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NOTES

Coinfection with Recombinant Vaccinia Viruses Expressing Poliovirus P1 and P3 Proteins Results in Polyprotein Processing and Formation of Empty Capsid Structures

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The assembly process of poliovirus occurs via an ordered proteolytic processing of the capsid precursor protein, P1, by the virus-encoded proteinase 3CD. To further delineate this process, we have isolated a recombinant vaccinia virus which expresses, upon infection, the poliovirus P1 capsid precursor polyprotein with an authentic carboxy terminus. Coinfection of HeLa cells with the P1-expressing vaccinia virus and with a second recombinant vaccinia virus which expresses the poliovirus proteinase 3CD resulted in the correct processing of P1 to yield the three individual capsid proteins VP0, VP3, and VP1. When extracts from coinfected cells were fractionated on sucrose density gradients, the VP0, VP3, and VP1 capsid proteins were immunoprecipitated with type 1 poliovirus antisera from fractions corresponding to a sedimentation consistent for poliovirus 75S procapsids. Examination of these fractions by electron microscopy revealed structures which lacked electron-dense cores and which corresponded in size and shape to those expected for poliovirus empty capsids. We conclude that the expression of the two poliovirus proteins P1 and 3CD in coinfected cells is sufficient for the correct processing of the capsid precursor to VP0, VP3, and VP1 as well as for the assembly of poliovirus empty capsid-like structures.

Poliovirus, like all members of the picornavirus family, is an RNA virus with a single-strand genome of positive polarity. The expression of the poliovirus genome occurs via the translation of a large precursor protein which is subsequently cleaved by two virus-encoded proteinases, $2A^{pro}$ and $3C^{pro}$, to produce the mature viral proteins (8, 26). The $2A^{pro}$ proteinase autocatalytically cleaves the capsid precursor (P1) from the polyprotein at a specific tyrosine-glycine amino acid pair (26) after translation of the polyprotein; once released from the polyprotein, a second function of this proteinase is to initiate the host cell protein shutoff characteristic of poliovirus infections (6, 13, 25). The second viral proteinase, $3C^{pro}$, further processes the P1 protein to yield the capsid proteins VP0, VP3, and VP1 (8, 9). Recent studies have demonstrated that this proteolytic process can occur in *trans* and is mediated predominantly by a precursor to the viral proteinase 3CD, which consists of a fusion of $3C^{pro}$ and the viral RNA polymerase $3D^{pol}$ (29).

Once the P1 polyprotein precursor is released from the polyprotein by the autocatalytic activity of the 2A proteinase, the process of viral morphogenesis and capsid assembly begins. Although the exact details of this process remain unknown, studies have suggested that the assembly pathway proceeds through several intermediates: a 5S protomer subunit consisting of one copy each of VP0, VP3, and VP1; a 14S pentameric subunit containing five copies each of VP0, VP3, and VP1; a 75S procapsid structure consisting of 60 copies each of VP0, VP3, and VP1; and a provirion intermediate in which the genomic RNA has become encapsidated but in which the capsid protein VP0 has not been completely processed to the mature virion proteins VP2 and VP4 (2, 11, 21, 23). In support of this model, previous studies by Phillips and colleagues have described a novel method in which isolated 14S pentamers are self-assembled in vitro to produce structures similar to the shell of the virion which are devoid of genomic RNA (18–20). A final step in the formation of the mature virion occurs with the processing of VP0 to VP4 and VP2 (1a, 10). The enzymatic activity responsible for this cleavage has not yet been identified.

