JOURNAL OF VIROLOGY, Mar. 1995, p. 1540–1547 Vol. 69, No. 3
0022-538X/95/$04.00+0

Amino Acid Substitutions in the Poliovirus Maturation Cleavage Site Affect Assembly and Result in Accumulation of Provirions

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Received 13 September 1994/Accepted 2 December 1994

The assembly of infectious poliovirus virions requires a proteolytic cleavage between an asparagine-serine amino acid pair (the maturation cleavage site) in VP0 after encapsidation of the genomic RNA. In this study, we have investigated the effects that mutations in the maturation cleavage site have on P1 polyprotein processing, assembly of subviral intermediates, and encapsidation of the viral genomic RNA. We have made mutations in the maturation cleavage site which change the asparagine-serine amino acid pair to either glutamine-glycine or threonine-serine. The mutations were created by site-directed mutagenesis of P1 cDNAs which were recombined into wild-type vaccinia virus to generate recombinant vaccinia viruses. The P1 polyproteins expressed from the recombinant vaccinia viruses were analyzed for proteolytic processing and assembly defects in cells coinfected with a recombinant vaccinia virus (VV-P3) that expresses the poliovirus 3CD protease. A *trans* complementation system using a defective poliovirus genome was utilized to assess the capacity of the mutant P1 proteins to encapsidate genomic RNA (D. C. Ansardi, D. C. Porter, and C. D. Morrow, J. Virol. 67:3684–3690, 1993). The mutant P1 proteins containing the glutamine-glycine amino acid pair (VP4-QG) and the threonine-serine pair (VP4-TS) were processed by 3CD provided in *trans* from VV-P3. The processed capsid proteins VP0, VP3, and VP1 derived from the mutant precursor VP4-QG were unstable and failed to assemble into subviral structures in cells coinfected with VV-P3. However, the capsid proteins derived from VP4-QG did assemble into empty-capsid-like structures in the presence of the defective poliovirus genome. In contrast, the capsid proteins derived from processing of the VP4-TS mutant assembled into subviral intermediates both in the presence and in the absence of the defective genome RNA. By a sedimentation analysis, we determined that the capsid proteins derived from the VP4-TS precursor encapsidated the defective genome RNA. However, the cleavage of VP0 to VP4 and VP2 was delayed, resulting in the accumulation of provirions. The maturation cleavage of the VP0 protein containing the VP4-TS mutation was accelerated by incubation of the provirions at 37°C. The results of these studies demonstrate that mutations in the maturation cleavage site have profound effects on the subsequent capability of the capsid proteins to assemble and provide evidence for the existence of the provirion as an assembly intermediate.

Poliovirus is a member of the *Picornaviridae*, which is a family of icosahedral nonenveloped plus-strand RNA viruses. The viral RNA genome is translated as a single long polyprotein which is subsequently processed by virus-encoded proteases to provide the structural (capsid) as well as nonstructural proteins (12, 15, 20). The capsid precursor protein, designated P1, is released from the polyprotein by autocatalytic activity of the viral proteinase $2A^{pro}$ (22). The viral proteinase, $3C^{pro}$, in the form of the fusion polyprotein with the viral polymerase 3D, designated 3CD, processes the P1 protein to release the capsid proteins VP0, VP3, and VP1 (14, 23).

Following cleavage of the capsid precursor protein, the process of viral assembly and morphogenesis begins. A single copy of VP0-VP3-VP1 forms a 5S protomer, which is the smallest identical subunit of the complete poliovirus capsid (20). The 5S protomers assemble into 14S pentamers, $(VP0\text{-}3\text{-}1)_5$, which then assemble into a 75S empty capsid, $(VP0\text{-}3\text{-}1)_{60}$, or a provirion structure that is found in poliovirus-infected cells (18, 20). The provirion differs from the empty capsid in that it contains the poliovirus RNA genome. The maturation of the provirion to an infectious particle is accomplished by the cleavage of VP0 to VP2 and a small capsid protein, VP4, which is located in the interior of the capsid (13, 20). This cleavage, termed maturation cleavage, occurs between an asparagine-serine amino acid pair and is believed to occur via an intermolecular event (6). It has been difficult to study the biochemical and physical features of the poliovirus provirion because of the rapid cleavage of VP0 and maturation to infectious virions.

Although the process of poliovirus morphogenesis has been studied for some time, the precise pathways leading to the formation of the subviral intermediates as well as the provirion have not been elucidated. The availability of an infectious clone of poliovirus has prompted the use of mutagenesis to generate specific mutations in regions of the poliovirus P1 protein (19). Transfection of the cDNA clones into susceptible cells results in the production of virus containing the original mutation in only a few cases; most of the time, revertants are generated or, if the original mutation is lethal, no virus is produced. While these studies have been informative, an in-depth analysis of the steps in which the mutant P1 proteins are blocked in subviral assembly and/or encapsidation is precluded because of the low levels of viral proteins generated upon transfection. To approach this problem, this laboratory has utilized recombinant vaccinia viruses to express the poliovirus capsid protein (VV-P1) and 3CD protease (VV-P3) (1–5). Coinfection of cells with VV-P1 and VV-P3 results in the proteolytic processing of the P1 protein and assembly of subviral intermediates, leading to the formation of a 75S empty capsid (3). In recent studies, we have utilized VV-P1 to complement a defective genome of poliovirus (which does not express functional capsid proteins) to produce a stock composed entirely of the encapsidated defective genomes (PVdefSM)

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(5). Coinfection of cells with VV-P1 and PVdefSM resulted in the processing of the P1 capsid protein followed by assembly and encapsidation of the defective genome. Mutations in the P1 protein which affect assembly and/or encapsidation can be evaluated with this system because of the low reversion frequency for the mutation in the P1 gene expressed from vaccinia viruses and because it is possible to coinfect enough cells with VV-P1 and PVdefSM to determine the step at which the assembly of the mutant P1 is blocked.

In this report, we have utilized recombinant vaccinia viruses to investigate the features of the maturation cleavage of VP0 that occurs during poliovirus morphogenesis. We have constructed two mutations which change the asparagine-serine amino acid pair cleaved during the maturation cleavage to either a glutamine-glycine or a threonine-serine. The latter mutation was based on recent studies in human rhinovirus which demonstrated that a threonine-serine substitution for asparagine-serine in the human rhinovirus VP0 cleavage site resulted in a delayed maturation cleavage (16). The mutants for the current studies were engineered into the poliovirus P1 gene and used to generate recombinant vaccinia viruses which express the mutant P1 upon infection. The mutant P1 proteins were analyzed for the capacity to be processed by 3CD, assemble into subviral intermediates, and encapsidate the defective genome RNA. The results of these studies are discussed with respect to the role that cleavage of VP0 plays in the assembly of subviral intermediates and mature virions.

