JOURNAL OF VIROLOGY, Sept. 1990, p. 4067–4075
0022-538X/90/094067-09$02.00/0

Vol. 64, No. 9

Temperature-Sensitive Poliovirus Mutant Fails To Cleave VP0 and Accumulates Provirions

SUSAN R. COMPTON,[1] BARBARA NELSEN,[2]† AND KARLA KIRKEGAARD[1]*

Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309,[1] and Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02138[2]

Received 19 January 1990/Accepted 15 May 1990

A temperature-sensitive mutant of poliovirus, VP2-103, was isolated and characterized. A single nucleotide change, resulting in the substitution of glutamine for arginine at amino acid 76 of the capsid protein VP2, prevented the maturation of virions at the nonpermissive temperature. Particles indistinguishable from the previously elusive provirions were observed; these particles have been proposed to be penultimate in virion morphogenesis. Cleavage of VP0 into VP2 and VP4, the products found in mature virions, was not observed in VP2-103-infected cells at the nonpermissive temperature. The cleavage of VP0 in wild-type poliovirus-infected cells is dependent on RNA packaging; this reaction has been postulated to be autocatalytic. The existence of RNA-containing provirionlike particles in VP2-103-infected cells shows that RNA packaging can be uncoupled from VP0 cleavage.

The existence and protein composition of potential intermediates in poliovirus assembly have been extensively documented, and likely assembly pathways have been suggested (21, 26, 36, 40). It is generally thought that the folding of P1, the precursor of the final capsid proteins, triggers the cleavages by the viral protease 3CD (2, 8, 10) that yield a 5S protomer particle composed of one copy each of VP1, VP3, and VP0. Five of these protomers are then thought to assemble into a structure sedimenting at 14S, termed the pentamer (40). The three-dimensional structures of poliovirus (13, 19) and other picornaviruses (1, 3, 30, 39) have revealed the apparent strength of the interactions at the fivefold symmetry axis of these viruses (36, 40).

The next steps in poliovirus assembly are less clear. The 14S pentamers can assemble into 75S empty icosahedral capsids both in vitro and in vivo (36, 40). During viral assembly within an infected cell, it has not been established whether the viral RNA genome is threaded into these preformed empty capsids (20, 21) or whether the 14S pentamers condense around the viral RNA (40).

The predicted product of either RNA packaging scheme is a particle containing 60 copies each of VP0, VP1, and VP3, as well as the 7,500-nucleotide genomic RNA, with the small protein VPg covalently attached to its 5′ end (40). In poliovirus-infected cells, particles with these properties were first observed by Fernandez-Tomas and Baltimore (11), and termed provirions. Additional properties of these particles included a sedimentation rate of 125S, instability in CsCl, sodium dodecyl sulfate (SDS), or EDTA, and lack of infectivity. The role of provirions as precursors to final mature virions was suggested by pulse-chase experiments, but the presence of large amounts of intact virions rendered these experiments inconclusive (11). Subsequent attempts to characterize the 125S provirion particles and to repeat the reported in vitro assembly of provirions (12) were unsuccessful (17); however, particles were found cosedimenting with 150S virions with some similar properties, including a preponderance of uncleaved VP0 and sensitivity to SDS and EDTA (17). Particles with some of the properties of the reported poliovirus provirions, including the presence of uncleaved capsid proteins, decreased stability, and lower sedimentation rate, have been observed in bovine enterovirus-infected cells (18) and in flock house virus-infected insect cells (15).

The maturation of the predicted poliovirus provirion into the final virion requires cleavage of an asparagine-serine peptide bond in VP0, yielding the individual capsid proteins VP2 and VP4. Only a few uncleaved VP0 proteins remain in preparations of infectious poliovirus; if these molecules are distributed evenly throughout the population of particles, one or two of the 60 VP0 proteins in each virion remain uncleaved (40). One of the several surprises that emerged from the determination of the three-dimensional structures of rhinovirus 14 (39), poliovirus (13, 19), and other picornaviruses (1, 30) was that the cleaved termini of VP2 and VP4 were found to be buried within the mature virion structures. Barring drastic rearrangement after VP0 cleavage, it thus appeared that the scissile bond is inaccessible to external proteases, leading to suggestions that VP0 cleavage is catalyzed by some aspect of the virion structure itself (3, 19, 39).

Another intriguing feature of the mechanism of VP0 cleavage is its apparent dependence on RNA packaging. In extracts from poliovirus-infected cells, the cleaved products VP2 and VP4 have been seen only in mature virions and not in any subviral particles that are not associated with the viral RNA (36, 40). It has been suggested that RNA nucleotide residues may function directly in the proteolysis of VP0, by contributing to the active site of a serine protease formed by conserved amino acid residues in VP0 (3). Whether the RNA acts directly as a component of the active site of a proteolytic activity or as an allosteric effector of a protein-based protease may be difficult to determine. However, the dependence of VP0 cleavage on RNA packaging may serve to make the RNA packaging step of viral assembly irreversible (3, 39).

We identified and characterized a temperature-sensitive poliovirus mutant, VP2-103, that shows no detectable VP0 cleavage at the nonpermissive temperature. That VP0 cleavage is the primary defect in VP0-103 is shown by the accumulation of particles identical to the proposed provirion

* Corresponding author.
† Present address: Department of Biology, Brandeis University, Waltham, MA 02254.

(11) at the nonpermissive temperature. The existence of these provirions in analyzable quantities demonstrates that whatever the precise mechanism of VP0 cleavage, the packaging of RNA can be uncoupled from the catalytic reaction.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in Dulbecco modified Eagle medium (DME) supplemented with 10% calf serum. Wild-type Mahoney type 1 poliovirus was derived from a single plaque resulting from transfection of an infectious poliovirus cDNA clone (37). Plaque assays were performed as described previously (23); incubations at 32.5°C were for 48 h, and incubations at 39.5°C were for 72 h. Titers of both wild-type and VP2-103 viruses were determined at 32.5°C on HeLa cells, and multiplicities of infection at both temperatures were calculated by using the viral titers determined at 32.5°C.

Mutagenesis and reconstruction of VP2-103. The initial isolate of a highly temperature-sensitive virus, here termed VP2-103i, was found in a genetic screen designed to recover temperature-sensitive, cold-sensitive, or host range mutants (24). The screen followed a mutagenesis procedure designed to introduce small deletions into the poliovirus genome (24). A deletion of nucleotides 632 to 657 relative to wild-type poliovirus RNA was first identified in VP2-103i by RNase protection (23, 24); the precise sequence was determined by chemical sequencing of cDNA primed from a 5′ end-labeled oligonucleotide complementary to the viral RNA 3′ of the mapped deletion. To reconstruct the deletion into an infectious cDNA encoding an otherwise wild-type poliovirus genome, a synthetic deoxyoligonucleotide (CCAGCAAA CAGATAGGGCCAATCCAATTCGC) was used to mutagenize (24) a 4.8-kilobase-pair plasmid, pSP61809, containing only the first 1,809 base pairs of the poliovirus cDNA (25, 31, 38); the mutant sequences were confirmed by chemical sequencing (32). A DNA fragment containing the 25-nucleotide deletion was then reconstructed into a plasmid containing the full-length poliovirus cDNA (24, 38). The resulting cDNA, p5NC-104, was transfected into HeLa cells at 32.5, 37, and 39.5°C to determine the phenotype of the encoded virus genome. All plaques resulting from duplicate transfections contained virus that was indistinguishable from the wild type, although the presence of the 25-nucleotide deletion in the 5NC-104 virus was confirmed by sequencing.