The further definition of the mechanism of assembly and morphogenesis of poliovirus would be facilitated by an experimental system in which the P1 protein and the viral proteinase 3CD could be manipulated independently. For these experiments, the expression of individual poliovirus genes from the poliovirus cDNA would be desirable. Recombinant vaccinia virus vectors are useful for this purpose because of the lack of mRNA splicing and the large capacity for foreign DNA (14). Recently, attempts to insert the cDNAs of noninfectious derivatives of full-length type 1 Mahoney and type 2 Lansing polioviruses into a recombinant vaccinia virus were made (27). Even though a recombinant was isolated, a low level of protein expression, most probably due to the instability of the recombinant, was observed. Further studies revealed that the expression of $2A^{pro}$ of poliovirus is lethal to vaccinia virus replication; thus, the isolation of a vaccinia virus recombinant with an intact $2A^{pro}$ gene is difficult, if not impossible. To circumvent this problem, a recombinant vaccinia virus which expressed the poliovirus P1 protein fused to two-thirds of the viral proteinase 2A was created (12). This fusion protein was efficiently processed to the mature P1 protein by coinfection with poliovirus, although further processing of the P1 polyprotein to VP0, VP3, and VP1 capsid proteins did not occur, for unknown reasons.

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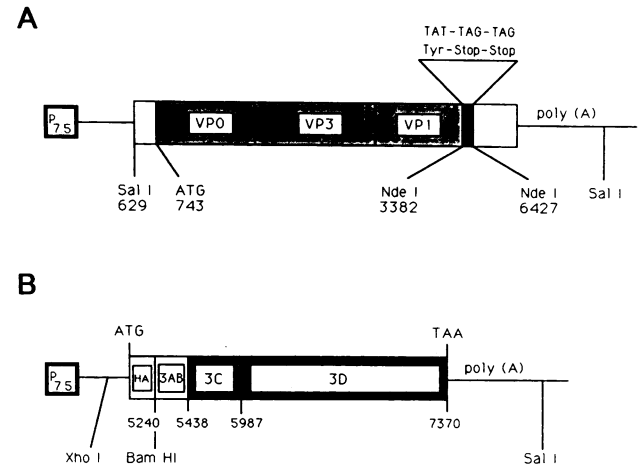

FIG. 1. Poliovirus sequences in vaccinia virus recombination plasmid pSC11. The poliovirus sequences encoding the P1 capsid region or the P3 gene were inserted into pSC11 under the control of the vaccinia virus 7,500-molecular-weight (7.5K) promoter ($P_{7.5}$). (A) The P1 construct contains sequences from nucleotides 629 to 3382, followed by a synthetic oligonucleotide encoding the carboxy-terminal tyrosine residue of P1, which is in turn followed by two stop codons (TAG). Poliovirus sequences between two *Nde*I sites at nucleotides 3382 and 6427 have been deleted. (B) The P3 construct contains a partial poliovirus 3AB region and complete 3C and 3D regions fused in frame to the influenza virus hemagglutinin gene (HA), which provides a 5′ nontranslated region and an AUG codon required for translation initiation of the desired P3 product.

In this report, we have utilized an experimental approach to express the individual poliovirus genes required for the assembly of capsids. We have constructed and characterized two individual recombinant vaccinia viruses which express authentic poliovirus P1 protein or the 3CD proteinase required for processing. Expression of an authentic poliovirus P1 polyprotein by using a recombinant vaccinia virus first required modification of the poliovirus cDNA (24) prior to its insertion into the vaccinia virus recombination plasmid pSC11 (3). The type 1 Mahoney poliovirus cDNA sequences were digested with restriction enzyme *Nde*I, thus releasing the sequences corresponding to poliovirus nucleotides 3382 to 6427 from the plasmid and deleting the P2- and much of the P3-encoding regions. Two synthetic oligonucleotides, 5′-TAT-TAG-TAG-ATC-TG-3′ and 5′-T-ACA-GAT-CTA-CTA-A-3′, were annealed together and ligated into the *Nde*I-digested DNA. The inserted synthetic sequences placed two translation termination codons (TAG) immediately downstream of the codon for the authentic P1 carboxy-terminal tyrosine residue. Thus, the engineered poliovirus sequences encode an authentic P1 polyprotein with a carboxy terminus identical to that generated when $2A^{pro}$ releases the P1 polyprotein from the nascent poliovirus polypeptide. Previous studies have clearly demonstrated that the P1 molecule with this carboxy terminus is a substrate for the viral proteinase ($3C^{pro}$) in in vitro translation reactions (30). Additionally, the first 628 nucleotides of the poliovirus 5′ nontranslated region were eliminated when the engineered sequences were ligated into the vaccinia virus recombination plasmid pSC11 to generate the plasmid pSC11-P1 (Fig. 1A).

