P1 can enter the encapsidation pathways of wild-type poliovirus (2, 3).

We first analyzed protein expression from the minireplicons transfected into cells previously infected with the recombinant vaccinia virus VV-P1 (Fig. 2). A protein of molecular mass 72 kDa, corresponding to the 3CD protein of poliovirus, was immunoprecipitated by anti-$3D^{pol}$ antibodies from cells transfected with the minireplicon RNA but not from mock-transfected cells. Under the same conditions for metabolic labeling, the 3CD protein, which is a fusion protein between the $3C^{pro}$ and $3D^{pol}$ proteins of poliovirus, is predominantly detected upon incubation of lysates from poliovirus-infected cells with the $3D^{pol}$ antisera. To determine whether the appropriate HIV-1–P1 fusion proteins

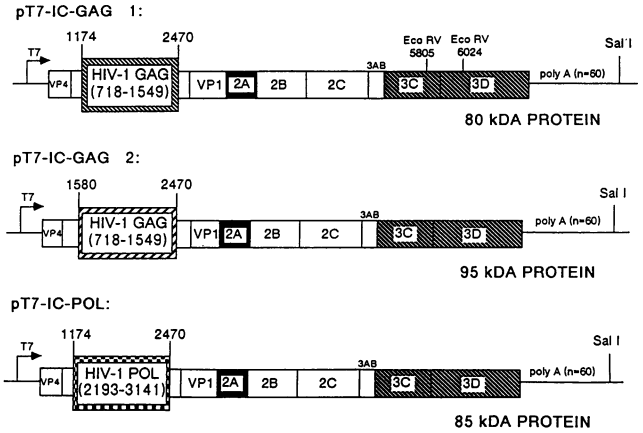

FIG. 1. Chimeric HIV-1–poliovirus genomes containing regions of the HIV-1 *gag* or *pol* gene substituted for the poliovirus P1 gene. Details of the construction of plasmids pT7-IC-GAG 1 and pT7-IC-POL have been described by Choi et al. and were presented as pT7IC-NheI-*gag* and pT7IC-NheI-*pol*, respectively (5). To construct pT7-IC-GAG 2, a unique *Sma*I site was created at nucleotide 1580 of the infectious cDNA of poliovirus, and the HIV-1 *gag* sequences were subcloned between nucleotides 1580 and 2470. Insertion of the HIV-1 genes maintains the translational reading frame with VP4 and VP1. In vitro transcription from these plasmids generates full-length RNA transcripts (linearized with *Sal*I). Transfection of full-length transcripts results in expression of the poliovirus 3CD protein, a fusion protein between the $3C^{pro}$ and $3D^{pol}$ proteins with a molecular mass of 72 kDa. The molecular masses of the HIV-1–P1 fusion proteins are indicated.

were also expressed, the extracts were incubated with pooled AIDS patient sera (Gag) or rabbit anti-RT antibodies (Pol). Expression of the HIV-1–Gag–P1 fusion proteins corresponding to the predicted molecular masses 80 and 95 kDa were detected from cells transfected with RNA genomes derived by in vitro transcription of pT7-IC-GAG 1 and pT7-IC-GAG 2, respectively. Similarly, an HIV-1–

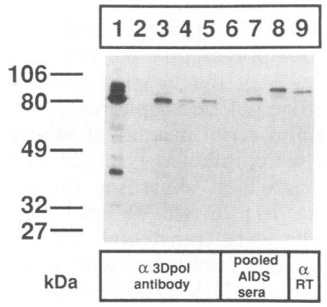

FIG. 2. Analysis of $3D^{pol}$ and HIV-1-P1 fusion protein expression from cells infected with VV-P1 and transfected with minireplicon RNAs. Cells were infected with VV-P1 at a multiplicity of infection of 20. At 2 h postinfection, cells were transfected with RNA derived from in vitro transcription of the designated plasmids. Cells were metabolically labeled, and cell extracts were incubated with anti-$3D^{pol}$ antibodies (lanes 1 to 5), pooled AIDS patient sera (lanes 6 to 8), or anti-RT antibodies (lane 9), and immunoreactive proteins were analyzed on SDS-polyacrylamide gels. Lanes: 1, cells infected with wild-type poliovirus; 2 and 6, mock-transfected cells; 3 and 7, cells transfected with RNA derived from pT7-IC-GAG 1; 4 and 8, cells transfected with RNA derived from pT7-IC-GAG 2; 5 and 9, cells transfected with RNA derived from pT7-IC-POL. The positions of molecular mass standards are indicated.

