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Complementation of a Poliovirus Defective Genome by a Recombinant Vaccinia Virus Which Provides Poliovirus P1 Capsid Precursor in *trans*

DAVID C. ANSARDI, DONNA C. PORTER, AND CASEY D. MORROW*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0007

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Defective interfering (DI) RNA genomes of poliovirus which contain in-frame deletions in the P1 capsid protein-encoding region have been described. DI genomes are capable of replication and can be encapsidated by capsid proteins provided in *trans* from wild-type poliovirus. In this report, we demonstrate that a previously described poliovirus DI genome (K. Hagino-Yamagishi and A. Nomoto, J. Virol. 63:5386–5392, 1989) can be complemented by a recombinant vaccinia virus, VVP1 (D. C. Ansardi, D. C. Porter, and C. D. Morrow, J. Virol. 65:2088–2092, 1991), which expresses the poliovirus capsid precursor polyprotein, P1. Stocks of defective polioviruses were generated by transfecting in vitro-transcribed defective genome RNA derived from plasmid pSM1(T7)1 into HeLa cells infected with VVP1 and were maintained by serial passage in the presence of VVP1. Encapsidation of the defective poliovirus genome was demonstrated by characterizing poliovirus-specific protein expression in cells infected with preparations of defective poliovirus and by Northern (RNA) blot analysis of poliovirus-specific RNA incorporated into defective poliovirus particles. Cells infected with preparations of defective poliovirus expressed poliovirus protein 3CD but did not express capsid proteins derived from a full-length P1 precursor. Poliovirus-specific RNA encapsidated in viral particles generated in cells coinfected with VVP1 and defective poliovirus migrated slightly faster on formaldehyde-agarose gels than wild-type poliovirus RNA, demonstrating maintenance of the genomic deletion. By metabolic radiolabeling with [^{35}S]methionine-cysteine, the defective poliovirus particles were shown to contain appropriate mature-virion proteins. This is the first report of the generation of a pure population of defective polioviruses free of contaminating wild-type poliovirus. We demonstrate the use of this recombinant vaccinia virus-defective poliovirus genome complementation system for studying the effects of a defined mutation in the P1 capsid precursor on virus assembly. Following removal of residual VVP1 from defective poliovirus preparations, processing and assembly of poliovirus capsid proteins derived from a nonmyristylated P1 precursor expressed by a recombinant vaccinia virus, VVP1 myr− (D. C. Ansardi, D. C. Porter, and C. D. Morrow, J. Virol. 66:4556–4563, 1992), in cells coinfected with defective poliovirus were analyzed. Capsid proteins generated from nonmyristylated P1 did not assemble detectable levels of mature virions but did assemble, at low levels, into empty capsids. These results contrast with an earlier study from our laboratory, in which assembly of processed capsid proteins generated from nonmyristylated P1 was not observed with cells coinfected with a recombinant vaccinia virus expressing poliovirus 3CD protease, and suggest that components of a poliovirus infection, such as poliovirus-induced membrane vesicles and poliovirus genomic RNA, facilitate the early stages of poliovirus capsid assembly.

Poliovirus is an icosahedral, nonenveloped RNA virus which encapsidates a single copy of an infectious RNA genome of approximately 7,500 nucleotides (26). The poliovirus capsid is composed predominantly of sixty copies of four proteins, VP1, VP2, VP3, and VP4, which are derived from proteolytic processing of a common precursor polyprotein, P1 (26). Poliovirus virions also contain a single copy of the small genome-linked protein, VPg, which is covalently linked to the 5′ terminus of poliovirus RNA through a phosphodiester linkage to a tyrosine residue (46). The P1 capsid protein precursor is translated as part of the long polyprotein encoded by the single open reading frame of the poliovirus genome (26) and is released from the genomic polyprotein by an intramolecular proteolytic cleavage mediated by viral protease 2A (45). The viral protease 3CD proteolytically processes P1 to generate three individual capsid proteins, VP0, VP3, and VP1, which remain associated together as the capsid promoter $(VP0\text{-}3\text{-}1)_1$ (5, 19, 47). Two types of well-characterized subviral capsid particles have been detected in poliovirus-infected cells: 14S pentamers $(VP0\text{-}3\text{-}1)_5$ and empty capsids or procapsids $(VP0\text{-}3\text{-}1)_{60}$ (40, 43). RNA encapsidation may take place by condensation of 14S pentamers around the RNA genome; however, the precise pathways of virion morphogenesis have not been elucidated. Upon RNA encapsidation, VP0 is cleaved, generating VP2 and VP4, although a few residual copies of uncleaved VP0 remain in the mature virion (43). Cleavage of VP0 is believed to be intramolecular and to occur after RNA encapsidation, since VP2 and VP4 are found only in mature viral particles (4, 21, 22, 43).