Expression of the poliovirus P3 gene by using a recombinant vaccinia virus required manipulation of the poliovirus cDNA sequences prior to their insertion into pSC11. The poliovirus P3 gene was derived from the plasmid pProt-Pol, which contains nucleotides 5240 to 7370, encoding a partial 3AB region and complete 3C and 3D regions. Previous studies from this laboratory have demonstrated that the expression of this region of the poliovirus genome in *Escherichia coli* results in the production of correctly processed, enzymatically active RNA polymerase (16). Since poliovirus is translated as a polyprotein, the expression of individual genes of poliovirus requires the addition of 5′ nontranslated regions and an AUG translation initiation codon. To do this, we created a gene that was a fusion of (i) the 5′ nontranslated region and the first 30 codons of the hemagglutinin gene of influenza virus and (ii) the poliovirus P3 gene. The engineered sequences were ligated into plasmid pSC11 to generate the plasmid pSC11-P3 (Fig. 1B). The details of the plasmid construction and complete characterization of this plasmid will be published elsewhere (20a).

Recombinant vaccinia viruses VV-P1 and VV-P3 were generated by using standard techniques (14). Briefly, confluent BSC-40 cells were infected with wild-type vaccinia virus and then transfected with pSC11-P1 or pSC11-P3 by the calcium phosphate method (7). The plasmid pSC11 contains vaccinia virus thymidine kinase (TK) gene sequences flanking either side of the site of foreign DNA insertion, thus allowing for recombination into the viral TK gene. Recombinant viruses have a TK^- phenotype, which was selected for in TK^- 143_B cells grown in culture media containing 5-bromo-3′deoxyuridine; recombinant viruses derived from pSC11 were identified by a blue plaque phenotype by using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in an overlay medium (3). All recombinants were verified by plaque hybridization with radiolabeled poliovirus-specific DNA probes prior to analysis for expression of poliovirus proteins (data not shown).

HeLa cells were infected with vaccinia virus recombinants (10 PFU per cell) containing P1 or P3 genes. The infections were allowed to proceed for 4 h before metabolic labeling with [^{35}S]methionine; the labeled cells were lysed in RIPA buffer (25 mM Tris-HCl [pH 7.8], 0.15 M NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Na deoxycholate, 1% Triton X-100), and antisera specific for either poliovirus type 1 Mahoney or poliovirus RNA polymerase (22) were added to the extracts for immunoprecipitation analysis of protein expression. The molecular weight of the immunoprecipitated protein from the VV-P1-infected HeLa cells corresponded to the molecular weight expected for authentic poliovirus P1 protein (Fig. 2A). The poliovirus-specific proteins expressed by recombinant VV-P3, as revealed by immunoprecipitation with anti-$3D^{pol}$ antibody, corresponded to the $3D^{pol}$ precursor 3CD when analyzed in parallel with poliovirus proteins immunoprecipitated from extracts of infected cells (Fig. 2B). No processed $3D^{pol}$ was detected under these experimental conditions.