MATERIALS AND METHODS

Chemicals and enzymes. Chemicals used for these studies were purchased from Sigma, unless otherwise indicated. Enzymes used in plasmid manipulations were purchased from New England Biolabs. DNA oligonucleotides were purchased from Cruachem, Fisher Co.

Cells and viruses. All experiments were conducted with HeLa cells purchased from American Type Culture Collection. HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) (GIBCO) supplemented with 5% fetal bovine serum and 1× GMS-G (GIBCO) (complete medium). Vaccinia virus stocks used for these studies were prepared as described previously (3–5). Stocks of encapsidated defective poliovirus genomes (PVdefSM) were generated as described previously (5).

Generation of recombinant vaccinia viruses that express mutant P1 polyproteins. Construction of mutations in the poliovirus P1 gene cDNA by site-directed mutagenesis was accomplished as described previously (1). For the studies reported here, two site-directed mutations were generated at the P1 cDNA sequences encoding the asparagine-serine (NS) dipeptide cleavage site at the junction of two of the mature poliovirus proteins, VP4 and VP2. One of these mutations resulted in substitution of a threonine residue for the asparagine at position 69 of VP4 and was previously reported under the designation VP4-N069T (2). In this report, the same construct is designated VP4-TS. A second mutation, which substituted the NS dipeptide cleavage site with a glutamine-glycine pair of amino acids, was constructed; this mutant construct was designated VP4-QG.

The VP4-VP2 cleavage site mutants were generated by site-directed mutagenesis using a uracil-enriched single-stranded DNA template generated in the CJ236 Dut^- Ung^- strain of *Escherichia coli*. The single stranded template was derived from the plasmid pUC119-P1, which contains a cDNA copy of the entire poliovirus P1 gene flanked by *Sal*I restriction sites (3). The primers used for the mutagenesis reactions contained the following sequences: 5′ CAATGCTAAC CTCGCCAAAC 3′ (VP4-TS) and 5′ GCCCCAATGCTACAGGGGGCCAAA CATAGAG 3′ (VP4-QG). The pUC119-P1 plasmids containing the desired mutations were isolated from individual colonies which arose upon transformation of the mutagenesis reactions, and the sequences were confirmed by direct dideoxy chain termination sequencing of the double-stranded plasmid DNA (21).

Upon confirmation of the mutant sequences, the mutant P1 genes were subcloned into the unique *Sal*I restriction site of the vaccinia virus recombination plasmid pSC11-*Sal I*, as has been described in previous reports (3), generating the plasmids pSC11-P1-VP4-TS and pSC11-P1-VP4-QG. Recombinant vaccinia viruses that express the mutant P1 polyproteins were generated by homologous recombination into the wild-type vaccinia virus genome, as described previously (3).

Metabolic radiolabeling and immunoprecipitations. Metabolic protein radiolabeling experiments were conducted by using [^{35}S]Translabel (ICN Biomedical), which contains a mixture of labeled methionine and cysteine. The labeling was conducted in methionine-cysteine-free DMEM (ICN Biomedical) with label concentrations of 100 to 500 μCi/ml. Immunoprecipitations were carried out with a rabbit antipoliovirus antiserum. Immunoprecipitates were collected on protein A-Sepharose, eluted from the Sepharose by boiling in an equal volume of 2× gel sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 5% glycerol, 0.7 M 2-mercaptoethanol, 0.025% bromphenol blue), and separated on SDS–10% polyacrylamide gels. To visualize immunoprecipitated proteins, the gels were treated with sodium salicylate, dried, and exposed to X-ray film at −70°C.

Intracellular cleavage of mutant P1 polyproteins by 3CD protease expressed by a second recombinant vaccinia virus. Intracellular analysis of mutant P1 polyprotein cleavage by 3CD was conducted by using cells coinfected with the mutant P1-expressing recombinant vaccinia viruses and a second recombinant vaccinia virus, VV-P3, which expresses the poliovirus 3CD protease (17). The recombinant vaccinia viruses (20 PFU per cell each) were adsorbed to the cell monolayers for 2 h in serum-free DMEM. Following adsorption, the cells were incubated in complete medium for 6 h. Proteins were then metabolically radiolabeled in the infected cells for 2.5 h. The infected cells were then lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate), and the lysates were clarified by centrifugation. The clarified lysates were adjusted to 0.2% SDS, and poliovirus capsid-related proteins in the extracts were recovered by immunoprecipitation using a rabbit polyclonal antibody to whole poliovirus virions (3) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Sucrose density gradient analysis of capsid particle assembly. Sucrose density gradient analyses of poliovirus capsid-related particles were conducted with extracts of cells that had been coinfected with the mutant P1-expressing recombinant vaccinia viruses and VV-P3 and extracts of cells that had been coinfected with the mutant P1-expressing recombinant vaccinia viruses and an encapsidated defective poliovirus genome (PVdefSM) (5).

For analysis of assembled particles from cells coinfected with the two vaccinia virus recombinants, cell monolayers were coinfected with 20 PFU of each virus per cell as described above. The cells were starved for methionine and cysteine from 5.5 to 6 h postinfection and then continuously incubated with [^{35}S]Translabel for 3 h (6 to 9 h postinfection). The medium was removed from the cells, and the cells were lysed in a buffer consisting of 10 mM Tris-HCl (pH 7.0), 10 mM NaCl, 1.5 mM $MgCl_2$, and 1% Triton X-100. The lysates were clarified by centrifugation for 5 min in a Microfuge and then were layered on linear, continuous sucrose density gradients generated in the same buffer containing 0.01% bovine serum albumin (BSA) but without Triton X-100. The gradient and centrifugation conditions were chosen according to the type of capsid or subviral particle to be analyzed. For separation of 14S pentamers from unassembled monomers, a 5 to 25% sucrose density gradient and centrifugation for 15.5 h at 187,000 × *g* (39,000 rpm) at 4°C in a TH-641 rotor (Sorvall) were used. For separation of empty capsids (75S) from pentamers and monomers, a 15 to 30% sucrose density gradient and centrifugation for 4.5 h at 197,000 × *g* (40,000 rpm) at 4°C in a TH-641 rotor were used. Poliovirus capsid-related particles were immunoprecipitated from alternating gradient fractions (adjusted to 1× RIPA buffer conditions) that had been collected from the bottom of the tube, and the immunoprecipitates were analyzed on SDS–10% polyacrylamide gels.