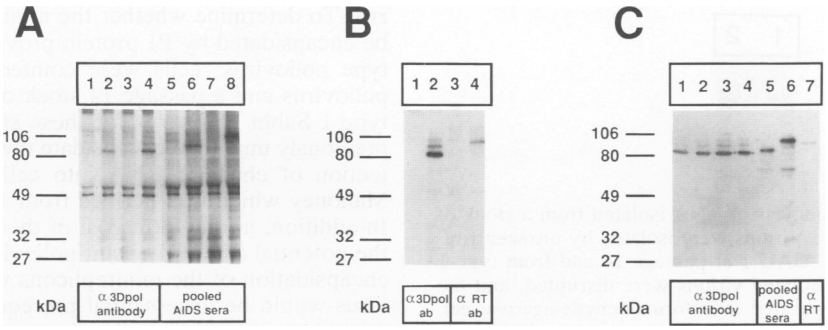

FIG. 3. Analysis of poliovirus- and HIV-1-specific protein expression from cells infected with minireplicons which were encapsidated and serially passaged with capsid proteins provided by VV-P1. Cells were infected with VV-P1 or wt VV at a multiplicity of infection of 20 and transfected with RNA derived from in vitro transcription of the designated plasmids. The cells were harvested for isolation of encapsidated genomes as described in Materials and Methods. The pelleted material was used to reinfect cells, which were metabolically labeled, and cell lysates were incubated with the designated antibodies. Immunoreactive proteins were analyzed on SDS-polyacrylamide gels. (A) Lanes: 1 and 5, cells infected with pelleted material derived from cells infected with wt VV and transfected with RNA derived from pT7-IC-GAG 1; 2 and 6, cells infected with pelleted material derived from cells infected with VV-P1 and transfected with RNA derived from pT7-IC-GAG 1; 3 and 7, cells infected with pelleted material derived from cells infected with wt VV and transfected with RNA derived from pT7-IC-GAG 2; 4 and 8, cells infected with pelleted material derived from cells infected with VV-P1 and transfected with RNA derived from pT7-IC-GAG 2. (B) Lanes: 1 and 3, cells infected with pelleted material derived from cells infected with wt VV and transfected with RNA derived from pT7-IC-POL; 2 and 4, cells infected with pelleted material derived from cells infected with VV-P1 and transfected with RNA derived from pT7-IC-POL. (C) Stocks of the encapsidated minireplicons were also used to infect cells which had been previously infected with VV-P1 for serial passage of the encapsidated genomes as described in Materials and Methods. Cells were infected with serially passaged stocks of minireplicons at passage 9 and metabolically labeled, and cell extracts were incubated with the designated antibodies (ab). Immunoreactive proteins were analyzed on SDS-polyacrylamide gels. Lanes: 1, cells infected with wild-type poliovirus; 2 and 5, cells infected with vIC-GAG 1; 3 and 6, Cells infected with vIC-GAG 2; 4 and 7, Cells infected with vIC-POL. The positions of molecular mass standards are indicated.

Pol–P1 fusion protein of the predicted molecular mass 85 kDa was immunoprecipitated from cells transfected with RNA derived from the in vitro transcription of pT7-IC-POL. These results demonstrate that transfection of the minireplicon RNA into VV-P1-infected cells results in the expression of appropriate HIV-1–P1 fusion proteins as well as $3D^{Pol}$-related proteins.