Previous studies have demonstrated that P1 is processed in *trans* by the 3CD polyprotein (29). For these studies, an in vitro translation system was developed for the desired P1 and 3CD precursors, and in vitro processing resulted in the production of VP0, VP3, and VP1. On the basis of these results, coinfection with VV-P1 and VV-3CD was performed to determine whether the processing of the P1 protein to the VP0, VP3, and VP1 proteins could be demonstrated. Parallel cultures of HeLa cells were infected with VV-P1 (10 PFU per cell) or coinfected with VV-P1 and VV-P3 (5 PFU per cell for each virus) for 6 h and then metabolically labeled for 30 min; complete growth medium was then added for chase times of 30 min, 1 h, and 2 h. Extracts were prepared from infected cells and immunoprecipitated with antisera to poliovirus type 1 Mahoney. The expression of P1 in VV-P1-

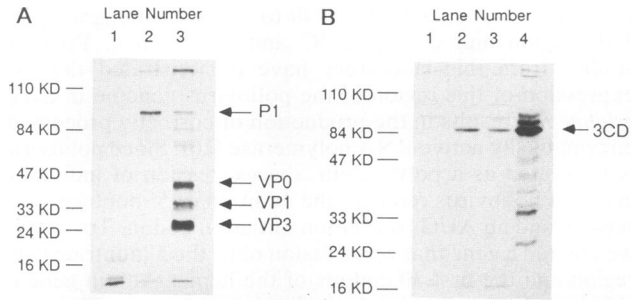

FIG. 2. Expression of poliovirus-related proteins by VV-P1 and VV-P3. (A) Autoradiogram of SDS-polyacrylamide gel of proteins immunoprecipitated with antisera to type 1 poliovirus from extracts of infected HeLa cells. Cells were infected with 10 PFU of wild-type vaccinia virus per cell (lane 1), 10 PFU of VV-P1 per cell (lane 2), or 30 PFU of type 1 poliovirus per cell (lane 3). The infected cells were labeled for 1 h at 4 h postinfection, followed by immunoprecipitation of the cell lysates. (B) Autoradiogram of SDS-polyacrylamide gel of proteins immunoprecipitated with antisera to poliovirus $3D^{pol}$ from extracts of infected HeLa cells. Cells were infected with 10 PFU of VV-P1 per cell (lane 1), 10 PFU of VV-P3 per cell (lane 2), 5 PFU each of VV-P1 and VV-P3 per cell (lane 3), or 30 PFU of type 1 poliovirus per cell (lane 4). The infected cells were labeled for 1 h at 4 h postinfection, followed by a 2-h chase in complete media. The cells were lysed and immunoprecipitated with antisera to $3D^{pol}$. KD, Kilodaltons.

infected cells did not change over the pulse-chase period, as is evident from a single protein band migrating at the molecular weight appropriate for unprocessed P1 protein (Fig. 3). When cells were coinfected with VV-P1 and VV-P3, the P1 protein was processed during the chase period, as is evident from the appearance of proteins which comigrated with authentic VP0, VP3, and VP1. The greater amount of label incorporation in the chase products is the result of a stronger reactivity of type 1 poliovirus antisera with the individual capsid proteins than with the P1 capsid precursor