Poliovirus replication is believed to rely on the activities of *cis*-acting nonstructural proteins; however, proteins encoded by the P1 region and RNA sequences of the P1 coding region are dispensable for RNA replication (18, 25). Cole et al. (16) were the first to describe defective interfering (DI) particles of type 1 poliovirus containing subgenomic RNA molecules. Subsequently, other investigators reported DI

* Corresponding author.

particles generated in poliovirus type 1 Mahoney and type 1 Sabin preparations passaged serially at high multiplicities of infection (24, 32). Poliovirus DI particles were shown to exhibit distinct characteristics, including interference with wild-type poliovirus production in cells coinfected with wild-type virus and DI particles and enrichment for DI populations during multiple passages of virus stocks containing DI particles (12–15). Characterization of poliovirus DI genomes recovered in nature has revealed that deletions occur in the P1 coding region with maintenance of the translational reading frame for proteins involved in replication of the genome (30, 32). Thus, poliovirus DI genomes depend on wild-type poliovirus for encapsidation, because they do not synthesize functional capsid proteins. In recent years, the infectious cDNA of poliovirus has been manipulated to generate subgenomic replicons in vitro (25). Furthermore, recent studies have demonstrated that P1 coding sequences can be replaced by sequences encoding foreign proteins, including human immunodeficiency virus *gag*, *pol*, and *env* proteins, and that these chimeric genomes replicate in transfected cells (10). Replacement of RNA sequences encoding the amino terminus of P1 by sequences encoding the chloramphenicol acetyltransferase gene resulted in production of a defective poliovirus which expressed chloramphenicol acetyltransferase upon infection (38). Further development of poliovirus defective genomes as research tools promises an advancement of understanding of poliovirus RNA replication and encapsidation as well as novel methods for expression of foreign proteins.

One drawback of working with defective poliovirus genomes is their requirement for wild-type poliovirus for encapsidation. Although defective poliovirus particles can be separated from wild-type virus particles by their lower buoyant density in cesium chloride, complete removal of contaminating wild-type virus is difficult (13). We have previously described a recombinant vaccinia virus, VVP1, which expresses the poliovirus P1 capsid precursor polyprotein upon infection (2). In a previous study, we reported that capsid proteins generated by proteolytic processing of P1 expressed by VVP1 were incorporated into mature poliovirus virions in cells coinfected with poliovirus type 1 Mahoney (3). This result suggested that VVP1 might complement a poliovirus defective genome containing a capsid gene deletion by supplying capsid proteins in *trans* and provide a unique method for generating defective poliovirus particles in the absence of wild-type poliovirus. To pursue this possibility, we used a previously reported and well-characterized defective poliovirus genome which contains an in-frame deletion of 816 nucleotides in the P1 coding region. This defective genome was derived from plasmid pSM1(T7)1, which contains cDNA for a poliovirus type 1 Mahoney–type 1 Sabin chimera constructed in vitro (18). The type 1 Sabin sequences were derived from a naturally occurring defective poliovirus genome lacking RNA sequences between nucleotides 1663 and 2478, which code for portions of VP2 and VP3 (30). RNA transcribed in vitro from pSM1(T7)1 template DNA was shown by Hagino-Yamagishi and Nomoto (18) to replicate upon transfection and could be passaged when wild-type poliovirus was used as a helper to provide capsid proteins.