The mutation responsible for the VP2-103 phenotype was then identified by making cDNA from VP2-103i viral RNA. RNA was prepared from VP2-103i virions as described previously (5). cDNA was made as described previously (16), except that the first strand was not primed with oligo(dT). Instead, the first strand of cDNA was primed with 100 μg of a deoxyoligonucleotide (AGTATGCTCTAGT TTC, termed g2seq) complementary to nucleotides 4431 to 4446 of the poliovirus genome (25, 38) per ml. Duplicate fragments containing sequences from nucleotides 66 to 2516 of the poliovirus cDNA were then cloned into a plasmid designed to reconstruct the 5′ end of the infectious poliovirus cDNA. These intact 5′ ends were then reconstructed into plasmids (p5, p43) containing infectious full-length poliovirus cDNA (24, 37). DNA from pPolio (24), p5, and p43 plasmids was transfected into HeLa cells at 32.5 and 39.5°C; plaques resulting from the p5 and p43 transfections contained virus displaying the initial temperature-sensitive phenotype of VP2-103i.

Further cloning revealed that infectious cDNA containing nucleotides 1 to 1118 of p43 DNA encoded wild-type viruses, whereas infectious cDNA with nucleotides 1118 to 1639 gave rise to temperature-sensitive virus. Chemical sequencing (32) between nucleotides 1118 and 1639 of p43 DNA revealed exclusively wild-type sequences, with the exception of the substitution of an A for the wild-type G at position 1178 (25, 38).

Analysis of virus yields. HeLa cells were grown in modified Eagle medium for suspension (MEM) supplemented with 7% horse serum in a spinner culture at 37° C to a density of 4.0×10^5 cells per ml. Cells (125 ml) were pelleted by low-speed centrifugation, suspended in 2 ml of phosphate-buffered saline (PBS), and infected with wild-type or VP2-103 virus at a multiplicity of infection of 20 PFU per cell. After 30 min of viral absorption, cells were washed twice in PBS, suspended in 20 ml of MEM supplemented with 10% fetal calf serum, and incubated with stirring at 32.5 or 39.5°C. Samples (1 ml) of cells were removed and washed twice with PBS at designated times. Cells were lysed by freezing and thawing three times, and nuclei were removed by centrifugation at $1,600 \times g$ for 10 min at 4°C. Plaque assays to determine virus yield were performed at 32.5°C.

Analysis of viral RNA synthesis. Total viral RNA synthesis was measured by incorporation of [^{3}H]uridine into trichloroacetic acid-precipitable material in the presence of dactinomycin as described previously (7).

Analysis of viral and subviral particles. For incorporation of [^{3}H]uridine into virus-specific particles, subconfluent HeLa cells in 100-mm dishes were infected with viruses at multiplicities of infection of 50 and incubated at 39.5°C in DME with 10% calf serum. After 3 h, dactinomycin was added to 5 μg/ml and [^{3}H]uridine was added to 20 μCi/ml. At 4 h postinfection, cells were washed with PBS and scraped into RSB (10 mM Tris [pH 7.4], 10 mM NaCl, 1.5 mM $MgCl_2$) with 1% Nonidet P-40, 1 mM phenylmethylsufonyl fluoride, and 40 U of RNasin per ml. Nuclei were pelleted by centrifugation at $1,600 \times g$ for 10 min at 4°C, and 0.5 ml of cytoplasmic extract was loaded directly onto an 11-ml 15 to 30% sucrose gradient in RSB. Particles were sedimented for 3 h at 27,500 rpm at 15°C in a Beckman SW41 rotor. Fractions from the gradient were analyzed for infectivity by plaque assay. For incorporation of [^{3}H]uridine, 0.1 ml of each fraction was precipitated with 4 ml of cold 5% trichloroacetic acid and collected by filtration through nitrocellulose. Radioactivity bound to each filter was measured by scintillation counting.

For incorporation of [^{35}S]methionine into virus-specific particles, infected HeLa cells were labeled as described previously (22). Cells were harvested 4 h after infection, extracts were prepared, and particles were sedimented through 15 to 30% sucrose gradients as indicated above.

Fractions from the sucrose gradients were analyzed for infectivity by plaque assay at 32.5°C, the permissive temperature for VP2-103.

Two-dimensional polyacrylamide gel electrophoresis. The products and amounts of viral protein synthesis were analyzed by two-dimensional electrophoresis of [^{35}S]methionine-labeled viral proteins. Plates (100 mm) of HeLa cells were infected separately with wild-type and VP2-103 viruses at multiplicities of infection of 50 PFU per cell and incubated at 32.5°C for 4 h or at 39.5°C for 2.5 h. Cells were then washed with PBS and incubated in 3 ml of DME without methionine or calf serum for 0.5 h. Subsequently, 100 μCi of [^{35}S]methionine per ml was added to each plate, and incubation was continued for 2.5 h. Thus, the total postinfection incubation times were 7 h at 32.5°C and 5.5 h at 39.5°C. Cells were washed with PBS and lysed in 0.5 ml of RSB with 1%