For analysis of assembled particles generated in cells coinfected with the P1-expressing recombinant vaccinia viruses and PVdefSM, HeLa cell monolayers were infected first with 20 PFU of the recombinant vaccinia virus per cell and incubated in a low volume of complete medium for 2 h. The cell monolayers were then infected with a preparation of PVdefSM (2 IU per cell) in a suspension of phosphate-buffered saline (PBS)–0.5% BSA (5). After 1 h of adsorption of PVdefSM (0 to 1 h postinfection), complete medium was added to the cultures, and the cells were incubated for an additional hour (1 to 2 h postinfection). The cells were then rinsed with methionine-cysteine-free DMEM and incubated with metabolic radiolabel for 3 h (2 to 5 h postinfection). After the 3-h incubation, the labeling medium was removed, and the cells were incubated in complete medium for another hour (5 to 6 h postinfection) or overnight (5 to 18 h postinfection). Extracts were generated by adding Triton X-100 directly to the culture medium at a final concentration of 1%. The extracts were clarified by centrifugation for 5 min in a Microfuge and then layered onto 15 to 30% sucrose density gradients prepared in the buffer described above. Since these gradients were used to separate more rapidly sedimenting virion (RNA-containing) particles from empty capsids, pentamers, and unassembled monomers, the gradient conditions were adjusted to 93,000 × *g* (27,500 rpm) for 4.5 h at 4°C in a Sorvall TH-641 rotor. Fractions were analyzed for presence of poliovirus capsid-related proteins by immunoprecipitation as described previously (5).

In vitro cleavage of VP0 to VP4 and VP2. For the in vitro cleavage experiment, a preparation of particles enriched for immature RNA-containing virions (provirions) was generated from cells coinfected with VV-VP4-TS and PVdefSM. The coinfections were conducted by infecting HeLa cells first with 20 PFU of VV-VP4-TS per cell for 2 h. Next, the cells were coinfected with a preparation of PVdefSM for 1.5 h. The coinfected cells were then rinsed with methionine-cysteine-free DMEM and incubated with [^{35}S]Translabel for 2 h. After the 2-h labeling, 0.5 volumes of complete medium was added to the labeling medium, and the cells were incubated for an additional 2.5 h. The cells and medium were then collected as described above, and the extract was clarified by centrifugation for 5 min in a Microfuge. To concentrate RNA-containing particles from the extracts, the extracts were layered over a sucrose cushion (30% sucrose, 25 mM

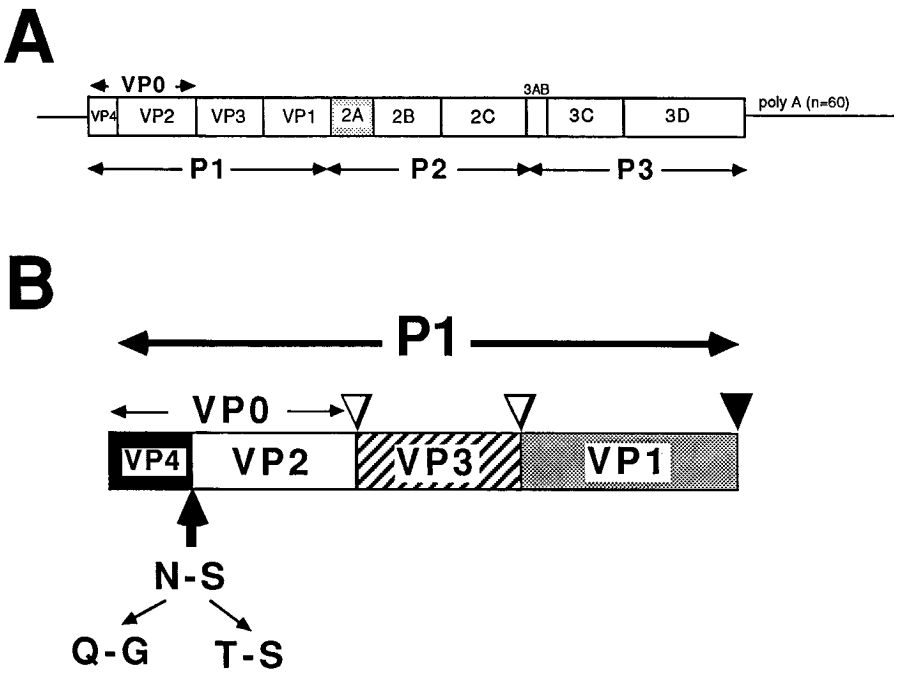

FIG. 1. Mutations in the poliovirus maturation cleavage site. (A) Organization of the poliovirus genome. The poliovirus genome is divided into three regions, P1 to -3. The P1 region encodes the capsid precursor protein. The P1 precursor consists of the VP0, VP3, and VP1 capsid proteins. Upon encapsidation of the genomic RNA, a cleavage occurs within VP0 to generate VP4 and VP2. (B) Mutations in the maturation cleavage site of VP0. An enlargement of the VP1 region of poliovirus is presented. The proteolytic cleavages between VP0, VP3, and VP1 are carried out by 3CD (open triangles); the cleavage of P1 from the P2 and P3 proteins is carried out by 2A (closed triangle). The VP0 protein is cleaved at an asparagine-serine amino acid pair after encapsidation of the genomic RNA, generating the capsid proteins VP4 and VP2 (thick arrow). In this study, we have utilized site-directed mutagenesis to change the asparagine-serine (NS) amino acid pair to either a glutamine-glycine (QG) or a threonine-serine (TS) amino acid pair. The mutant P1 genes containing these mutations were subcloned into a plasmid that was used for recombination into vaccinia virus.

Tris-HCl [pH 7.0], 50 mM NaCl, 10 mM $MgCl_2$, 0.1% BSA), which was centrifuged in an SW55-Ti rotor for 2.5 h at 45,000 rpm at 4°C. The material in the pellet was resuspended in Dulbecco's PBS–0.1% BSA containing 1.5 mM $MgCl_2$. The resuspended material was divided into aliquots which were stored at −70°C.

For the in vitro maturation experiment, aliquots of the radiolabeled material were thawed on ice and then incubated for different periods at either 4°C, room temperature, or 37°C. Following the incubation period, the suspensions were adjusted to 1× RIPA buffer conditions, and poliovirus capsid-related proteins were recovered by immunoprecipitation and analyzed on SDS–10% polyacrylamide gels.