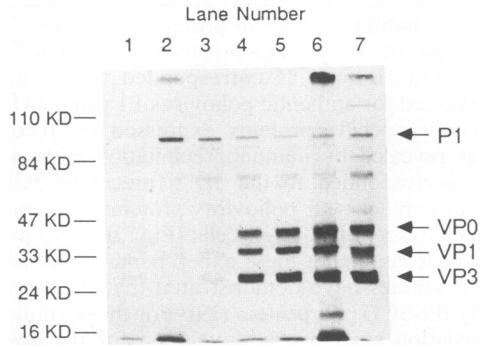

FIG. 3. Processing of P1 to VP0, VP3, and VP1 in cells coinfected with VV-P1 and VV-P3. Autoradiogram of SDS-polyacrylamide gel of proteins immunoprecipitated with antisera to poliovirus type 1 from extracts of HeLa cells that were infected for 6 h and labeled for 30 min and treated for each lane as indicated. Lanes: 1, wild-type vaccinia virus-infected cells (10 PFU per cell) labeled and then chased in complete media for 2 h; 2, VV-P1-infected cells (10 PFU per cell) labeled and chased for 2 h; 3, cells coinfected with VV-P1 and VV-P3 (5 PFU per cell each) and harvested immediately after labeling; 4, cells coinfected with VV-P1 and VV-P3 labeled and chased for 30 min in complete media; 5, coinfected cells chased for 1 h; 6, coinfected cells chased for 2 h; 7, poliovirus-infected cells metabolically labeled for 1 h at 4 h postinfection. KD, Kilodaltons.

(1). The expression of the P3 protein in coinfected cells was confirmed by the immunoprecipitation of the polymerase-related proteins from these extracts with the anti-$3D^{pol}$ antibody (data not shown).

To further characterize the expression and proteolytic processing of the P1 protein, extracts from cells infected with VV-P1 and from cells coinfected with VV-P1 and VV-P3 were analyzed by sucrose density gradients. HeLa cells were infected with VV-P1 alone or coinfected with VV-P1 and VV-P3, metabolically labeled for 1 h at 6 h postinfection, and chased overnight in complete media. Lysates of the cells were prepared by sonication in RSBK buffer (10 mM Tris-HCl [pH 6.8], 10 mM KCl, 1.5 mM $MgCl_2$) and fractionated on a 15 to 30% continuous RSBK-sucrose gradient. Fractions from the gradient were removed from the bottoms of the tubes and immunoprecipitated with antisera to poliovirus type 1 Mahoney. In a parallel experiment, HeLa cells were infected with poliovirus type 1 Mahoney for 3 h and metabolically labeled, and extracts were fractionated in identical gradients (Fig. 4A). Two peaks from the poliovirus gradient with sedimentation patterns consistent with those for virus particles (150S) and subviral empty capsids (75S) were identified. Further analysis of these peaks by immunoprecipitation with anti-type 1 poliovirus antibodies revealed that the capsid protein composition of the 150S peaks was VP2, VP3, and VP1, with a small amount of VP0; the 75S peak capsid protein composition consisted mainly of VP0, VP3, and VP1 (data not shown). The results of this analysis of poliovirus viral and subviral particles correlated with previous studies using identical sucrose gradient fractionation methods (17). Following density gradient separation of extracts from VV-P1-infected HeLa cells, the P1 protein was detected in fractions near the top of the gradient, consistent with the expected migration of a protein having a molecular mass of approximately 100,000 Da (Fig. 4B).

When the extracts from cells coinfected with VV-P1 and VV-P3 were fractionated by sucrose density gradients, capsid proteins VP0, VP3, and VP1 were immunoprecipitated in fractions 6 and 7, which corresponded to those fractions from poliovirus-infected-cell extracts that contained the 75S subviral empty capsid peak (Fig. 4C). This result suggested that the processed P1 proteins had assembled into empty capsid-like structures in cells coinfected with VV-P1 and VV-P3. The fractions from these gradients were concentrated by ultracentrifugation, negatively stained with 1% phosphotungstic acid, and examined by electron microscopy. Structures resembling empty capsid poliovirus particles in both size (approximately 27 nm) and shape and in their lack of the electron-dense core were readily identified (Fig. 5). We have also observed similar structures in intact HeLa cells coinfected with VV-P1 and VV-P3 (data not shown). No such structures were observed from experiments in which extracts from cells infected with VV-P1 alone were analyzed by gradient density analysis and electron microscopy (data not shown).