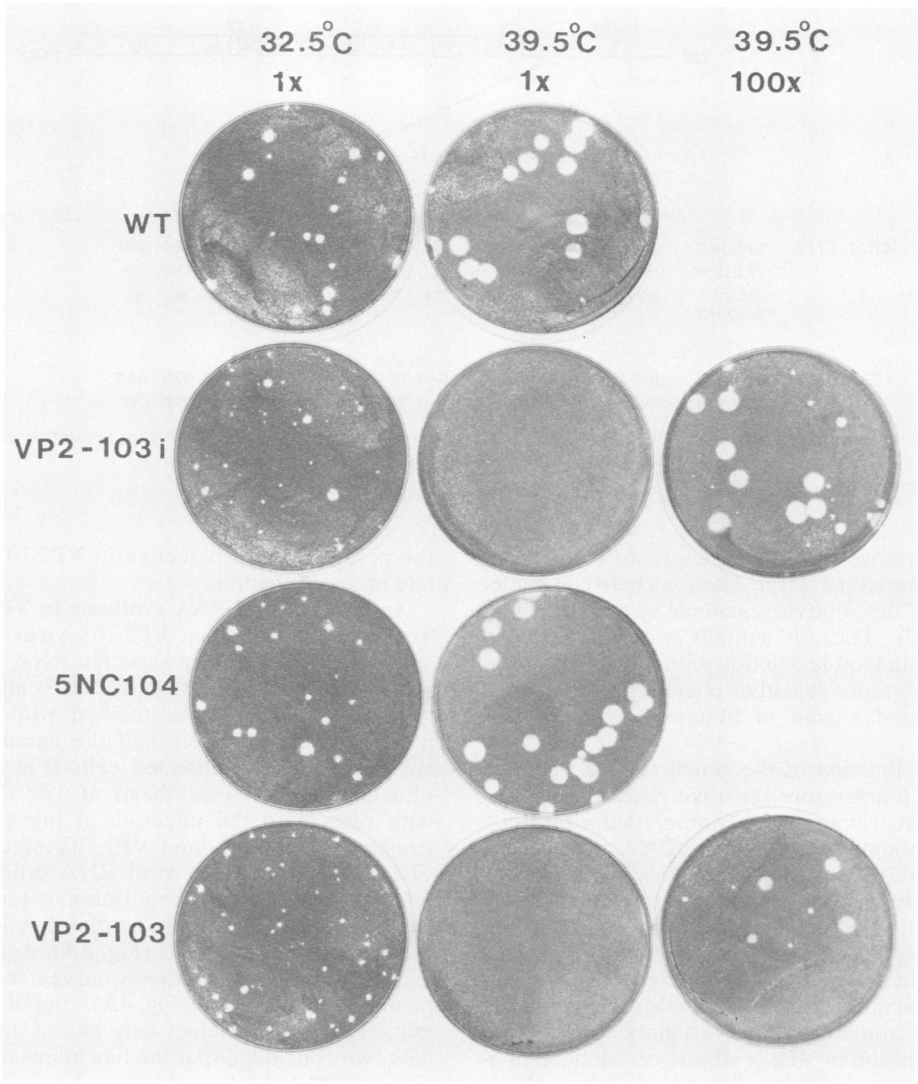

FIG. 1. Plaque phenotypes of wild-type poliovirus, VP2-103i, reconstructed virus 5NC-104, and VP2-103 at 32.5 and 39.5°C. 1× and 100× denote the relative concentrations of the viral inocula.

Nonidet P-40 per plate. Nuclei were pelleted by centrifugation at 1,600 × *g* for 10 min at 4°C, and 20 μl of each cytoplasmic extract was applied to 4% acrylamide tube gels containing 9.2 M urea, 2% Nonidet P-40, and 2% ampholine (pH 4 to 9; Pharmacia). Electrophoresis was for 16 h at 400 V (34). Tube gels were then loaded on SDS–15% polyacrylamide gels and electrophoresed. Labeled proteins were visualized by autoradiography. The assignments of the mobilities of the capsid proteins VP0 and VP2 were made by comparison of the mobilities in the first dimension with those of proteins from purified virions and from the expected net charges (25).

RESULTS

Isolation of the temperature-sensitive mutant VP2-103 and its genetic reconstruction. During a genetic screen for conditional poliovirus mutants (24), a virus isolate that displayed a very pronounced temperature-sensitive phenotype on HeLa, CV1, cos-7, and Vero cells was identified. The plaque phenotype of this mutant, now termed VP2-103*initial* (VP2-103i), is shown in Fig. 1. The VP2-103i isolate produced more than 100-fold fewer plaques at 39.5°C than at 32.5°C, and the 32.5°C plaques were smaller than wild-type plaques. Virus from plaques picked at 39.5°C no longer displayed the mutant phenotype and thus consisted of phenotypically revertant viruses with either intragenic or extragenic second-site mutations.

The mutagenesis procedure used to generate the viral cDNAs examined in the genetic screen involved the introduction of random small deletions into infectious poliovirus cDNA (24). RNase protection mapping (Materials and Methods) of the VP2-103i RNA revealed the presence of a 25-nucleotide deletion spanning nucleotides 632 through 657 in the 5′ noncoding region of the poliovirus genome. By using deoxyoligonucleotide-directed mutagenesis (Materials and Methods), this deletion was reconstructed into a plasmid containing a full-length copy of otherwise wild-type poliovirus cDNA; the mutant plasmid was termed p5NC-104. However, the 5NC-104 virus that resulted from transfection of this plasmid displayed a completely wild-type phenotype (Fig. 1), showing that the 25-nucleotide deletion was not the

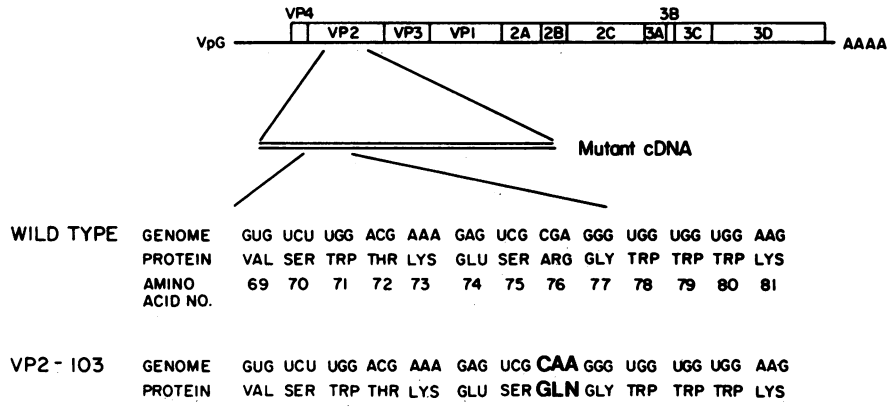

FIG. 2. Portions of the nucleotide sequence of the type 1 Mahoney poliovirus genome and the protein sequence of VP2 in wild-type and VP2-103 mutant viruses.

cause of the temperature-sensitive phenotype. It has been previously demonstrated that large deletions between nucleotides 600 and 720 of the poliovirus genome have no effect on viral replication (27). Thus, it actually would have been surprising if the 25-nucleotide deletion within this region had conferred the temperature-sensitive phenotype; this result would have suggested a gain of function in the mutant genome.

To determine the location of the genetic lesion that was responsible for the temperature-sensitive phenotype of the isolated virus, cDNA was prepared from mutant viral RNA (Materials and Methods). We found that cDNA representing nucleotides 1118 to 1639 of the VP2-103i viral RNA was sufficient to create the mutant phenotype in the transfection products of an otherwise wild-type poliovirus cDNA (Fig. 1). Sequencing of this cDNA fragment revealed the presence of only one genetic deviation from the wild-type sequence: a transition mutation at nucleotide 1178, altering amino acid 76 in VP2 of poliovirus from an arginine to a glutamine (Fig. 2). Thus, this point mutation in VP2 is almost certainly responsible for the temperature-sensitive phenotype of the isolated virus; the mutant was therefore termed VP2-103, according to the nomenclature suggested by Bernstein et al. (6).