RESULTS

Expression of P1 capsid precursors containing VP4/VP2 cleavage site mutations. The poliovirus assembly process includes a series of proteolytic cleavage events and assembly steps which culminate with encapsidation of the RNA genome. To shed light on the molecular details of these steps, we have developed unique systems for analyzing these processes. In previous reports, our laboratory described the intracellular expression of poliovirus capsid proteins by using recombinant vaccinia virus vectors (1–5). These recombinant vaccinia viruses serve as useful tools for analysis of the poliovirus capsid assembly and RNA encapsidation processes. The recombinant vaccinia viruses can be used in conjunction with a second recombinant vaccinia virus which expresses the poliovirus protease 3CD or with a defective poliovirus replicon (PVdefSM) which expresses functional poliovirus proteins outside of the capsid region and which can be encapsidated by poliovirus capsid proteins provided in *trans*. For the current study, we constructed two site-directed mutants of the poliovirus P1 capsid precursor with the aim of understanding the importance of the proteolytic cleavage between VP4 and VP2 in the assembly and encapsidation processes (Fig. 1A). A diagram of the P1 capsid protein is depicted in Fig. 1B and shows the ordered arrangement in the precursor of the mature virion proteins, VP4, VP2, VP3, and VP1. The figure also depicts the cleavage events resulting in the generation of the mature capsid proteins. The VP0 protein, present in subviral capsid particles, is the precursor to VP4 and VP2. The proteolytic cleavage which releases VP4 from VP2 occurs at an asparagine-serine (NS) amino acid pair in the P1 capsid precursor. Two site-directed mutants of the poliovirus P1 capsid precursor were constructed as shown in Fig. 1B. The first mutant, VP4-TS, contains a threonine substitution for asparagine at the NS cleavage site. This mutant has been previously reported by our laboratory as VP4-N069T (2) and was designed to correspond to an identical mutation reported for human rhinovirus 14 which was found to result in accumulation of provirion particles (16). The second mutant capsid precursor generated contained a glutamine substitution for the last amino acid of VP4 and a glycine substitution for the amino-terminal residue of VP2 (VP4-QG). This mutation resulted in substitution of a glutamine-glycine (QG) amino acid pair for the wild-type NS cleavage site. Since all poliovirus proteolytic cleavages catalyzed by the viral proteases $3C^{pro}$ or 3CD occur at QG dipeptides, the principal aim in construction of this mutation was to stimulate cleavage of VP4 from VP2 to occur in the absence of or before RNA encapsidation. Both mutations were constructed and recombined into vaccinia viruses by using previously described procedures (3, 4).

Cleavage of mutant P1 precursors by 3CD protease. As a first step in characterizing the VP4-TS and VP4-QG mutant P1 precursors, we compared the proteolytic cleavage products of these precursors generated in cells coinfected with mutant

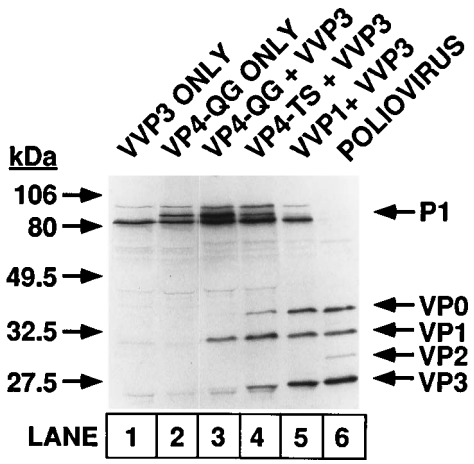

FIG. 2. Expression and proteolytic processing of mutant P1 proteins. Recombinant vaccinia viruses expressing P1 precursors containing either the glutamine-glycine substitution (VV-VP4-QG [VP4-QG]) or the threonine-serine substitution (VV-VP4-TS [VP4-TS]) were used to infect cells in the presence of a recombinant vaccinia virus which expresses poliovirus 3CD (VV-P3). The infected cells were metabolically labeled, and poliovirus capsid-related proteins were immunoprecipitated with antibodies to poliovirus. The immunoprecipitated proteins were analyzed by SDS-PAGE; an autoradiogram of the gel is shown. Lane 6, poliovirus-infected cells labeled at 4.5 h postinfection and immunoprecipitated as a marker. The migrations of the poliovirus P1 capsid precursor and the individual proteins VP0, VP1, VP2, and VP3 are noted on the right. The positions of the molecular mass markers are indicated on the left. The levels of expression for VV-VP4-TS and VV-P1 alone were similar to that of VV-VP4-QG. Note also that two additional vaccinia virus-derived proteins that have molecular masses slightly greater or slightly less than that of the authentic P1 protein are present.

P1-expressing recombinant vaccinia viruses and a second recombinant vaccinia virus (VV-P3), which expresses the 3CD protease, with those generated in cells coinfected with VV-P1 (expressing the wild-type precursor) and VV-P3 (17). This experiment was conducted by coinfecting cells with the recombinant vaccinia viruses, incubating the coinfected cells with [^{35}S]Translabel (methionine-cysteine) from 6 to 8.5 h postinfection, and then immunoprecipitating radiolabeled poliovirus capsid-related proteins from lysates of the infected cells (Fig. 2). From each set of vaccinia virus-infected cells, two nonspecific vaccinia virus proteins which migrated at positions consistent with molecular masses slightly greater and slightly lower than 80 kDa were detected (lanes 1 to 5). The P1 capsid precursor was not detected from cells infected with VV-P3 alone but was detected from cells infected with VV-VP4-QG alone and from cells coinfected with VV-P3 and either VV-VP4-QG, VV-VP4-TS, or VV-P1. In addition to the P1 capsid precursor, VP0, VP3, and VP1 cleavage products clearly were generated in cells coinfected with VV-VP4-TS and VV-P3 and VV-P1 and VV-P3. The levels of VP0 and VP3 were lower in cells infected with VV-VP4-TS and VV-P3 than in cells infected with VV-P1 and VV-P3. In contrast, VP1 was generated in cells coinfected with VV-VP4-QG and VV-P3, whereas VP0 and VP3 were detected at much lower levels. No significant accumulation of uncleaved VP0-VP3 was detected, however, suggesting that, although the cleavage did occur, the VP0 and VP3 proteins were either unstable or not immunoprecipitated as efficiently as VP1 from this mutant. We have observed this type of cleavage pattern previously from assembly-defective mutant precursors (2). Importantly, even upon extended exposure of the autoradiogram, we did not detect a VP2 cleavage product from the VP4-QG mutant (the migration of VP2 is shown for poliovirus in lane 6), which would have suggested that the 3CD protease was catalyzing a cleavage at the QG site engineered between VP4 and VP2.