For our initial experiments, pSM1(T7)1 RNA was transcribed in vitro by using T7 RNA polymerase and transfected into cells using four sets of conditions: mock-infected cells transfected with pSM1(T7)1 RNA, wild-type vaccinia virus (WTVV)-infected cells transfected with pSM1(T7)1 RNA, VVP1-infected cells transfected with pSM1(T7)1 RNA, and VVP1-infected cells which were then mock transfected. HeLa cell monolayers were infected with 20 PFU of the vaccinia viruses per cell and allowed to incubate for 2 h prior to transfection. RNA transcripts derived from linearized pSM1(T7)1 template DNA were prepared as described by Hagino-Yamagishi and Nomoto (18), and transfections of in vitro-transcribed RNA were performed as previously described by using a modified DEAE-dextran method (10). After overnight incubation, freeze-thaw extracts of the infected, transfected cells were microcentrifuged at 14,000 rpm for 20 min to clarify the majority of vaccinia virus present in the extracts, as previously reported by Li et al. (31). This method allowed control of levels of vaccinia virus introduced onto cell monolayers upon subsequent passage of the extracts. The clarified extracts were also treated with RNase A (50 μg/ml, 15 min at 37°C) prior to the first passage to digest unencapsidated RNA. The extracts were then passaged onto BSC-40 cell monolayers which had been infected 2 h earlier with 20 PFU of VVP1 per cell. The passage process was repeated nine times with a gradual increase in the number of cells used for infection. During passage of the extracts, we noted poliovirus-like cytopathic effects marked by dead cells 12 to 18 h postpassage in cells infected with material derived from VVP1-infected, pSM1(T7)1 RNA-transfected cells (1). In contrast, cells infected with passage extracts from the negative controls displayed cytopathic effects consistent with vaccinia virus infection, typified by rounded cells that were still attached to the tissue culture dishes after 12 to 18 h of incubation. After the ninth passage, viral particles from the infected cells and culture medium were solubilized by addition of 1% Triton X-100 and 0.25% sodium dodecyl sulfate (SDS) to the culture medium and were recovered by ultracentrifugation through a 30% sucrose cushion (30% sucrose, 1 M NaCl, 20 mM Tris acetate [pH 7.4], 0.1% bovine serum albumin) (41). The methods used to prepare viral particles took advantage of the resistance of poliovirus virions to detergent treatment and high salt concentrations (28). Residual infectious VVP1 could not be detected in the samples, either by plaque assay or by analyzing for P1 expression by immunoprecipitation of radiolabeled proteins using anti-poliovirus antiserum (1). Portions of the pelleted material from each extract were used to infect new monolayers of BSC-40 cells, which were subsequently incubated in methionine-cysteine-free medium containing 100 μCi of [^{35}S]Translabel (methionine-cysteine) from 4.5 to 6.5 h postinfection to metabolically radiolabel proteins. Lysates of the infected cells were divided into two portions and analyzed by immunoprecipitation for expression of 3CD protein or poliovirus capsid-specific proteins, by using previously described antisera (3, 20). Cells infected with material derived from serial passage of the VVP1-infected, pSM1(T7)1 RNA-transfected cell extract clearly expressed 3CD (Fig. 1A), whereas 3CD expression was not detected with cells infected with material recovered after serial passage of the negative-control extracts. Capsid proteins P1, VP0, VP1, VP2, and VP3 were not detected by immunoprecipitation with antipoliovirus antiserum from cells infected with material derived from serial passage of the VVP1-infected, pSM1(T7)1 RNA-transfected cell extract (Fig. 1B). However, a protein which migrated at a rate consistent with that expected for a P1 precursor with the 272-amino-acid deletion encoded by the defective poliovirus genome, corresponding to a molecular mass of approximately 67 kDa, was immunoprecipitated by antipoliovirus antiserum. The deletion-containing P1 protein migrated at a rate similar to that of the approximately 63-kDa VP0-VP3