In conclusion, the results of this study demonstrate that the proteolytic processing and assembly of poliovirus capsids can be unlinked from the replication of the viral genome. We have used vaccinia virus to express independent regions of the poliovirus genome encoding the capsid precursor protein, P1, and the proteinase required for processing, 3CD. Recombinant vaccinia viruses VV-P1 and VV-P3 were isolated and found to express these individual viral proteins and, upon coinfection, to produce the processed P1 capsid proteins VP0, VP3, and VP1. More importantly, sucrose gradient density analysis and electron microscopy revealed that the initial steps of virus assembly occurred in cells

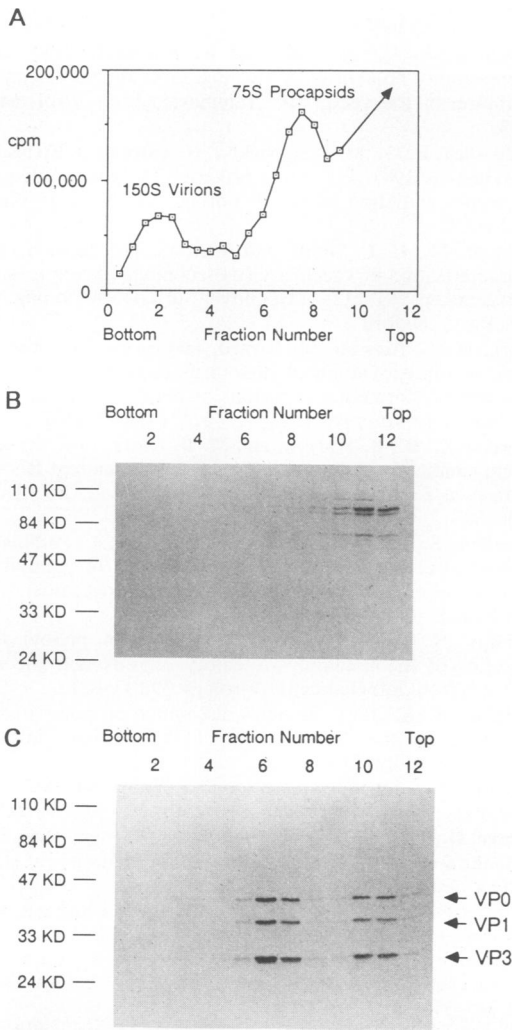

FIG. 4. Sucrose density gradient analysis of infected-cell extracts. Infected HeLa cells were metabolically labeled at the times indicated below, resuspended in RSBK buffer, and lysed by sonication. Cell debris was pelleted by brief centrifugation, and the supernatants were treated as indicated below. (A) To standardize the 15 to 30% linear sucrose density gradients for the 75S procapsid fractions and the 150S virion fractions, HeLa cells were infected with 30 PFU of type 1 poliovirus per cell and labeled for 1.5 h at 3 h postinfection. The lysate in RSBK buffer was prepared as described in the text and layered onto a linear continuous 15 to 30% sucrose gradient with a 40% sucrose cushion. The gradient was centrifuged at 16,000 rpm for 16 h at 4°C in an SW-41 rotor. Fractions were collected from the bottoms of the tubes, and the amount of radioactivity present in equal portions of each fraction was determined with a scintillation counter. (B) VV-P1-infected cells were labeled for 1 h at 6 h postinfection and chased overnight in complete media. Lysates were prepared and fractionated on a 15 to 30% sucrose gradient as described in the text. Each fraction was immunoprecipitated with type 1 poliovirus antisera. An autoradiogram of an SDS-polyacrylamide gel of the immunoprecipitated proteins from each fraction is shown; fractions separated by the sucrose gradient are from left (bottom of gradient) to right (top of gradient). (C) Sucrose density gradient analysis of HeLa cells coinfected with VV-P1 and VV-P3. HeLa cells were coinfected with 5 PFU each of VV-P1 and VV-P3 per cell and subjected to density gradient and immunoprecipitation analysis as described for panel B. KD, Kilodaltons.