Assembly of capsid cleavage products in the presence of 3CD. We next compared the assembly of the cleavage products of the mutant P1 precursors with those generated from cleavage of the wild-type P1 expressed by VV-P1. In previous reports, we have shown that VP0, VP3, and VP1 generated in cells coinfected with VV-P1 and VV-P3 assemble into 14S pentamers and empty-capsid particles, and we have identified assembly defects of other mutant P1 precursors by using this system (1, 2, 4, 5). To compare the assembly phenotypes of these mutants with that of the wild type, HeLa cells were coinfected with either VV-P1 and VV-P3, VV-VP4-QG and VV-P3, or VV-VP4-TS and VV-P3 and incubated with [^{35}S]Translabel to metabolically radiolabel proteins. Lysates of the infected cells were fractionated on either 5 to 25% sucrose density gradients (Fig. 3) to detect 14S pentamer capsid particles or 15 to 30% sucrose density gradients (Fig. 4) to detect empty capsids. Poliovirus capsid-specific proteins were immunoprecipitated from individual fractions of the gradients, and the sedimentation of these proteins within the gradients was compared to the protein sedimentation profiles of radiolabeled proteins from poliovirus-infected cells to identify 14S pentamer and empty-capsid peak fractions (data not shown).

A peak of VP0, VP3, and VP1 proteins in fractions 5 and 7 from the VV-P1–VV-P3 5 to 25% sucrose density gradient which corresponded to the 14S pentamer peak from poliovirus-infected cells was evident (Fig. 3C). VP0, VP3, and VP1 were also detected in higher fractions of the gradient, as in our previous studies (5). VP1 was immunoprecipitated in abundance over VP0 and VP3 in these higher fractions. A 14S pentamer peak of VP0, VP3, and VP1 was not detected on the gradient from VV-VP4-QG–VV-P3-coinfected cells (Fig. 3A). VP1 and smaller amounts of VP0 and VP3 were detected in the higher fractions of the gradient, especially fractions 15 and 17. In contrast, a peak of 14S pentamers (fractions 5 and 7) was detected on the gradient from cells coinfected with VV-VP4-TS–VV-P3 (Fig. 3B), although it was less pronounced than on the VV-P1–VV-P3 gradient.

Similar results were obtained when the assembly of empty capsids from cleavage products of the three precursors was analyzed (Fig. 4). Empty capsids derived from the VP4-QG precursor could not be detected (Fig. 4A), whereas empty capsids derived from the VP4-TS precursor were detected in fractions 5 and 7 from the 15 to 30% sucrose density gradient (Fig. 4B). The empty-capsid peak from the VP4-TS precursor was less pronounced, though, than that from the wild-type P1 precursor (Fig. 4C). Together, the results of these two sets of experiments demonstrated that mutations at the VP4-VP2 cleavage site of the P1 precursor affected assembly of subviral capsid particles. In the case of the VP4-QG mutant, assembly of pentamer and empty-capsid particles was prevented completely in this system.

Assembly of capsid proteins in the presence of a defective poliovirus RNA genome. Our laboratory has described a system which uses recombinant vaccinia viruses that express P1 capsid precursors in conjunction with a defective poliovirus genome which provides the viral protease required for capsid maturation. With this system, the use of a defective poliovirus RNA allows the processes of RNA encapsidation to be assessed as well. The defective poliovirus genome contains an in-frame deletion in the P1 capsid gene and thus requires a P1 protein for encapsidation. We have described the generation of helper-free stocks of these defective viruses for use in assembly and encapsidation analyses (5). To further characterize

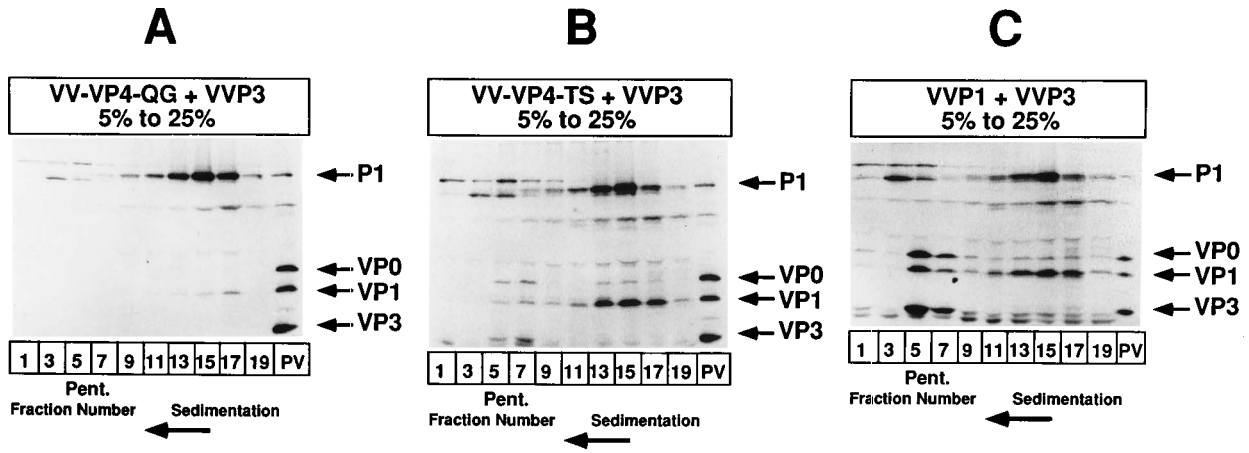

FIG. 3. Sucrose density gradient (5 to 25%) analysis of capsid proteins derived from the VV-VP4-QG and VV-VP4-TS P1 proteins. Cells were coinfected with recombinant vaccinia viruses that express either the wild-type or mutant P1 precursors and with VV-P3. After 6 h of infection, the cells were metabolically labeled. The lysates were fractionated on 5 to 25% linear sucrose density gradients. Twenty 0.5-ml fractions were recovered. The capsid proteins present in each fraction were immunoprecipitated with antibodies to poliovirus. Autoradiograms depicting the SDS-PAGE analysis of the capsid-specific proteins immunoprecipitated from the odd-numbered fractions of the gradients are presented. Gradient fractions are numbered from the bottom to the top of the gradient. The migrations of the poliovirus (PV) proteins P1, VP0, VP1, and VP3 are noted. The identity of the fractions containing the 14S pentamers (Pent.) was confirmed from a similar fractionation of a lysate from poliovirus-infected cells.