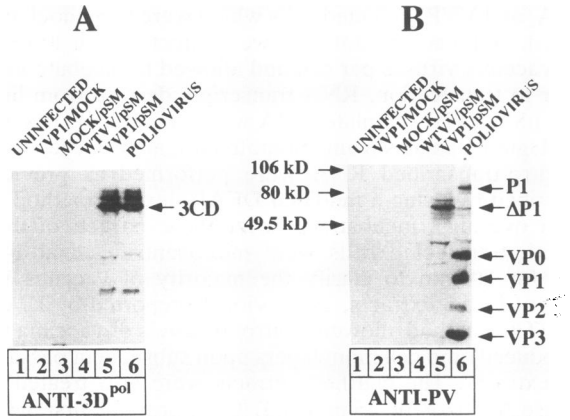

FIG. 1. Poliovirus-specific protein expression in cells infected with material derived from serial passage of extracts from vaccinia virus-infected, defective RNA genome-transfected cells. (A) Autoradiogram of an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of metabolically radiolabeled proteins immunoprecipitated by anti-3D^{pol}. The samples for this experiment were generated by initially mock infecting HeLa cells or infecting them with WTVV or VVP1 and then either mock transfecting or transfecting with in vitro-transcribed defective poliovirus RNA derived from a pSM1(T7)1 DNA template. Freeze-thaw extracts of the infected, transfected cells were serially passaged in VVP1-infected BSC-40 cells. After the ninth passage, viral particles were solubilized from the culture medium and infected cells by using detergents and were recovered by ultracentrifugation through a 30% sucrose cushion. Under the conditions used for preparation, infectious vaccinia viruses were no longer detectable, so P1 expression from residual VVP1 in the preparations was not observed. BSC-40 cells were infected with the pelleted material and were metabolically radiolabeled with [^{35}S]Translabel. One-half of lysates of the radiolabeled, infected cells were analyzed for poliovirus-specific protein expression by immunoprecipitation with anti-3D^{pol}, which recognizes a linear stretch of amino acids in the 3D^{pol} protein (20). The immunoprecipitated proteins were separated by SDS-PAGE, and visualization of the separated proteins was enhanced by sodium salicylate fluorography (9). Cells were infected with material derived from serial passage of the extract from VVP1-infected, mock-transfected cells (lane 2); mock-infected, pSM1(T7)1 RNA-transfected cells (lane 3); WTVV-infected, pSM1(T7)1 RNA-transfected cells (lane 4); or VVP1-infected, pSM1(T7)1 RNA-transfected cells (lane 5) or with 10 PFU of poliovirus type 1 per cell. The migration of poliovirus protein 3CD is indicated. (B) Autoradiogram of an SDS-PAGE analysis of poliovirus-specific proteins immunoprecipitated by anti-poliovirus antiserum from one-half of the cell extracts used for the immunoprecipitations shown in panel A. The migrations of poliovirus capsid-specific proteins are indicated. ΔP1 denotes the putative deletion-containing P1 protein encoded by the defective poliovirus genome. The migrations of protein molecular mass standards are indicated.

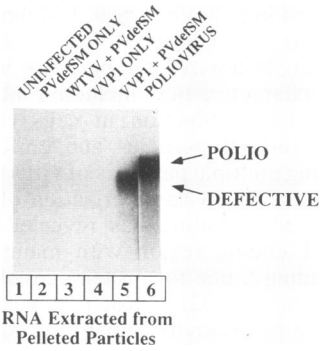

FIG. 2. Northern blot analysis of poliovirus-specific RNA encapsidated in viral particles. An autoradiogram of a Northern blot analysis of RNA extracted from pelleted particles is shown. BSC-40 cells were infected with the indicated viruses. Following overnight incubation, rapidly sedimenting material was recovered from culture medium and cells by ultracentrifugation through a 30% sucrose cushion. The pelleted material was treated with RNase A, and protected RNA was then extracted with phenol-chloroform and ethanol precipitated. Extracted RNA samples were separated on a formaldehyde–1% agarose gel and subjected to Northern blot analysis using an [α-^{32}P]UTP-labeled riboprobe complementary to poliovirus plus-strand RNA between nucleotides 671 and 1174 (10). Infections: WTVV and VVP1, 20 PFU per cell each; poliovirus type 1, 10 PFU per cell. Migrations of poliovirus-specific RNA extracted from pelleted material derived from cells coinfected with VVP1 and PVdefSM (defective cells) and that from cells infected with poliovirus type 1 are indicated. Lanes 1 to 5 were exposed to X-ray film six times as long as lane 6.

(1ABC) cleavage intermediate immunoprecipitated from poliovirus-infected cells. These data demonstrated serial passage of pSM1(T7)1-derived RNA in the presence of VVP1, which provided the capsid proteins required for virion formation. Characterization of the poliovirus-specific proteins expressed upon infection of cells with the material derived from VVP1-infected, pSM1(T7)1 RNA-transfected cells demonstrated that an unexpected recombination event regenerating a full-length poliovirus genome had not occurred, because such an event would have resulted in expression of P1 and the products of its proteolytic processing. The defective poliovirus particles generated in this experiment were designated PVdefSM and were subsequently maintained by passage in the presence of VVP1. Preparations of PVdefSM were solubilized in the presence of detergents and recovered by ultracentrifugation as described above to remove detectable infectious VVP1. Prior to further experiments, 5×10^5 BSC-40 cells were infected with several concentrations of the stock of PVdefSM, and the minimum amount resulting in complete cytopathic effect after overnight incubation was selected as the concentration to be used in subsequent studies.