Temperature sensitivity of VP2-103 in single-cycle infections. The production of infectious wild-type and VP2-103 virus during single-cycle infections initiated at the same multiplicity of infection was measured at 39.5 and 32.5°C. In accordance with the plaque phenotype, VP2-103-infected cells produced 1,000-fold less virus than did wild type-infected cells at the nonpermissive temperature (Fig. 3A). At 32.5°C, however, VP2-103-infected cells produced only 10-fold less virus than did the wild type, and the mutant viral titer increased with time, showing that an infection was occurring at the permissive temperature (Fig. 3B).

Temperature shift experiments were performed to determine whether the process that is defective in VP2-103 is required throughout the infection. Cells infected with VP2-103 at 39.5°C for 4 h began to produce mutant virus when shifted to the permissive temperature (Fig. 3C). Therefore, the defect could be reversed even when the infection was begun at the nonpermissive temperature. Conversely, when cells were infected with VP2-103 at 32.5°C for 6 h and then shifted to 39.5°C, viral production quickly ceased (Fig. 3D). Wild-type viral production was relatively unaffected by the temperature shifts, except for the slower growth that occurred at the lower temperature. Therefore, it appears that the process that is defective in VP2-103 virus is important late in viral infection.

Analysis of viral RNA synthesis in VP2-103-infected cells. To determine whether VP2-103 virus is defective in the synthesis of RNA, virus-specific RNA synthesis was measured at 39.5 and 32.5°C in VP2-103- and wild-type poliovirus-infected cells. Cells infected with VP2-103 at 39.5°C produced approximately half the amount of RNA synthesized by wild type-infected cells (Fig. 4). The amount of virus-specific RNA synthesis at 32.5°C, although delayed with respect to the infection at higher temperature, was similar in wild type- and VP2-103-infected cells (Fig. 4B). The small decrease in viral RNA production in VP2-103-infected cells at the nonpermissive temperature does not seem sufficient to account for the drastic reduction in virus production seen at 39.5°C (Fig. 3A). Actually, the wild-type and VP2-103 RNA synthesis curves are identical until 4 h postinfection at 39.5°C (Fig. 4A), supporting the idea that the mutant displays a defect only late in infection. From these data, we concluded that the biochemical event that is defective in VP2-103-infected cells occurs after viral RNA synthesis.

Analysis of assembly of mutant and wild-type viruses. After macromolecular synthesis, the next step in the formation of infectious viral progeny is the assembly of newly synthesized viral proteins and RNA into the infectious virion and its precursors. To determine whether any viral particles could be assembled from VP2-103 proteins made at 39.5°C, sucrose gradients of viral and subviral particles were analyzed. To aid in the identification of the particles that were formed, similar gradients were studied in which either the viral RNA or the viral proteins were labeled.

[^{35}S]methionine-labeled viral and subviral particles formed at 39.5°C by VP2-103 and wild-type poliovirus are shown in Fig. 5A. Two peaks of radioactivity were observed in the sucrose gradients of particles from wild type-infected cells; the positions of these peaks suggested their identities as the 150S virions and 75S empty capsids, respectively. These assignments of the peaks were confirmed by analyzing the corresponding fractions for infectivity (see below) and protein composition (data not shown). These peaks correlated well with the 150S and 75S peaks documented in the literature (36, 40). Two peaks of [^{35}S]methionine-containing particles were also observed in extracts from cells infected with VP2-103 at the nonpermissive temperature (Fig. 5A). One of these peaks corresponded to the 75S empty capsids in

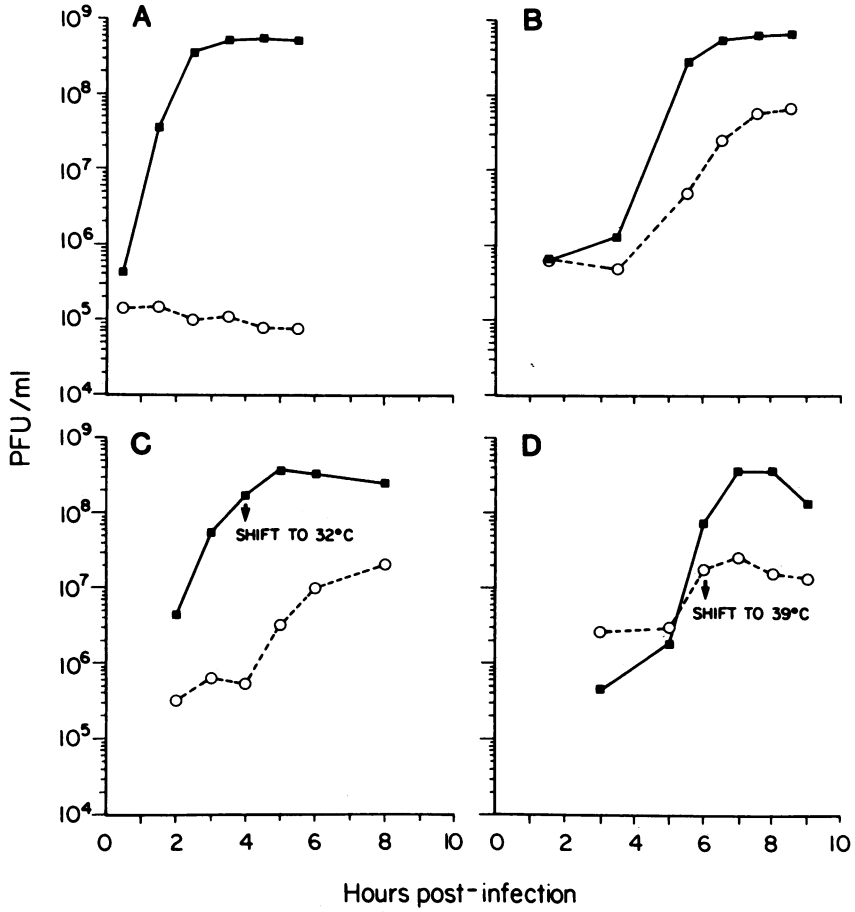

FIG. 3. Growth curves of VP2-103 (○) and wild-type (■) poliovirus at 39.5°C (A) and 32.5°C (B). (C) Effect of temperature shift to 32.5°C at 4 h postinfection on the 39.5°C growth curves. (D) Effect of shifting to 39.5°C at 6 h postinfection on the 32.5°C growth curves.

the wild type-infected cell extracts. Another peak in the mutant-infected cell extracts had an approximate sedimentation rate of 125S, intermediate between procapsids and mature virions and identical with the originally reported provirion of Fernandez-Tomas and Baltimore (11). Comparison of the distributions of infectious particles and ^{35}S-labeled particles made at 39.5°C in VP2-103 (Fig. 5B) and wild-type (Fig. 5C) infections revealed that the 125S particles made in the mutant-infected cells were more than 10 times less infectious than mature wild-type virions. Like the