the mutant P1 precursors generated for this study, the cleavage products generated from these precursors in cells coinfected with a recombinant vaccinia virus and encapsidated defective poliovirus genomes (PVdefSM) were compared on sucrose density gradients. For these experiments, lysates were fractionated as described previously to allow separation of virions (fractions 5 to 7) from empty capsids (fractions 13 to 15) and pentamers and unassembled protomers or monomers at the top of the gradient. Lysates of infected cells were recovered either at 6 h postinfection with defective poliovirus genomes or after an overnight incubation. In the case of the VP4-QG mutant, capsid proteins VP0, VP3, and VP1 derived from this precursor were detected in fractions 11 to 19 when harvested 6 h postinfection and primarily only in fractions 11 and 13 after an overnight incubation (Fig. 5A). Fractions 11 and 13 corresponded to the empty-capsid peak fractions in a parallel gradient analysis of radiolabeled proteins from poliovirus-infected cells (data not shown). Detection of empty capsids derived from this mutant was in marked contrast to the result obtained in the coinfection experiment using VV-P3 to provide 3CD protease (Fig. 4A). An additional observation made from these studies was the appearance of a capsid-related protein in the gel in the upper panel of Fig. 5A (lane UF) that migrated at a position consistent with that of VP2; however, mature virions derived from this mutant were not detected. This protein might be a product of an inefficient cleavage of the QG site separating VP4 and VP2 in this mutant precursor by 3CD or $3C^{pro}$.

In the case of the VP4-TS mutant, processed capsid proteins VP0, VP3, and VP1 derived from this precursor were detected in both virion and empty-capsid fractions when harvested either at 6 h postinfection or after an overnight incubation (Fig.

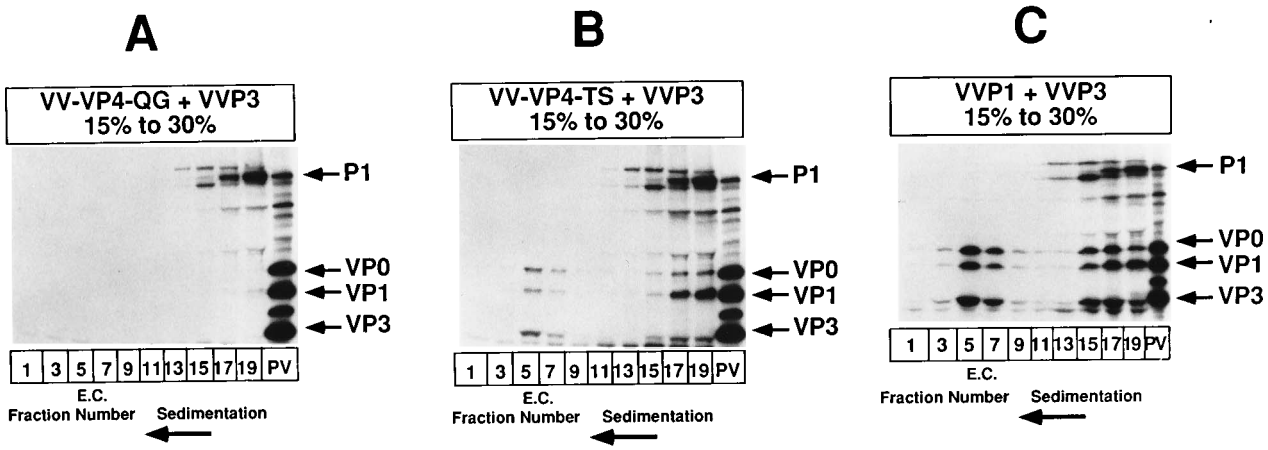

FIG. 4. Sucrose density gradient (15 to 30%) analysis of capsid proteins derived from the mutant P1 proteins. Cells were coinfected with recombinant vaccinia viruses expressing either the wild-type or mutant P1 precursors and with VV-P3. After 6 h of infection, the cells were metabolically labeled. The lysate was fractionated on a 15 to 30% sucrose density gradient. Twenty 0.5-ml fractions were recovered from the gradient, and poliovirus capsid-related proteins were immunoprecipitated from the odd-numbered fractions with an antipoliovirus antiserum. Autoradiograms depicting the SDS-PAGE analysis of the poliovirus capsid-specific proteins immunoprecipitated from the fractions are presented. The migrations of the poliovirus (PV) capsid proteins P1, VP0, VP1 and VP3 are noted. The fractions containing empty capsids (E.C.) (fractions 5 and 7) were identified by analysis of a parallel fractionation of a lysate from poliovirus-infected cells.