Encapsidation and serial passage of the chimeric HIV-1–poliovirus genomes with VV-P1. We next determined whether transfection of the chimeric genomes into VV-P1-infected cells would result in encapsidation of the minireplicons. For these studies, the minireplicon RNAs were transfected into either VV-P1 or wt VV-infected cells, and the encapsidated genomes were isolated as described in Materials and Methods. The pelleted material was then used to reinfect cells; this procedure was followed by metabolic labeling of viral proteins and incubation with anti-$3D^{Pol}$ or HIV-1 antisera (Fig. 3A and B). The poliovirus 3CD protein was immunoprecipitated from cells infected with pelleted material derived from transfection of the minireplicon RNA into VV-P1-infected cells. The molecular masses of the HIV-1–P1 fusion proteins immunoprecipitated from the infected cells were consistent with the predicted molecular masses and those observed from expression of the minireplicons in transfected cells (Fig. 1). No $3D^{Pol}$ or HIV-1–P1 proteins were detected from cells infected with material derived from transfection of the chimeric genomes into wt VV-infected cells, demonstrating a requirement for the poliovirus P1 protein for encapsidation of the minireplicons.

To determine whether the encapsidated minireplicons could be serially passaged, passage 1 stock of the encapsidated minireplicons was used to infect cells that had been previously infected with VV-P1. After 24 h, the encapsidated minireplicons were isolated as described in Materials and Methods and subsequently used to reinfect cells which had been previously infected with VV-P1; this procedure was repeated for an additional nine passages. By convention, we will refer to the stocks of serially passaged minireplicon RNA as vIC-GAG 1, vIC-GAG 2, or vIC-POL. Cells were infected with passage 9 material and metabolically labeled, and the lysates were incubated with antisera to poliovirus $3D^{Pol}$ protein or antibodies to HIV-1 proteins (Fig. 3C). The poliovirus 3CD protein was immunoprecipitated from cells infected with poliovirus and the encapsidated minireplicons. The HIV-1–Gag–P1 and HIV-1–Pol–P1 fusion proteins were also immunoprecipitated from cells infected with the serially passaged minireplicons. In contrast, no immunoreactive proteins were detected from cells which were infected with VV-P1 alone and immunoprecipitated with the same antisera (Fig. 2).

To determine whether the encapsidated minireplicons had undergone any significant deletion of genome size as a result of serial passage with VV-P1, RNA isolated from vIC-GAG 1 at passage 14 was analyzed by Northern blotting (Fig. 4). For these studies, a riboprobe complementary to nucleotides 671 to 1174 of poliovirus, present in the HIV-1–poliovirus chimeric genomes, was used. RNA isolated from vIC-GAG 1 was compared with RNA isolated from type 1 Mahoney poliovirions. The migration of the RNA isolated from vIC-GAG 1 was slightly faster than that of the wild-type poliovirus RNA, consistent with the predicted 7.0-kb size for RNA from pT7-IC-GAG 1 versus the 7.5-kb size for wild-type poliovirus RNA. Furthermore, we detected a single predominant RNA species from vIC-GAG 1, indicating that no significant deletions of the RNA had occurred during the serial passages.

Antibody neutralization of minireplicons encapsidated by VV-P1. To confirm that the minireplicon RNA passaged with VV-P1 was encapsidated in poliovirions, we analyzed the capacity of poliovirus-specific antisera to prevent expression of the HIV-1–P1 fusion proteins and poliovirus 3CD protein. The results of this experiment are important to exclude the

FIG. 4. Northern blot analysis of RNA isolated from a stock of encapsidated minireplicons. Virions were isolated by ultracentrifugation from a stock of vIC-GAG 1 at passage 14 and from type 1 Mahoney poliovirus. The isolated virions were disrupted, and the RNA was precipitated, separated in a formaldehyde-agarose gel, and transferred to nitrocellulose. For these studies, RNA was detected by using a riboprobe complementary to nucleotides 671 to 1174 of poliovirus. Lanes: 1, RNA isolated from vIC-GAG 1 stock; 2, RNA isolated from poliovirions. Note that the exposure time for the sample in lane 1 of the gel was six times longer than that for lane 2.