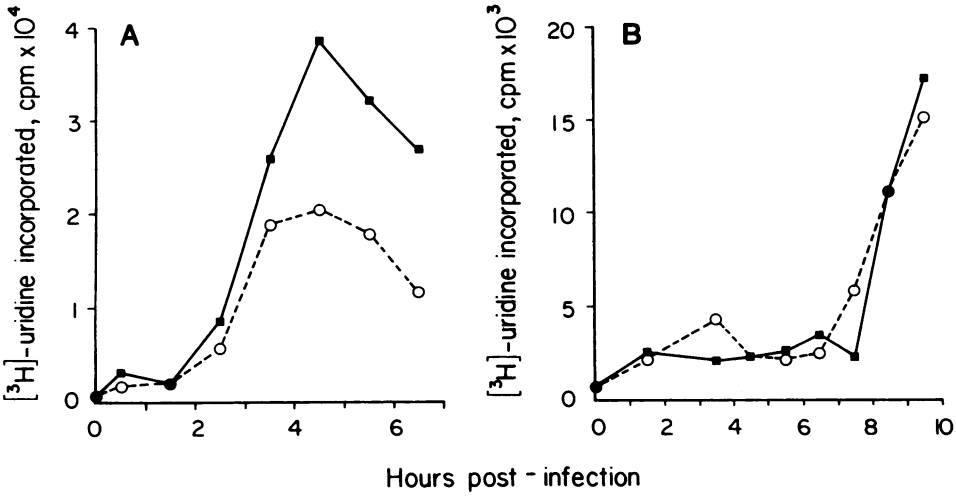

FIG. 4. Time course of viral RNA synthesis in cells infected with VP2-103 (○) and wild-type (■) poliovirus at 39.5°C (A) and 32.5°C (B).

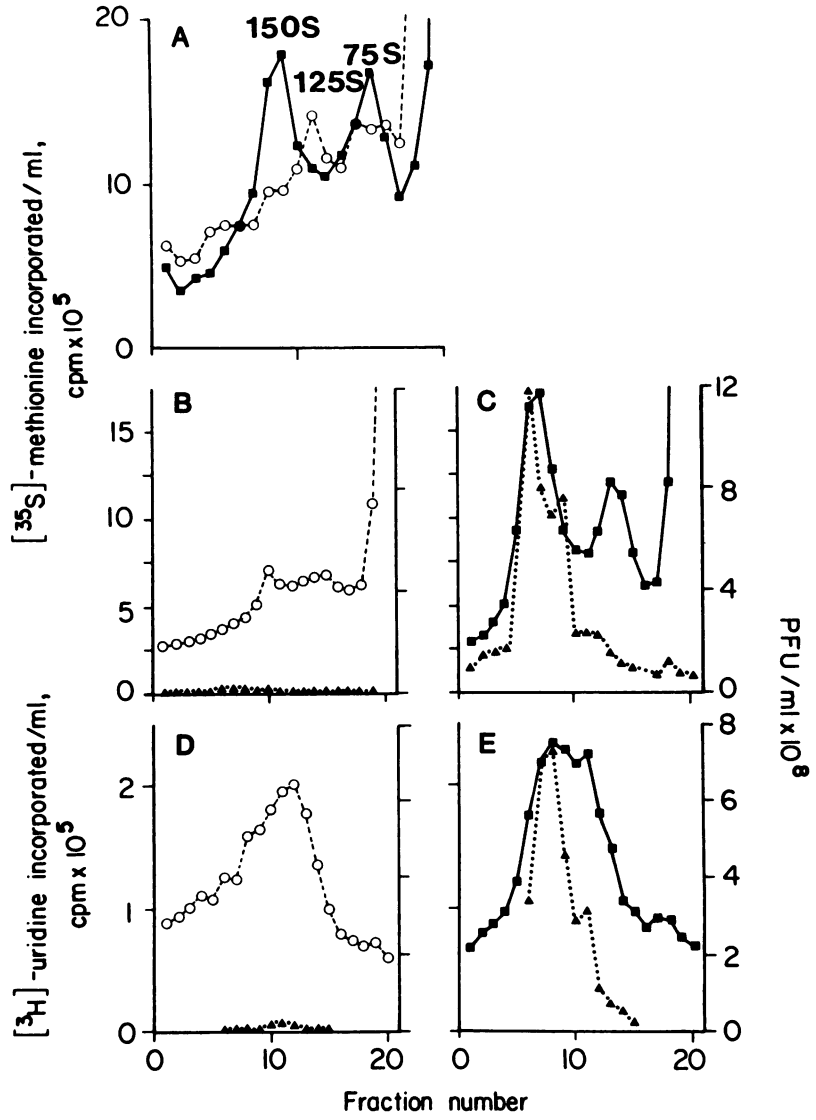

FIG. 5. Analysis of subviral particles formed at 39.5°C in VP2-103- and wild-type poliovirus-infected cells by sedimentation through 15 to 30% sucrose gradients. The heaviest fractions were collected first; 20 0.5-ml fractions were collected from each gradient. (A) [^{35}S]methionine-labeled particles from HeLa cells infected with VP2-103 (○) and the wild type (■). (B) [^{35}S]methionine (○) and PFU (▲) in particles from VP2-103 infected cells. (C) [^{35}S]methionine (■) and PFU (▲) in particles from wild type-infected cells. (D) [^{3}H]uridine (○) PFU (▲) in particles from VP2-103-infected cells. (E) [^{3}H]uridine (■) and PFU (▲) in particles from wild type-infected cells. Gradients B and C were run simultaneously and are thus directly comparable; gradients D and E were also run simultaneously.

previously reported provirion, the 125S material that accumulated in VP2-103-infected cells at 39.5°C was much more sensitive to SDS and EDTA treatment than was the 150S wild-type material (data not shown).

Did the 125S peak contain viral RNA? Cells infected with either VP2-103 or wild-type poliovirus at 39.5°C were labeled with [^{3}H]uridine in the presence of dactinomycin, so that only viral RNA should incorporate radioactivity (4). Analysis of the resulting particles on sucrose gradients revealed one large peak of radioactivity in extracts from cells infected with both VP2-103 (Fig. 5D) and the wild type (Fig. 5E). The positions of the RNA-containing fractions from wild type- and VP2-103-infected cells in the sucrose gradients were similar to those of the 150S and 125S particles, respectively, observed during [^{35}S]methionine labeling. Like the 125S particles in Fig. 5B, the RNA-containing particles from VP2-103-infected cells were more than 10 times less infectious than the 150S peak of mature virions resulting from the wild-type infection (Fig. 5D and E). The shoulder of material sedimenting more slowly than the 150S virions in Fig. 5E may be provirionlike particles as well.