We further characterized defective poliovirus particles produced in cells coinfected with VVP1 and PVdefSM by Northern (RNA) blot analysis of encapsidated poliovirus-specific RNA. For this experiment, BSC-40 cells were infected with VVP1 or WTVV and then superinfected 2 h later with PVdefSM or mock infected. After an overnight incubation, virus particles were recovered by ultracentrifugation and the pelleted material was treated with RNase A to digest any unencapsidated RNA. After inactivation of the RNase A by treatment with SDS and proteinase K (44), viral RNA was extracted with phenol-chloroform, separated on a formaldehyde-agarose gel, and subjected to Northern blot analysis using an [α-^{32}P]UTP-labeled riboprobe complementary to poliovirus plus-strand RNA between nucleotides 671 and 1174 (10). Poliovirus-specific RNA species were detected for pelleted material derived from poliovirus-infected cells and for cells coinfected with VVP1 and PVdefSM (Fig. 2). In contrast, we did not detect poliovirus-specific RNA in pelleted and RNase A-treated material derived from cells infected with VVP1 alone or PVdefSM alone or coinfected with WTVV and PVdefSM. The virion RNA recovered from VVP1/PVdefSM-coinfected cells migrated slightly faster on the formaldehyde-agarose gel than poliovirus virion RNA. The RNase A resistance of these

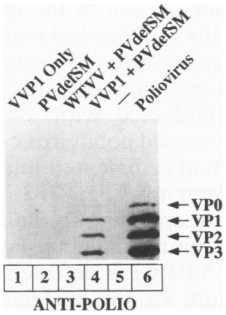

FIG. 3. Immunoprecipitation analysis of poliovirus capsid-specific proteins generated in cells coinfected with vaccinia viruses and defective poliovirus. BSC-40 cells were infected as indicated below and metabolically radiolabeled with [^{35}S]Translabel. Following overnight incubation, rapidly sedimenting material from the culture medium and cells was recovered by ultracentrifugation through a 30% sucrose cushion. Poliovirus capsid-specific proteins contained within the pelleted material were immunoprecipitated with anti-poliovirus antiserum and separated by SDS-PAGE. An autoradiogram of the gel is shown. Infections: WTVV and VVP1, 20 PFU per cell each; poliovirus type 1, 10 PFU per cell. Lane 5 was empty (—). The migrations of poliovirus capsid proteins VP0, VP1, VP2, and VP3 are indicated.

samples prior to extraction with phenol-chloroform and the requirement for cells to be infected with both VVP1 (to provide capsid proteins) and PVdefSM (to provide the defective RNA genome) for detection by this procedure further demonstrated that the RNA samples were obtained from intact virions. The migration of RNA extracted from particles produced in VVP1/PVdefSM-coinfected cells was consistent with that of in vitro-transcribed RNA derived from pSM1(T7)1, indicating that no gross deletions of the defective RNA genome had occurred during passage (1).

A primary goal in developing the *trans*-complementation system described in this report was to use the system to analyze poliovirus capsid mutants that might provide insight into the poliovirus assembly process. Therefore, it was important to demonstrate that we could trace formation of mature-capsid proteins from the P1 precursor expressed in VVP1/PVdefSM-coinfected cells. To analyze capsid-specific proteins produced in coinfected cells, BSC-40 cells were infected with WTVV or VVP1 or mock infected for 2 h and then superinfected with PVdefSM or mock superinfected. Beginning 1.5 h after inoculation with PVdefSM, the cells were continuously incubated with [^{35}S]Translabel overnight. After overnight incubation, virus particles were recovered by ultracentrifugation, and pelleted material was solubilized and incubated with anti-poliovirus antiserum to immunoprecipitate capsid-specific proteins. Poliovirus capsid proteins VP0, VP1, VP2, and VP3 were clearly recovered from cells coinfected with VVP1 and PVdefSM (Fig. 3). VP1, VP2, and VP3 were the major species of capsid proteins immunoprecipitated, indicating that the pelleted material was enriched in mature virions. Processed capsid proteins were not, however, detected in cells infected with VVP1 alone or PVdefSM alone or coinfected with WTVV and PVdefSM. The production of mature poliovirus virion protein VP2 further substantiated the formation of authentic poliovirus virion particles in cells coinfected with VVP1 and PVdefSM. In addition, we found that passage of the defective poliovirus particles was prevented by prior incubation with anti-poliovirus antiserum (1). In summary, the defective poliovirus particles generated by *trans*-complementation of the defective RNA genome using VVP1 exhibited many of the same properties as wild-type poliovirus, including mature capsid protein composition and resistance to detergents and high salt concentrations (28).