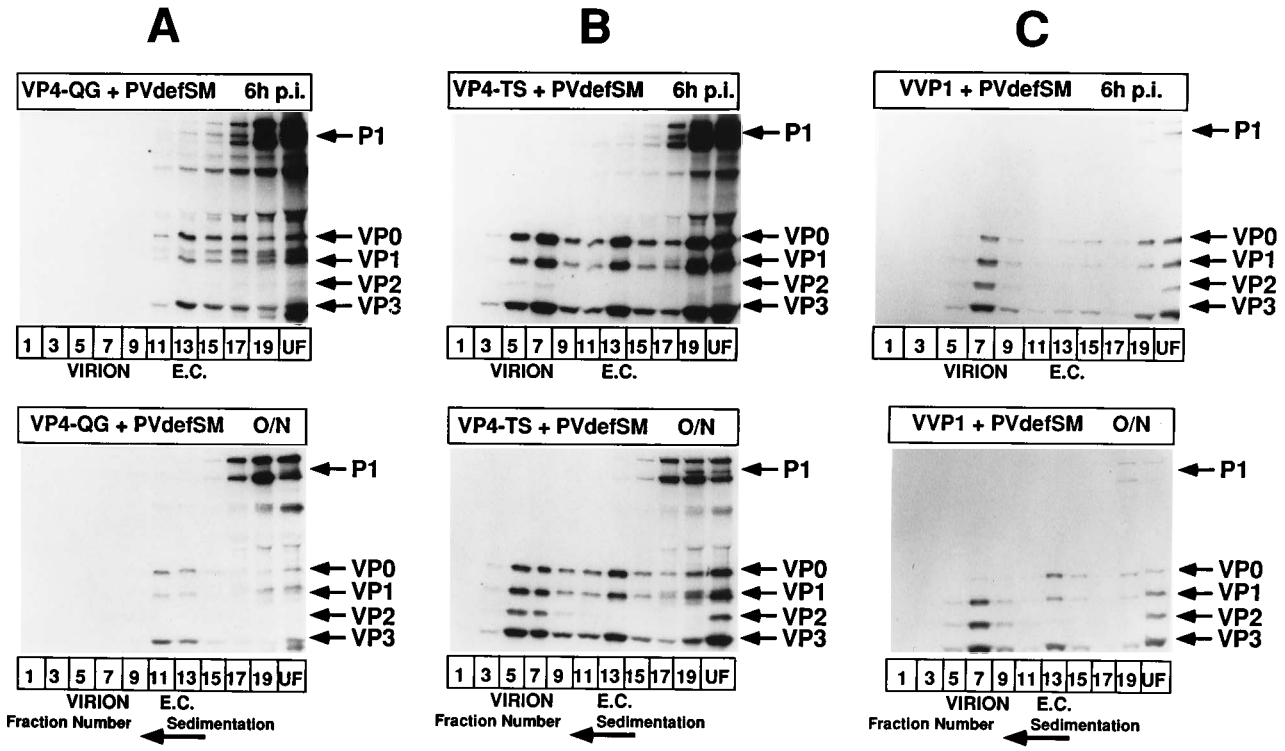

FIG. 5. Sucrose density analysis of capsid proteins generated in cells coinfected with PVdefSM and recombinant vaccinia viruses. Cells were coinfected with PVdefSM and recombinant vaccinia viruses that express either the VV-VP4-QG (VP4-QG), the VV-VP4-TS (VP4-TS), or the wild-type P1 precursor. From 2 to 5 h postinfection, the cells were metabolically labeled. The lysates were either harvested 1 h after the metabolic labeling (6 h postinfection) or incubated with complete medium for an additional 12 h (O/N). The lysates from these cells were fractionated on 15 to 30% linear sucrose density gradients. The capsid-related proteins in the odd-numbered fractions were immunoprecipitated with antibodies to poliovirus. The immunoprecipitates were then analyzed by SDS-PAGE. The migrations of the poliovirus P1, VP0, VP1, VP2, and VP3 capsid proteins are noted. The sedimentation of poliovirus empty capsids (E.C.) and virions was determined by analysis of a parallel gradient fractionation of an extract from poliovirus-infected cells. Lane UF, portion of the unfractionated lysate.

5B). After an overnight incubation, however, the amount of VP2 relative to VP0 in the virion fractions clearly increased. The accumulation of VP0 in the virion fractions from the VP4-TS mutant contrasted with the pattern of capsid proteins observed for the wild-type P1 precursor (Fig. 5C). In virion-corresponding fractions from the VV-P1–PVdefSM gradient, VP2 is present in abundance over uncleaved VP0, especially after an overnight incubation. In summary, these results indicated that the VP4-QG mutation prevented assembly of virion particles; assembly of empty capsids, or particles with a sedimentation velocity similar to that of empty capsids, did occur at low levels. In the case of the VP4-TS mutation, assembly of virion particles was not prevented, but the maturation cleavage of VP0 to VP2 and VP4 was clearly delayed. This effect was less pronounced after prolonged incubations but did indicate that the mutation present in the capsid particles derived from this precursor resulted in accumulation of provirions.

In vitro cleavage of VP0 to VP2 and VP4. The results of the experiment presented in Fig. 5B led us to further characterize the VP4-TS mutant to determine whether the delayed maturation cleavage of VP0 derived from this precursor could be observed in vitro at different temperatures. For this experiment, HeLa cells were coinfected with VV-VP4-TS and PVdefSM and incubated with metabolic radiolabel from 1.5 to 6 h postinfection with the encapsidated defective poliovirus genomes. A lysate of the coinfected cells was layered over a 30% sucrose cushion and centrifuged at 4°C under conditions which allowed sedimentation of virion and provirion particles through the cushion. Material in the pellet was resuspended in a buffer consisting of PBS–0.1% BSA and was aliquoted into seven individual fractions. The seven fractions were kept at −70°C until being thawed for individual incubations. Following incubation, capsid-specific proteins were immunoprecipitated from the aliquots and separated by SDS-PAGE. Figure 6 shows the separation of the immunoprecipitated proteins from aliquots kept at −70°C or incubated at 4°C for 24 h, at room temperature for 24 h, or at 37°C for 3, 7, 11, or 24 h. Little change in levels of VP2 was observed after the incubation at 4°C; however, VP2 was observed after incubation at room temperature or 37°C. A gradual increase in VP2 levels was observed upon incubation at 37°C for increasing amounts of time. Several proteins of larger molecular mass were also observed upon incubation at room temperature or 37°C for extended times. At present, we do not know the origin of these proteins, although we speculate that the proteins might be derived from vaccinia virus that copelleted with the poliovirions. Taken together, these results demonstrated that the maturation cleavage of VP0 derived from VP4-TS occurs slowly at permissive temperatures (i.e., 23 or 37°C). In a related set of experiments, we have also compared the capacity of VV-VP4-TS with VV-P1 to support serial passage of PVdefSM. From our preliminary studies, it was clear that VV-VP4-TS supported passage of PVdefSM but was much less effective, as measured by the amount of encapsidated PVdefSM, than VV-P1 (data not shown).

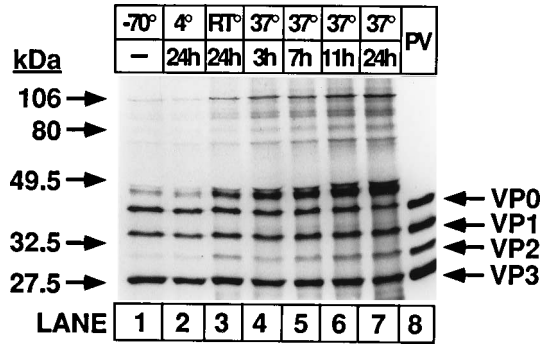

FIG. 6. Maturation cleavage of provirions containing the VP4-TS mutation. To generate a supply of provirion particles, cells were coinfected with the recombinant vaccinia virus which expresses the VP4-TS mutant P1 precursor (VV-VP4-TS) and PVdefSM. The coinfected cells were incubated with radiolabel from 1.5 to 3.5 h postinfection with PVdefSM. Following labeling, RNA-containing poliovirus-like particles were recovered from the medium and the cells and concentrated by centrifugation through a 30% sucrose cushion. The pelleted material was resuspended and divided into seven aliquots which were stored at −70°C prior to incubation under the indicated conditions. Following incubation, the samples were mixed with RIPA buffer, and capsid-related proteins were immunoprecipitated with anti-poliovirus antibodies. The immunoprecipitates were analyzed by SDS-PAGE. An autoradiogram of the fluor-treated gel is shown. Lane 8, radiolabeled virus particles recovered from cells infected with wild-type poliovirus (PV). The migrations of the poliovirus capsid proteins VP0 to VP3 are noted on the right. The positions of the molecular mass markers are indicated on the left. RT, room temperature.