possibility that the minireplicons were being passaged by inclusion into VV-P1 rather than poliovirions. For these studies, passage 9 material of vIC-GAG 1 was preincubated with preimmune and immune type 1 poliovirus antisera as described in Materials and Methods. After incubation, the samples were used to infect cells, which were then metabolically labeled, and cell lysates were analyzed for expression of poliovirus- and HIV-1-specific proteins after incubation with anti-3D^{pol} antisera and pooled AIDS patient sera, respectively (Fig. 5). No expression of the poliovirus 3CD or HIV-1–Gag–P1 fusion protein was detected from cells infected with vIC-GAG 1 which had been preincubated with the antipoliovirus antibodies. Expression of 3CD protein and HIV-1–Gag–P1 fusion protein was readily detected from cells infected with vIC-GAG 1 which had been preincubated with normal rabbit serum (preimmune). These results demonstrate that the minireplicons were encapsidated by P1 protein provided in *trans* by VV-P1 which could be neutralized by antipoliovirus antibodies.

Encapsidation of serially passaged minireplicons by poliovirus. To determine whether the minireplicon genomes could be encapsidated by P1 protein provided in *trans* from wild-type poliovirus, cells were coinfected with type 1 Sabin poliovirus and a passage 14 stock of vIC-GAG 1. We used type 1 Sabin poliovirus for these studies because we were previously unable to encapsidate the minireplicons by transfection of chimeric RNA into cells infected with type 1 Mahoney which was derived from an infectious cDNA (4). In addition, a long-term goal of these studies is to evaluate the potential of recombinant poliovirus vaccines; therefore, encapsidation of the minireplicons with type 1 Sabin poliovirus would be an essential prerequisite for these studies. After 24 h, the coinfected cells were harvested as described in Materials and Methods, and the extracted material was serially passaged 10 additional times at a high multiplicity of infection. Cells were infected with passage 10 material of vIC-GAG 1 and type 1 Sabin poliovirus and metabolically labeled, and cell extracts were incubated with antibodies to type 1 Sabin poliovirus (Fig. 6A), pooled sera from AIDS patients (Fig. 6B), and anti-p24 antibodies (Fig. 6C). Poliovirus capsid proteins were detected from cells infected with type 1 Sabin poliovirus alone and from cells infected with material derived from passaging vIC-GAG 1 with type 1 Sabin poliovirus. No HIV-1-specific proteins were detected from cells infected with type 1 Sabin poliovirus alone. A slight cross-reactivity of the HIV-1–Gag–P1 fusion protein with antipoliovirus antisera was detected in extracts of cells infected with material derived from passaging vIC-GAG 1 with type 1 Sabin poliovirus (Fig. 6A). Although the HIV-1–Gag–P1 fusion protein was clearly detected from cells infected with material derived from passaging vIC-GAG 1 with type 1 Sabin poliovirus after incubation with pooled AIDS patient sera, some cross-reactivity of the poliovirus capsid proteins was also detected (Fig. 6B). To confirm that we had immunoprecipitated the HIV-1–Gag–P1 fusion protein from extracts of cells infected with material derived from passaging vIC-GAG 1 with type 1 Sabin poliovirus, we also incubated extracts with rabbit anti-p24 antiserum (Fig. 6C). Again, detection of the HIV-1–Gag–P1 fusion protein was evident from cells infected with material derived from passaging vIC-GAG 1 with type 1 Sabin poliovirus but not from cells infected with type 1 Sabin alone. Furthermore, HIV-1–Gag–P1 fusion protein expression was detected after each serial passage (1 to 10) of vIC-GAG 1 with type 1 Sabin poliovirus (30). We conclude from these results that the chimeric minireplicons can be encapsidated by P1 protein provided in *trans* from type 1 Sabin poliovirus under the appropriate experimental conditions and are stable upon serial passage.