Since formation of mature-virion proteins from VVP1-expressed P1 polyprotein could be demonstrated with VVP1/PVdefSM-coinfected cells, we wanted to further characterize sedimentation of these proteins on sucrose density gradients to show similarity with the sedimentation profiles observed with poliovirus-infected cells. To demonstrate the utility of the VVP1/PVdefSM coinfection system for studying poliovirus assembly, we analyzed assembly of capsid proteins generated from myristylated and nonmyristylated P1 precursors expressed by recombinant vaccinia viruses in cells coinfected with PVdefSM. Previous studies have determined that myristylation of P1 is required for virus infectivity (29, 33), and we found that VVP1myr− did not substitute for VVP1 in passage of PVdefSM (1). In previous studies, the requirement for myristylation of P1 for virus infectivity was traced to assembly defects, and the myristate moiety was also speculated to play a role in virus entry or uncoating upon a new round of infection (3, 11, 29, 33–37).

In preliminary experiments, we determined that nonmyristylated P1 expressed by VVP1myr− was processed to individual capsid proteins VP0, VP3, and VP1 in cells coinfected with PVdefSM; however, the levels of capsid-specific material recovered by ultracentrifugation were considerably lower than that from VVP1/PVdefSM-coinfected cells (1). In contrast to capsid proteins derived from myristylated P1, mature-virion protein VP2 was not detected, suggesting that nonmyristylated promoters were defective for mature-virion formation. To characterize assembly of proteolytically processed capsid proteins derived from VVP1 and VVP1myr−, poliovirus capsid-specific proteins produced in VVP1/PVdefSM- and VVP1myr−/PVdefSM-coinfected cells were analyzed on sucrose density gradients. BSC-40 cells were infected for 2 h with either VVP1 or VVP1myr− and then superinfected with PVdefSM. The coinfected cells were continuously metabolically radiolabeled with [^{35}S]Translabel beginning 2 h after infection with PVdefSM and allowed to incubate overnight. VVP1myr−/PVdefSM coinfections were conducted in four times as many cells as VVP1/PVdefSM coinfections, and lysates of the VVP1myr−/PVdefSM-coinfected cells were pooled together and layered over a single gradient to enhance detection of low levels of rapidly sedimenting capsid proteins. Parallel cultures of poliovirus-infected cells were incubated with [^{35}S]Translabel or [^{3}H]uridine to standardize gradients for sedimentation of virions and empty capsids. Lysates of the infected cells were fractionated on 15 to 30% sucrose gradients, and fractions were collected from the bottoms of the tubes. Even-numbered fractions from VVP1/PVdefSM and VVP1myr−/PVdefSM gradients were analyzed for capsid-specific proteins by immunoprecipitation, whereas radioactivity present in sucrose gradient fractions from the poliovirus gradients was directly quantitated by liquid scintillation.

Gradient fractionation of the lysate from poliovirus-infected cells that had been incubated with [^{35}S]Translabel resulted in two distinct peaks of radioactivity (Fig. 4A). Only one peak of radioactivity was detected upon fractionation of the lysate of poliovirus-infected cells that had been incubated with [^{3}H]uridine. This peak corresponded to the more rapidly sedimenting ^{35}S-labeled material and was, thus, identified as the mature-virion peak (155S). The second peak