Some infectious virus was always seen after growth of VP2-103 at high temperature (Fig. 5D). This infectivity was usually not centered under the 125S radioactive peak, and we presume that it results from the presence of the inevitable phenotypically revertant viruses, which could grow under these conditions. Thus, we conclude that VP2-103 viruses can accomplish morphogenesis of subviral particles, at least to the point of assembling particles that sediment at approximately 125S and display several properties reminiscent of the elusive poliovirus provirion, including lack of infectivity (11).

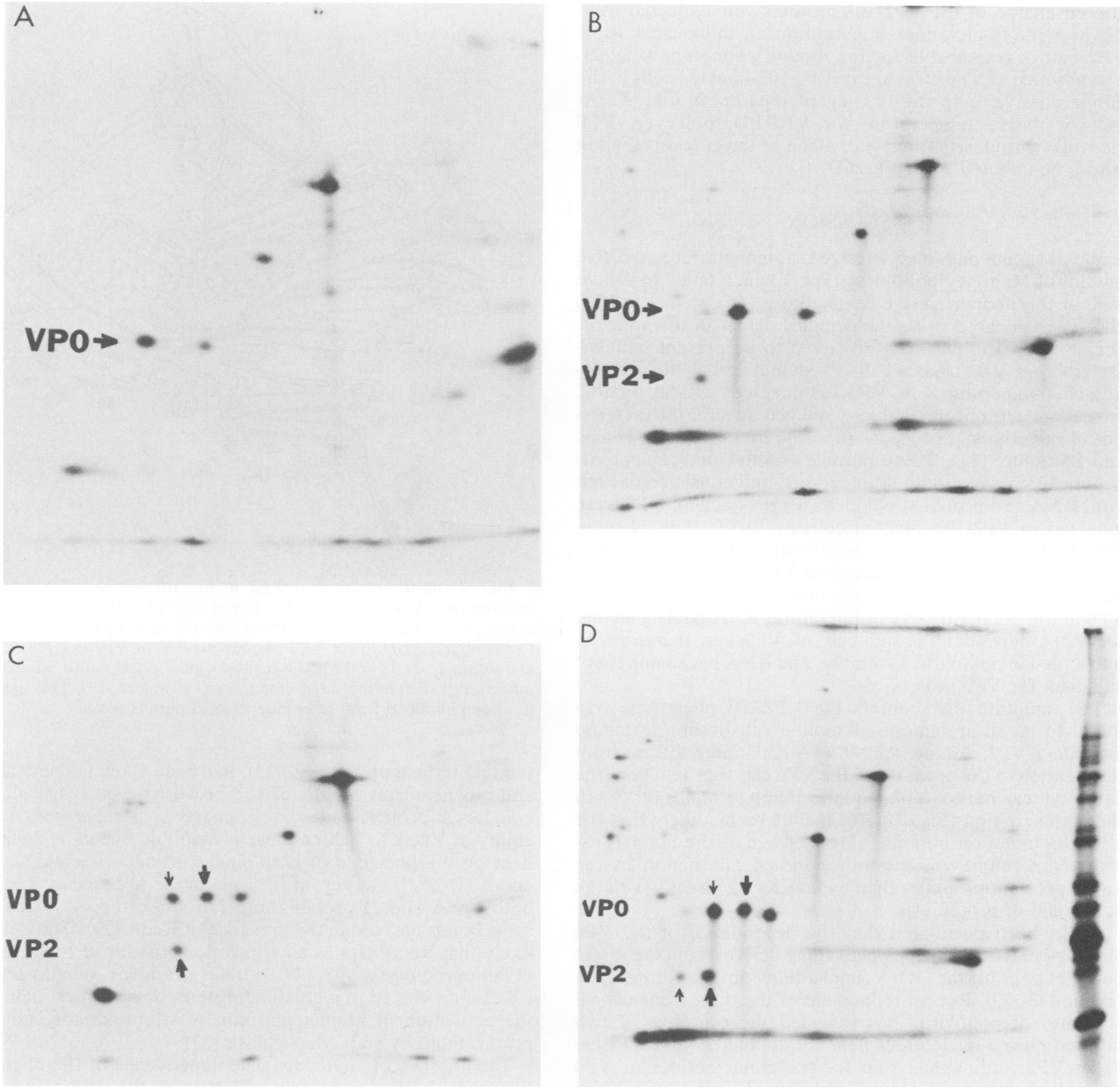

FIG. 6. Two-dimensional gels of VP2-103 and wild-type proteins produced at 32.5 and 39.5°C. Proteins were labeled from 4.5 to 7.0 h postinfection at 32.5°C and from 3.0 to 5.5 h postinfection at 39.5°C. Along the horizontal axis, the pH increases from left to right. (A) VP2-103 proteins at 39.5°C. (B) VP2-103 proteins at 32.5°C. (C) Mixture of wild-type and VP2-103 proteins from 39.5°C infections. (D) Mixture of wild-type and VP2-103 proteins from 32.5°C infections. Large and small arrows denote wild-type and VP2-103 proteins, respectively. Labeled proteins from wild-type infected cells are displayed in the SDS-polyacrylamide gel electrophoresis dimension in panel D.

VP2-103 is defective in VP0 cleavage at the nonpermissive temperature. To determine the efficiency of protein processing in VP2-103-infected cells, two-dimensional polyacrylamide gel electrophoresis (34) of mutant and wild-type viral proteins was performed. Proteins from cells infected with VP2-103 at 39.5°C (Fig. 6A) or 32.5°C (Fig 6B) were labeled with [^{35}S]methionine. Assignment of the spots whose mobilities corresponded to the viral capsid proteins were made by comparison with one-dimensional SDS-gel electrophoretic patterns of labeled proteins from extracts of wild-type poliovirus-infected cells (Fig. 6D), from purified virions (data not shown), and from the predicted net charges of the proteins (25). At 39.5°C, VP2-103 proteins were synthesized, but no processing of VP0 to VP2 (Fig. 6A) was apparent. In VP2-103-infected cells at 32.5°C, however, cleavage of VP0 to VP2 was readily observed (Fig. 6B).

The electrophoretic patterns of mixtures of wild-type and VP2-103 proteins from separate infections at 39.5°C (Fig. 6C) and 32.5°C (Fig. 6D) were also determined. Both VP0 and VP2 from VP2-103-infected cells (light arrows) displayed slightly more acidic isoelectric points than the corresponding proteins from wild type-infected cells (heavy arrows). The

altered charge of the VP2-103 proteins was expected from the arginine-to-glutamine substitution at amino acid 76 of VP2 that is responsible for the mutant phenotype (Fig. 2). The absence of VP0 cleavage in VP2-103-infected cells at the nonpermissive temperature is again apparent in Fig. 6C. At the permissive temperature for VP2-103, however, VP0 cleavage was clearly observed, albeit at lower levels than in wild-type-infected cells (Fig. 6D).