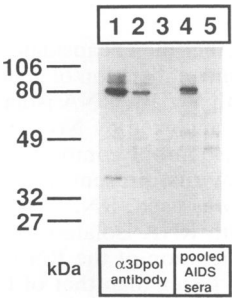

FIG. 5. Neutralization of minireplicons encapsidated by VV-P1 with antipoliovirus antibodies. Cells were infected with a passage 9 stock of vIC-GAG 1 that had been preincubated with anti-poliovirus type 1 antisera or preimmune sera as described in Materials and Methods. Infected cells were metabolically labeled, cell lysates were incubated with anti-3D^{pol} antibodies (lanes 1 to 3) or pooled AIDS patient sera (lanes 4 and 5), and immunoreactive proteins were analyzed on SDS-polyacrylamide gels. Lanes: 1, cells infected with wild-type poliovirus (no neutralization); 2 and 4, cells infected with vIC-GAG 1 which had been preincubated with preimmune sera; 3 and 5, cells infected with vIC-GAG 1 which had been preincubated with anti-poliovirus type 1 antisera. The positions of molecular mass standards are indicated.

DISCUSSION

In this report, we have described the methodology for the encapsidation of chimeric HIV-1–poliovirus minireplicons by using P1 protein provided in *trans* from the recombinant vaccinia virus VV-P1. Infection of cells with the encapsidated minireplicons resulted in efficient expression of the HIV-1–Gag–P1 or HIV-1–Pol–P1 fusion protein. The encapsidated material could be propagated by serial passage into cells coinfected with VV-P1, resulting in stocks of the encapsidated minireplicons. RNA from the minireplicons encapsidated by VV-P1 was analyzed by Northern blotting, confirming that significant deletions had not occurred in the genome during serial passage. The encapsidated minireplicons could be neutralized by antipoliovirus antisera, indicating that the genomes were encapsidated into poliovirions.

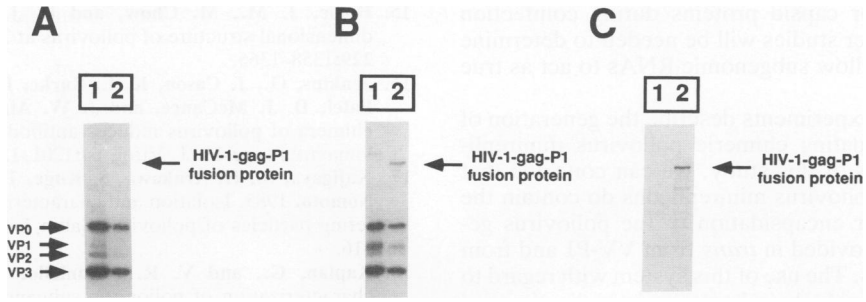

FIG. 6. Analysis of poliovirus- and HIV-1-specific protein expression from cells infected with a stock of minireplicons encapsidated by type 1 Sabin poliovirus. Cells were infected with passage 10 material derived from serial passaging of vIC-GAG 1 and type 1 Sabin poliovirus and then metabolically labeled. Viral proteins were incubated with antibodies to type 1 poliovirus (A), pooled AIDS patient sera (B), or anti-p24 antibodies (C), and immunoreactive proteins were analyzed on SDS-polyacrylamide gels. Lanes: 1, cells infected with type 1 Sabin poliovirus alone; 2, cells infected with material derived from passage 10 of vIC-GAG 1 and type 1 Sabin poliovirus. The positions of relevant proteins are indicated.

Finally, the encapsidated minireplicons could be serially passaged with type 1 Sabin poliovirus.