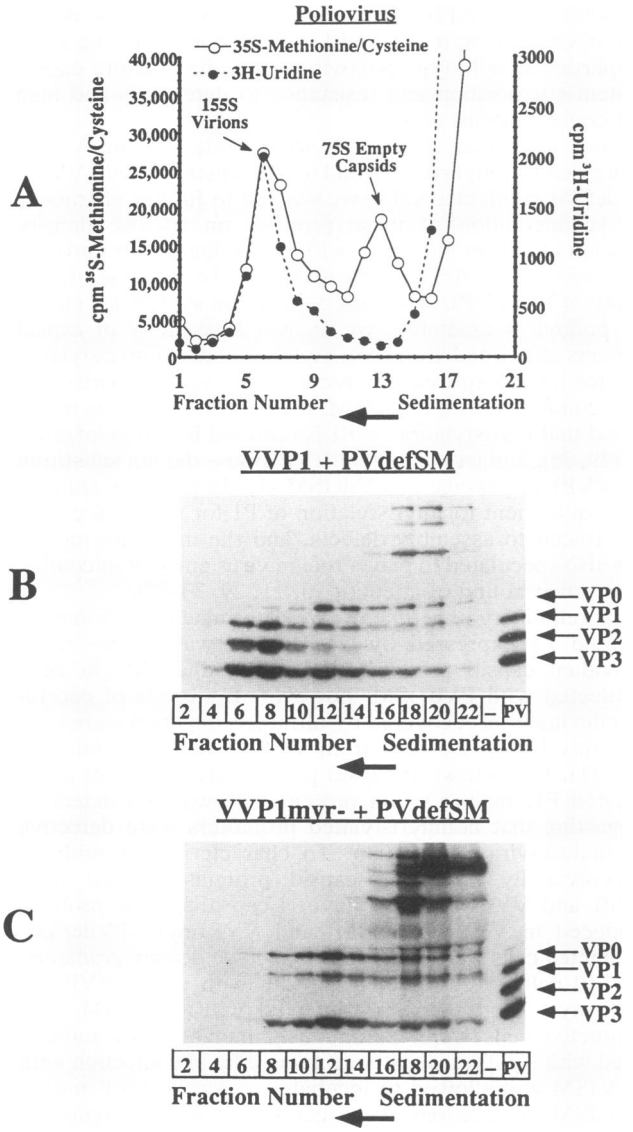

FIG. 4. Sucrose density gradient analysis of poliovirus capsid proteins generated in cells coinfected with defective poliovirus and 20 PFU of either VVP1 or VVP1myr− per cell. Soluble material from the culture medium and infected cells, which had been continuously metabolically radiolabeled overnight, was recovered by addition of 1% Triton X-100 to the culture medium and was fractionated on a 15 to 30% sucrose density gradient. Fractions were collected from the bottom of the tube and analyzed as described below. Fraction 1 corresponds to the bottom of the gradient, and fraction 22 corresponds to the top. (A) To standardize the gradients for sedimentation of mature poliovirus virions and 75S empty capsids, sucrose gradient fractionation was carried out on extracts of the cells and culture medium from poliovirus-infected cells which had been metabolically radiolabeled with either [^{35}S]Translabel or [^{3}H]uridine. Gradient fractions were analyzed directly for radioactivity by liquid scintillation counting, and the resulting profiles are shown. Radioactivity peaks corresponding to the 155S mature-virion and 75S empty-capsid peaks are indicated. (B) Immunoprecipitation of poliovirus capsid-specific proteins from sucrose density gradient fractions derived from VVP1/PVdefSM-coinfected cells. Poliovirus capsid-specific proteins were immunoprecipitated from even-numbered sucrose gradient fractions by using anti-poliovirus antiserum and separated by SDS-PAGE. An autoradiogram of the polyacrylamide gel is shown, and migrations of poliovirus capsid proteins VP0, VP1, VP2, and VP3 are indicated. Sample PV contains proteins immunoprecipitated by anti-poliovirus antiserum from fraction 8 of the poliovirus ^{35}S gradient. (C) Immunoprecipitation of poliovirus capsid-specific proteins from sucrose density gradient fractions derived from VVP1myr−/PVdefSM-coinfected cells. Poliovirus capsid-specific proteins present in even-numbered sucrose density gradient fractions were immunoprecipitated and analyzed as described for panel B.

from the gradient fractionation of the lysate of ^{35}S-labeled poliovirus-infected cells represented material that had sedimented a distance expected for 75S empty capsids or procapsids. Immunoprecipitation of capsid-specific proteins generated in cells coinfected with VVP1 and PVdefSM demonstrated that processed poliovirus capsid proteins VP0, VP1, VP2, and VP3 had sedimented into the gradient (Fig. 4B). Mature-virion proteins VP1, VP2, and VP3 were detected in fractions corresponding to the mature-virion peak identified for the poliovirus gradients. Primarily capsid proteins VP0, VP1, and VP3 were detected in fractions above those containing mature virions, including fractions 12 and 14, which corresponded to the 75S empty-capsid peak identified for the poliovirus gradient. Rapidly sedimenting processed capsid proteins were also detected by sucrose density gradient fractionation of the pooled lysates from VVP1myr−/PVdefSM-coinfected cells (Fig. 4C). In contrast to the gradient from VVP1/PVdefSM-coinfected cells, the proteins from VVP1myr−/PVdefSM-coinfected cells were primarily VP0, VP1, and VP3 and were localized in higher gradient fractions, some of which corresponded to the empty-capsid peak from the poliovirus gradient. These results demonstrated that processed capsid proteins generated in VVP1myr−/PVdefSM-coinfected cells were defective in production of mature poliovirus virions, although they did assemble at low levels into empty capsids.