DISCUSSION

We isolated and characterized a temperature-sensitive mutant of Mahoney poliovirus type 1 which failed to cleave VP0 at the nonpermissive temperature. The defect in VP0 cleavage appeared to be the primary defect in this mutant, because subviral particles predicted to be present immediately before VP0 cleavage also accumulated at the nonpermissive temperature. In VP2-103-infected cells at 39.5°C, particles were observed that displayed several characteristics of the original provirions reported by Fernandez-Tomas and Baltimore (11). These particles sedimented at approximately 125S, were quite uninfectious, and consisted of both viral RNA and proteins. No cleavage products of VP0 were detected in VP2-103-infected cells at 39.5°C (Fig. 6) or in isolated 125S particles (data not shown). Thus, the presence of a single amino acid change in VP2 still allowed RNA packaging to occur but prevented the maturation cleavage of VP0 at the nonpermissive temperature. RNA packaging and the RNA-dependent proteolysis of VP0 can therefore be uncoupled in poliovirus assembly, and RNA packaging is not sufficient for VP0 proteolysis.

The mutation that confers the VP2-103 phenotype was found to be an arginine-to-glutamine substitution at amino acid 76 of VP2. Amino acid 76 of VP2 is quite distant from the location of the products of the VP0 cleavage reaction, the carboxyl terminus of VP2, and the amino terminus of VP4 in the mature virion (Fig. 7) (19). It thus seems likely that the VP2-103 mutation inhibits VP0 cleavage at the nonpermissive temperature via a conformational alternation in the virion precursors, rather than by interfering directly with the active site of proteolysis.

It has been postulated that the dependence of the VP0 cleavage reaction on RNA packaging derives from the direct participation of the RNA nucleotides in the proteolysis reaction (3, 39). Recent refinement of the three-dimensional structure of poliovirus has revealed the presence of two ordered purine nucleotides near tryptophan 38 and tyrosine 41 in VP2, although a role for these nucleotides in VP0 proteolysis remains highly speculative (13). Direct involvement of RNA nucleotides in a serine protease active site that includes serine 10 of VP2 (Fig. 7) has been suggested (3). Regardless of the precise mechanism of a catalytic activity containing both amino acids and nucleotides, or only nucleotides, in its active site, the interesting possibility of a direct role for RNA bases, phosphates, or copackaged divalent cations or polyamines (14) in VP0 cleavage remains extant.

Another model for the RNA dependence of VP0 cleavage is that packaged RNA causes a conformational change in the capsid proteins, allowing exclusively protein-catalyzed autoproteolysis to occur. In the VP2-103 mutant, for example, it may be impossible at the nonpermissive temperature for the mutant capsid to undergo the conformational changes that normally accompany viral RNA encapsidation, thereby blocking VP0 cleavage. Several examples of autoproteolytic events mediated by the binding of a noncatalytic effector exist. For example, *Escherichia coli* RecA protein, originally

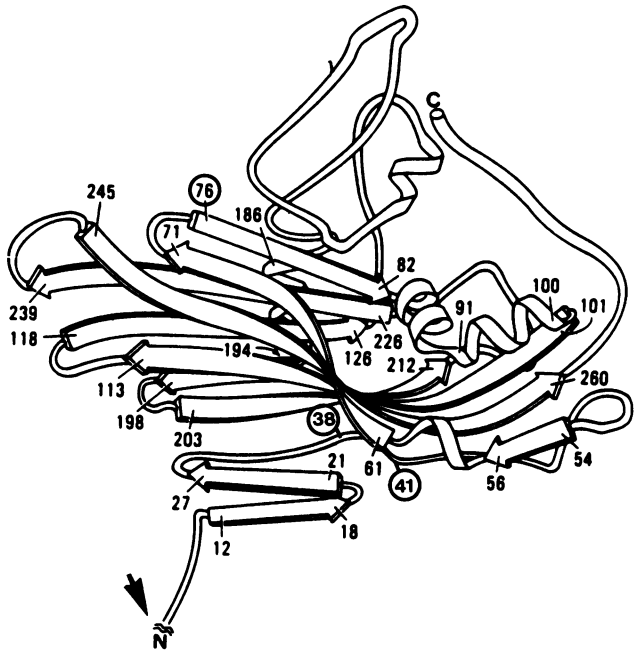

FIG. 7. Representation of VP2 in mature virions. The amino terminus resulting from VP0 cleavage is marked with an arrow. VP4 is not shown, but the newly formed carboxy terminus is quite close to the amino terminus of VP2. Amino acid 76 of VP2 is circled, as are amino acids 38 and 41, the residues most proximal to the RNA nucleotides seen in the three-dimensional structure (13). This figure has been modified from reference 19 with permission.

thought to be a protease (9, 35), has been shown genetically and biochemically to induce the proteolytic cleavage of *E. coli* LexA protein and bacteriophage λ *c*I repressor. The ability of RecA to induce these proteolytic events is dependent on the presence of both single-stranded nucleic acids and ATP (9). However, at high pH in the absence of RecA, both LexA and *c*I proteins undergo autoproteolysis at the same bonds cleaved in the presence of RecA (29). Thus, it is likely that RecA acts as an allosteric activator of LexA and *c*I autoproteolysis (28, 29). Other examples of allosteric effectors involved in regulating proteolytic activities include the activation of human plasmin by streptokinase and of prothrombin by staphylcoagulase (33).

The absence of any host-range dependence of the phenotype of VP2-103 is consistent with the idea that VP0 cleavage is either autoproteolytic or exclusively mediated by viral constituents. The role of packaged viral RNA in VP0 cleavage remains a subject of investigation. The existence of a poliovirus mutant that accumulates provirionlike particles at the nonpermissive temperature may facilitate the purification and analysis of poliovirus provirions and inquiry into the mechanism of their proposed maturation into infectious virions.

ACKNOWLEDGMENTS

We are grateful to Peter Sarnow for introducing us to two-dimensional gel electrophoresis and for numerous suggestions throughout the course of this work. The experiments presented here were initiated in the laboratory of David Baltimore, and his support is greatly appreciated. We thank Thale Jarvis and Constance Nugent for their contributions to the manuscript and Jim Hogle for his frequent communication of unpublished results.

This work was supported by Public Health Service grant AI-25166 from the National Institutes of Health, Junior Faculty Award JFRA-171 from the American Cancer Society, and the Searle Scholar's Fund.