Recent studies from this laboratory as well as others have suggested the possibility of using poliovirus as a system in which to express foreign proteins (5, 13, 29). Poliovirus is attractive for this purpose because of the well-characterized biology, including the three-dimensional structure of the virion (15), as well as the availability of transgenic animals expressing the poliovirus receptor which can be used for pathogenesis studies (32). The relatively small genome and availability of infectious cDNA clone (31, 36) have prompted the use of recombinant DNA techniques to construct novel recombinant poliovirus genomes designed to express foreign proteins. Early studies described the construction of recombinant poliovirus genomes in which the expressed foreign gene segment was restricted to several amino acids that could be accommodated by the exposed antigenic site 1 (11, 16, 21). While these recombinant polioviruses proved useful for generating monospecific antibodies, the technology is limiting because only a small amount of genetic material can be expressed. Previous studies from this laboratory have described the construction of poliovirus minireplicons which expressed considerably larger amounts of genetic information by substitution of foreign genes between nucleotides 1174 and 2950 of the P1 coding region (5). Transfection of in vitro-transcribed RNA into cells resulted in the expression of the foreign proteins as fusion proteins with the P1 region. However, the levels of expression with this system were restricted to the number of cells transfected because the minireplicons were not encapsidated when transfected into type 1 Mahoney poliovirus-infected cells. Recently Percy et al. have described the encapsidation of a minireplicon containing the CAT gene (29). For these studies, the minireplicon was transfected into cells previously infected with type 3 poliovirus, and then virus stocks containing the minireplicon were encapsidated and propagated. A potential drawback to this system is the difficulty in estimating the amounts of encapsidated minireplicons relative to the type 3 poliovirus genomes. The results of our studies are unique because we have generated stocks composed entirely of encapsidated minireplicons which express a foreign gene upon infection. These stocks of encapsidated minireplicons maintain the same characteristics as wild-type poliovirus with regard to the capacity to be neutralized by antipoliovirus antisera. Furthermore, no evidence of recombination between the chimeric HIV-1–poliovirus minireplicon RNA and RNA derived from VV-P1 was detected in our studies as measured by production of infectious poliovirus (30). This result is consistent with the fact that poliovirus recombination has been shown to require two replicating RNA genomes (19). Finally, the maximum size of foreign genes which can be incorporated into the P1 coding region of the minireplicons and still be encapsidated is not known. Studies are under way to examine this question.

Previous studies have described the generation and characterization of poliovirus defective interfering (DI) particles (6, 7, 10, 13, 17, 23, 26, 27). DI particles have been isolated from preparations of type 1 Mahoney strain of poliovirus (6, 10, 26, 27) as well as Sabin type 1 (17). Kuge et al. reported precise descriptions of the size of Sabin DIs, with deletions corresponding to 4.2 to 16% of the wild-type genome (23). The sizes of the chimeric genomes described in this report are consistent with that reported for DI particles. Previous attempts to encapsidate the chimeric HIV-1–poliovirus minireplicons by transfection of the chimeric RNA into type 1 Mahoney poliovirus-infected cells were unsuccessful (4). The reason that we were unable to demonstrate encapsidation when the minireplicon genomes were transfected into type 1 Mahoney poliovirus-infected cells is unclear. One possibility is that the transfected minireplicon RNA is partitioned into a different intracellular compartment than the wild-type poliovirus genome. This seems unlikely because we did not observe encapsidation of the minireplicon RNA when cells were cotransfected with type 1 Mahoney poliovirus RNA (4). A second possibility is that the encapsidation of the chimeric RNA is unique to type 1 Sabin poliovirus. However, this is unlikely because we were previously unable to encapsidate the minireplicon genomes by transfection of the chimeric RNA into cells infected with type 1 Sabin poliovirus (28). A more likely explanation is that because we transfected the minireplicon genomes into cells either cotransfected with type 1 poliovirus cDNA or infected with type 1 Mahoney poliovirus, the minireplicon genomes were unable to compete efficiently for capsid proteins under these conditions. Earlier studies have demonstrated that true defective genomes were amplified during coinfection with poliovirus (6, 8, 9). However, passaging a stock of the chimeric poliovirus minireplicons with type 1 Sabin poliovirus did not result in a significant amplification of the chimeric genomes in our studies, as measured by expression of the HIV-1–P1 fusion protein. It appears, then, that true DI genomes have additional properties which allow them to

efficiently compete for capsid proteins during coinfection with poliovirus. Further studies will be needed to determine the properties which allow subgenomic RNAs to act as true DI particles.

In summary, these experiments describe the generation of a system for encapsidating chimeric poliovirus minireplicons. From the results of this study, we can conclude that the chimeric HIV-1–poliovirus minireplicons do contain the appropriate signals for encapsidation of the poliovirus genome by P1 protein provided in *trans* from VV-P1 and from type 1 Sabin poliovirus. The use of this system with regard to defining the role of individual viral proteins in replication and the requirements for RNA encapsidation, as well as the use of this system as a potential means to deliver antigens to the immune system, is now possible.

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