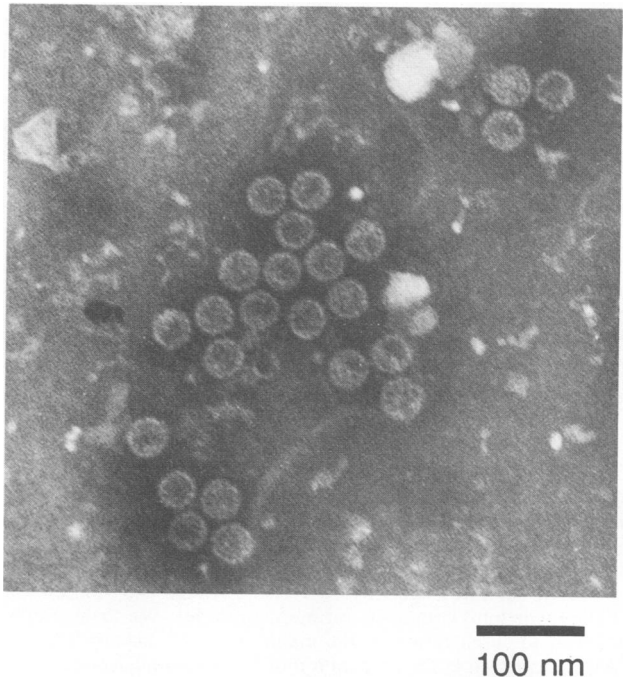

FIG. 5. Identification of empty poliovirus capsid-like structures by electron microscopy. An electron micrograph (original magnification, ×131,000) of material pelleted from sucrose density gradient fractions from HeLa cells coinfected with VV-P1 and VV-P3, corresponding to 75S-procapsid-containing fractions from poliovirus-infected cells, is shown. Samples were stained with 1% phosphotungstic acid (pH 6.5) and examined at 80 kV with a Philips EM 301 electron microscope.

coinfected with VV-P1 and VV-P3. We believe that these empty shells are devoid of RNA because of the lack of an electron-dense core and, obviously, because the full-length genomic poliovirus RNA was not present in the infected cells. Recently, the production of empty poliovirus capsids was also demonstrated by using a recombinant baculovirus containing the entire poliovirus coding sequences (28). Thus, we conclude from our studies that the steps in assembly prior to encapsidation of the RNA can be accomplished by poliovirus P1 and 3CD expressed independently from recombinant vaccinia viruses.

Previous studies on the assembly pathway of poliovirus have used poliovirus mutants (5, 15) or in vitro reconstitution of disrupted particles (18–21) to elucidate some of the features of the assembly pathway. The assembly of isolated 14S pentamers in vitro is highly concentration dependent unless they are supplemented with an extract prepared from infected HeLa cells, giving rise to the suggestion of a poliovirus morphopoietic activity (20). From the results presented in this paper, it is clear that specific protein-protein interaction required for the assembly of poliovirus is inherent in the P1 protein and the 3CD proteinase. Under most experimental conditions, nearly all of the expressed P1 is processed by the 3CD proteinase in coinfected cells. Given the fact that the molecules are expressed from different recombinant vaccinia viruses, these results suggest that an affinity must exist between the P1 substrate and the 3CD proteinase. In support of this concept is the fact that, in poliovirus-infected cells, P1 is processed from the polyprotein after the 2A proteinase is synthesized and is thus

presumably free to diffuse away from the majority of the poliovirus proteins (26, 30). Second, the assembly of the processed VP0, VP3, and VP1 proteins into empty capsid-like structures demonstrates that a poliovirus infection per se is not required for this process. The mechanism by which this protein interaction takes place is unknown, although it is tempting to speculate that the complex of P1 and 3CD proteinase might form a nucleating center required for virus assembly. Interestingly, previous studies have established that the P1 polyprotein is myristylated and have suggested that this property facilitates the protein interaction required for the assembly of 14S pentamers (4). To address this point, we are currently analyzing the kinetics of P1 processing by 3CD and the assembly process in greater detail by using this vaccinia virus expression system with defined mutants in the P1 protein (1a).

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