LITERATURE CITED

1. **Acharya, R., E. Fry, D. Stuart, G. Fox, D. Rowlands, and F. Brown.** 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 A resolution. Nature (London) **337**:709–716.
2. **Argos, P., G. Kamer, M. J. H. Nicklin, and E. Wimmer.** 1984. Similarity in gene organization and homology between proteins of animal picornaviruses and a plant comovirus suggest common ancestry of these virus families. Nucleic Acids Res. **12**:7251–7267.
3. **Arnold, E., M. Luo, G. Vriend, M. G. Rossmann, A. C. Palmenberg, G. D. Parks, M. J. H. Nicklin, and E. Wimmer.** 1987. Implications of the picornavirus capsid structure for polyprotein processing. Proc. Natl. Acad. Sci. USA **84**:21–25.
4. **Baltimore, D., M. Girard, and J. E. Darnell.** 1966. Aspects of the synthesis of poliovirus RNA and the formation of virus particles. Virology **29**:179–189.
5. **Baron, M. H., and D. Baltimore.** 1982. Antibodies against the chemically synthesized genome-linked protein of poliovirus react with native virus-specific proteins. Cell **28**:395–404.
6. **Bernstein, H. D., P. Sarnow, and D. Baltimore.** 1986. Genetic complementation among poliovirus mutants derived from an infectious cDNA clone. J. Virol. **60**:1040–1049.
7. **Bernstein, H. D., N. Sonenberg, and D. Baltimore.** 1985. Poliovirus mutant that does not selectively inhibit host cell protein synthesis. Mol. Cell. Biol. **5**:2913–2923.
8. **Burns, C. C., M. A. Lawson, B. L. Semler, and E. Ehrenfeld.** 1989. Effects of mutations in poliovirus $3D^{pol}$ on RNA polymerase activity and on polyprotein cleavage. J. Virol. **63**:4866–4874.
9. **Craig, N. L., and J. W. Roberts.** 1980. *E. coli* rec A protein-directed cleavage of phage lambda repressor requires polynucleotide. Nature (London) **283**:26–30.
10. **Dewalt, P. G, and B. L. Semler.** 1987. Site-directed mutagenesis of proteinase 3C results in a poliovirus deficient in synthesis of viral RNA polymerase. J. Virol. **61**:2162–2170.
11. **Fernandez-Tomas, C. B., and D. Baltimore.** 1973. Morphogenesis of poliovirus. II. Demonstration of a new intermediate, the provirion. J. Virol. **12**:1181–1183.
12. **Fernandez-Tomas, C. B., N. Guttman, and D. Baltimore.** 1973. Morphogenesis of poliovirus. III. Formation of provirion in cell-free extracts. J. Virol. **12**:1181–1183.
13. **Filman, D. J., R. Syed, M. Chow, A. J. Macadam, P. D. Minor, and J. M. Hogle.** 1989. Structural factors that control conformational transitions and serotype specificity in type 3 poliovirus. EMBO J. **8**:1567–1579.
14. **Fout, G. S., K. C. Medappa, J. E. Mapoles, and R. R. Rueckert.** 1984. Radiochemical determination of polyamines in poliovirus and human rhinovirus 14. J. Biol. Chem. **259**:3639–3643.
15. **Gallagher, T. M., and R. R. Rueckert.** 1988. Assembly-dependent maturation cleavage in provirions of a small icosahedral insect ribovirus. J. Virol. **62**:3399–3406.
16. **Gubler, U., and B. J. Hoffman.** 1983. A simple and very efficient method of generating cDNA libraries. Gene **25**:263–269.
17. **Guttman, N., and D. Baltimore.** 1977. Morphogenesis of poliovirus. IV. Existence of particles sedimenting at 150S having the properties of provirions. J. Virol. **23**:363–367.
18. **Hoey, E. M., and S. J. Martin.** 1974. A possible precursor containing RNA of a bovine enterovirus: the provirion. J. Gen. Virol. **24**:515–524.
19. **Hogle, J. M., M. Chow, and D. F. Filman.** 1985. Three-dimensional structure of poliovirus at 2.9 A resolution. Science **229**:1358–1365.
20. **Jacobson, M. F., J. Asso, and D. Baltimore.** 1970. Further evidence on the formation of poliovirus proteins. J. Mol. Biol. **49**:657–669.
21. **Jacobsen, M. F., and D. Baltimore.** 1968. Morphogenesis of poliovirus. I. Association of viral RNA with coat protein. J. Mol. Biol. **33**:369–378.
22. **Kirkegaard, K.** 1990. Mutations in VP1 of poliovirus affect both encapsidation and release of viral RNA. J. Virol. **64**:195–206.
23. **Kirkegaard, K., and D. Baltimore.** 1986. The mechanism of RNA recombination in poliovirus. Cell **47**:433–443.
24. **Kirkegaard, K., and B. Nelsen.** 1990. Conditional poliovirus mutants made by random deletion mutagenesis of infectious cDNA. J. Virol. **64**:185–194.
25. **Kitamura, N., B. Semler, P. Rothberg, G. Larsen, C. Adler, A. Dorner, E. Emini, R. Hanecak, J. Lee, S. van der Werf, C. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization, and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
26. **Koch, F., and G. Koch.** 1985. The molecular biology of poliovirus. Springer-Verlag, Vienna.
27. **Kuge, S., and A. Nomoto.** 1987. Construction of viable deletion and insertion mutants of the Sabin strain of type 1 poliovirus: function of the 5′ noncoding sequence in viral replication. J. Virol. **61**:1478–1487.
28. **Lin, L.-L., and J. W. Little.** 1988. Isolation and characterization of noncleavable (Ind^-) mutants of the LexA repressor of *Escherichia coli* K-12. J. Bacteriol. **170**:2163–2173.
29. **Little, J. W.** 1984. Autodigestion of lex A and phage lambda repressors. Proc. Natl. Acad. Sci. USA **81**:1375–1379.
30. **Luo, M., G. Vriend, G. Kamer, I. Minor, E. Arnold, M. G. Rossmann, U. Boege, D. G. Scraba, G. M. Duke, and A. C. Palmenberg.** 1987. The atomic structure of mengo virus at 3.0 A resolution. Science **235**:182–191.
31. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
33. **Neurath, H.** 1984. Evolution of proteolytic enzymes. Science **224**:350–357.
34. **O'Farrell, P. H.** 1975. High-resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
35. **Phizicky, E. M., and J. W. Roberts.** 1980. Kinetics of *recA* protein-directed inactivation of repressors of phage lambda and phage P22. J. Mol. Biol. **139**:319–328.
36. **Putnak, J. R., and B. A. Phillips.** 1981. Picornaviral structure and assembly. Microbiol. Rev. **45**:287–315.
37. **Racaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science **214**:916–919.
38. **Racaniello, V. R., and D. Baltimore.** 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. USA **78**:4887–4891.
39. **Rossmann, M. G., E. Arnold, J. W. Erickson, E. A. Frankenberger, J. P. Griffith, H. J. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, R. R. Rueckert, B. Sherry, and G. Vriend.** 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature (London) **317**: 145–153.
40. **Rueckert, R. R.** 1986. Picornaviruses and their replication, p. 357–390. *In* B. N. Fields and D. M. Knipe (ed.), Fundamental virology. Raven Press. New York.