The results from these experiments were in marked contrast to those of earlier studies from our laboratory using VVP1myr−. In those studies, we found that nonmyristylated P1 was processed by poliovirus 3CD protein provided in *trans* by another recombinant vaccinia virus; however, we did not detect assembly of subviral capsid particles from nonmyristylated capsid promoters (3). Under the same conditions, capsid proteins derived from a myristylated precursor assembled 14S pentamers and 75S empty capsids. Those results demonstrated that nonmyristylated 5S promoters of VP0, VP3, and VP1 were unable to form stable 14S pentamers. Detection of empty capsids in cells coinfected with VVP1myr− and PVdefSM suggests that factors other than protein 3CD present in VVP1myr−/PVdefSM cells facilitated assembly of nonmyristylated promoters. Assembly of poliovirus capsid proteins has been reported to be associated with membrane vesicles induced during poliovirus infection (6–8, 43). In a recent study, 14S pentamers were shown to be located in replication complexes associated with poliovirus-induced membrane vesicles and were speculated to be maintained in these vesicles in a conformation competent for encapsidation of viral RNA (39). The molecular nature of capsid protein interaction with membrane vesicles is not understood; however, association of poliovirus proteins with a membranous support might facilitate assembly and account for subviral-particle formation in VVP1myr−/PVdefSM-coinfected cells. The possibility that defective poliovirus RNA present in VVP1myr−/PVdefSM-coinfected cells aided assembly of nonmyristylated promoters into subviral particles also exists. Although 14S pentamers are

considered to be precursors to RNA-containing virions (40, 42, 43), RNA-protein interactions may occur early in the morphogenesis pathway and facilitate formation of subviral particles.

In this report, we have described the use of a recombinant vaccinia virus which expresses poliovirus P1 capsid precursor protein to complement a defective poliovirus genome containing a capsid gene deletion. Our studies are the first demonstration of *trans*-complementation of capsid gene-deficient poliovirus genomes by a source other than wild-type poliovirus. Generation of encapsidated, deletion-containing poliovirus genomes provides a new approach for the study of problems in poliovirus assembly and RNA encapsidation. Most previous intracellular studies of poliovirus assembly have relied on characterization of mutant and/or temperature-sensitive viruses or assembly of virus in cells transfected with in vitro-transcribed poliovirus genomes encoding capsid protein mutants. Interpretation of experimental results from these approaches has been complex, because mutations introduced into full-length genomes revert during replication. In a previous report, we described the development of a recombinant vaccinia virus-based system for studying poliovirus assembly which depended on expression of poliovirus P1 and 3CD proteins from separate recombinant vaccinia viruses (2). In cells coinfected with both recombinant vaccinia viruses, the P1 polyprotein was processed by the 3CD protease and the resulting VP0-3-1 protomers assembled into 14S pentamers and empty capsids, thus permitting study of the early stages in the poliovirus assembly process. Using the VVP1/PVdefSM coinfection system, we can now extend the study of poliovirus assembly through the processes of RNA encapsidation. The recombinant vaccinia virus-defective poliovirus coinfection system allows efficient analysis of mutant capsid protein assembly defects, since all of the cells in a monolayer can be productively infected with both viruses, thus overcoming problems with transfection efficiencies. Providing P1 from a recombinant vaccinia virus significantly reduces the possibility that observed phenotypes from mutant capsid proteins arise from reversion of the mutation during replication of poliovirus RNA. The recombinant vaccinia virus system also overcomes the need for recovering viable mutant polioviruses and permits study of capsid protein mutations which are not lethal for assembly but which might be lethal for initiation of a new round of infection.

Finally, the technology developed in this study may also be applied to expression of foreign proteins. Infectious chimeric polioviruses displaying foreign antigenic epitopes have previously been presented as candidates for new vaccine strategies, since the chimeric viruses elicited antibody responses against the foreign antigenic determinants displayed on the surface of the virus (17, 23, 27). The technology presented here provides a method for generating defective polioviruses which display foreign antigenic epitopes but which are capable of initiating only one round of infection, thus reducing the possibility of reversion and eliminating the potential introduction of recombinant polioviruses into the environment. Experiments testing the utility of defective polioviruses generated by using the recombinant vaccinia virus-mediated complementation system described in this report are under way.

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