DISCUSSION

In this study, we have utilized oligonucleotide site-directed mutagenesis to construct two separate mutations in the P1 gene of poliovirus in the region encoding the Asn-Ser site of maturation cleavage between VP4 and VP2. The first mutation substituted a glutamine-glycine amino acid pair (VP4-QG) at the site of maturation cleavage. A second mutation was constructed to change the asparagine to threonine, creating a threonine-serine (VP4-TS) cleavage site. Both of the mutant P1 genes were subcloned into a plasmid and used to generate a recombinant vaccinia virus which expressed the mutant P1 protein upon infection. Coinfection of cells with the vaccinia virus expressing the mutant P1 protein and with a second vaccinia virus, VV-P3, resulted in the proteolytic processing of P1 by 3CD. A defect in the assembly of subviral particles was observed for the VP4-QG mutant in this system. In contrast, the VP4-QG mutant assembled into empty-capsid structures in the presence of PVdefSM. The VP4-TS mutant was processed by 3CD provided in *trans*, assembled into subviral intermediates, and encapsidated the viral genomic RNA. The maturation cleavage of VP0 to VP4 and VP2 was delayed compared with that observed for the wild type, resulting in the accumulation of provirions.

The results of our studies are significant because this is the first demonstration that mutations in the maturation cleavage site of poliovirus have drastic effects on the subsequent capability of the P1 protein to assemble into subviral particles. Previous studies have used the infectious clone of either poliovirus or, recently, rhinovirus to investigate the effects that mutations in the maturation cleavage site have on virus assembly and release of infectious virions (11, 16). Although the results of these studies demonstrated a role for the maturation cleavage in the generation of infectious virus, it was difficult to analyze the transfected cells to ascertain the step in assembly at which the mutants were blocked. It was only recently that studies of Lee et al. were able to address this question using a highly efficient transfection system (16). The use of the vaccinia virus system provides an alternative approach and allows the identification of the step in assembly or encapsidation affected by the mutation in the P1 proteins. This was most evident in the analysis of the VP4-QG mutation. On the basis of the analysis of the proteolytic processing of the P1 precursor by 3CD provided in *trans* from VV-P3, we believe that the glutamine-glycine substitutions did not affect the capacity of P1 to be proteolytically processed by 3CD. Once processed though, the capsids from the VP4-QG mutant were deficient in assembly, as evidenced by the lack of 14S pentamers in cells coinfected with VV-P3. The assembly defect was not complete, because capsid proteins derived from the VP4-QG mutant precursor assembled into empty-capsid-like structures in the presence of the defective poliovirus (PVdefSM) RNA genome. In this case, low levels of assembly into empty-capsid-like structures were detected at early times postcoinfection; if the chase period was extended for an additional 12 h, smaller amounts of the empty capsid-like structures were detected, indicating that the structures were unstable. These results suggest that the genome RNA from PVdefSM might have stabilized the proteolytically processed VP4-QG mutant proteins in structures which had a sedimentation profile similar to that of empty capsids. Because of the nature of the mutations in the maturation cleavage site, the subviral particle cannot progress towards the formation of a stable provirion and is thus susceptible to proteolytic degradation, similar to what we have found for other mutants that lack the capacity to assemble (1, 2). An alternative interpretation is that the additional nonstructural proteins encoded by PVdefSM might stabilize the subviral particles. Further studies will be required then to delineate the mechanisms by which the VP4-QG mutant is stabilized in the presence of PVdefSM.

One of the unique findings of our study was that the VP4-TS mutation resulted in a provirion which could be studied by sedimentation analysis. Previous studies have shown the existence of provirions isolated from poliovirus-infected cells by using a sedimentation analysis. In many cases the provirions were unstable or contaminated with infectious particles, precluding an analysis of their physical features (8–10). Studies by Bishop and Anderson have characterized the hepatitis A provirion because of the slow VP0 cleavage (7). Recently, Lee et al. have described a mutation in the rhinovirus P1 maturation cleavage site, a threonine-serine change that allowed the isolation of a rhinovirus provirion (16). To isolate a poliovirus provirion, we have made the same mutation in the poliovirus P1 precursor, VP4-TS. Expression of the P1 precursor containing the VP4-TS mutation in the presence of the defective poliovirus (PVdefSM) RNA resulted in the processing and encapsidation of the defective genome. The encapsidated genome was contained in a provirion structure that sedimented at a position similar to that for mutant poliovirus virions; the sedimentation of the provirions was clearly distinct from that of the empty-capsid structures, which do not contain genomic RNA. Thus, in agreement with previous studies of rhinovirus provirions, the poliovirus provirion has a sedimentation profile that is similar to that of the mature virion. One of the interesting features of VP4-TS described in this report is the fact that the maturation cleavage is not completely prevented but takes place at a much slower rate than that observed for the wild-type capsid. This is evident from the analysis presented in Fig. 5 and was further highlighted by the incubation of the isolated provirions at different temperatures for various times (Fig. 6). A similar result was also noted by Lee et al. with the threonine-serine mutation in human rhinovirus (16). Incubation of the rhinovirus virions containing the threonine-serine cleavage site at 35°C for 24 to 48 h resulted in the gradual

increase of the VP4 protein. In our studies, incubation at 37°C for 24 h resulted in a significant increase in the level of VP2 compared with provirions incubated at 4°C for 24 h. These findings are also consistent with those of previous studies of hepatitis A virus, which demonstrated that the cleavage of VP0 was temperature dependent, with little or no cleavage occurring at 40°C (7). Taken together, the results of the studies on hepatitis A, rhinovirus, and poliovirus support the concept that a series of conformational changes occur during the maturation cleavage of VP0. These conformational changes might occur as a result of the genomic RNA coming into contact with the amino acid at the cleavage site or with the catalytic amino acid involved in maturation cleavage. The mechanism by which this occurs throughout the entire poliovirion (i.e., the 60 copies of VP0 to be cleaved) is not clear. In a recent study, Zlotnick et al. suggested that intrasubunit communication within the particle is important during the maturation cleavage (24). The kinetics of the maturation cleavage for nodaviruses, as well as hepatitis A virus, suggest a cooperative effort between subunits during the cleavage event. Whether this is the case for the maturation cleavage of poliovirus will require further experimentation. The analysis of additional mutants at or near the maturation cleavage site using the vaccinia virus system might provide important insights into the actual mechanism of the complex process of maturation cleavage.

ACKNOWLEDGMENTS

We thank Marie Anderson for helpful comments and Dee Martin for preparation of the manuscript.

This work was supported by a grant from Public Health Service (AI25005) to C.D.M.

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