JOURNAL OF VIROLOGY, Mar. 1990, p. 1102–1107
0022-538X/90/031102-06$02.00/0

Vol. 64, No. 3

An Intragenic Revertant of a Poliovirus 2C Mutant Has an Uncoating Defect

JING-PO LI AND DAVID BALTIMORE*

Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142, and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

Received 18 July 1989/Accepted 16 November 1989

A revertant was isolated from a temperature-sensitive poliovirus 2C mutant, 2C-31, which is defective in viral RNA synthesis. This revertant, called 2C-31R1, grew well at 39°C and was not defective in RNA synthesis. However, in contrast to its parental mutant, 2C-31R1 was cold sensitive and could hardly grow at all at 32°C. Analysis of a single-cycle growth revealed that 2C-31R1 was defective in virion uncoating at 32°C, and a substantial amount (more than 30%) of input viruses could be recovered as infectious particles from an infected cell lysate up to 6 h postinfection. The uncoating defect and the inability to grow at cold temperatures could be overcome by a brief incubation at the permissive temperature (39°C) before the infection was continued at 32°C. cDNA cloning and mix-and-match recombination experiments indicated that the defect in uncoating was the result of two secondary point mutations, seven nucleotides apart, in the 2C-coding sequence downstream of the inserted linker which is the original mutation in the parental 2C-31 genome. Another revertant, 2C-31R3, isolated from the same 2C-31 stock, was not defective in uncoating and appeared to be a secondary revertant that contained an intragenic suppressor for the uncoating defect. The uncoating defect of 2C-31R1 could be complemented by type 2 poliovirus. These results suggested that protein 2C, in addition to its role in viral RNA synthesis, has a function in determining virion structure.

Poliovirus is a single-stranded RNA virus that belongs to the picornavirus family. The plus-sense RNA genome is about 7.5 kilobases and encodes a single polyprotein which is proteolytically cleaved to give rise to various structural and nonstructural viral proteins (16, 17, 22, 23).

During poliovirus infection, the first step is the binding of virions to specific cellular receptors found only on the surface of certain primate cells (12, 13, 21). The virus then enters the cell, probably through receptor-mediated endocytosis, with a low pH requirement (20). At this time, the configuration of the virion is altered drastically, and all copies of the capsid protein VP4 are lost, making the viral RNA genome accessible to RNase (15, 19). What triggers this reaction is unknown, but the binding of the virion to its cellular receptor and the subsequent changes in virion configuration must play a key role. For this report, we call this alteration reaction uncoating, recognizing that it is only the first step toward liberating the RNA from its protein coat.

The cloning of poliovirus genome into an infectious cDNA form (26) offered a way to study various virus-encoded proteins that may have important functions during different stages in the viral replication cycle (2, 3, 18). One of these proteins is 2C, which is highly conserved among all members of picornaviruses (1, 14, 24). Through studies of 2C mutants made by site-directed mutagenesis, we showed previously that 2C is involved in viral RNA synthesis (18). To further study the function of 2C, we isolated several 2C revertants. Surprisingly, through the study of revertant viruses derived from one of these 2C mutants, we found that 2C may also have a function in determining virion structure.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension culture in the Joklik modification of minimal essential medium supplemented with 7.5% horse serum (GIBCO Laboratories, Grand Island, N.Y.). Wild-type virus and mutant 2C-31 were isolated from single plaques (18). For revertant isolation, about 10^4 PFU of 2C-31 were used to infect each 60-mm plate of HeLa cell monolayer. After adsorption, the plates were covered with medium containing 1% agar and incubated for 2 days at 39°C. Revertant viruses were then isolated from single plaques under agar and plaque purified once again before their propagation in large liquid cultures. Type 2 poliovirus was isolated from a single plaque from HeLa cells transfected with the plasmid pVR-204 (25), a gift from V. Racaniello, that contains the infectious cDNA clone of type 2 poliovirus. Both [^{35}S]methionine and [^{3}H] myristic acid labeling of poliovirus-infected HeLa cells have been described previously (11, 18). For heat or acid treatment, about 10^8 PFU were included in 1 ml of buffer (50 mM Tris chloride, 0.1 M NaCl, 1.5 mM $MgCl_2$) adjusted to either pH 7.0 or pH 5.0 and incubated at different temperatures for periods specified in the text.

cDNA cloning and plasmid constructions. Molecular cloning of revertant viruses into cDNAs was done by the method described previously (26). After cDNA synthesis, the preparation was double digested with restriction enzymes *Aat*II and *Hin*dIII and an *Aat*II-*Hin*dIII fragment from the revertant cDNA encompassing the viral genomic sequence from nucleotide 1118 to nucleotide 6056 was cloned into the corresponding sites in the pGEM3 vector (Promega Biotec, Madison, Wis.). Plasmid p2C-31 contains a cDNA insert from the parental virus 2C-31, which has an *Eco*RI linker insertion in the 2C-encoding sequence (18). For the construction of pAH-R1, p2C-31 was double digested with *Aat*II and *Hin*dIII (partial) and ligated with the *Aat*II-*Hin*dIII cDNA fragment of 2C-31R1 described above. Further constructions of the plasmids for the mix-and-match analysis were recombinations between p2C-31 and pAH-R1, except for pSpEB-R31 (see text for the construction of pSpEB-R31); pAE-R1, pEH-R1, and pEB-R1 contained an *Aat*II-*Eco*RI (4886), an *Eco*RI-*Hin*dIII, or an *Eco*RI-*Bgl*I (5318)

* Corresponding author.

fragment, respectively, from the 2C-31R1 cDNA, and the rest was p2C-31 sequence.

Virus infections. About 2×10^6 to 5×10^6 HeLa cells were collected from suspension culture and infected with various amounts of virus in 0.2 ml. After adsorption at room temperature for 30 min, fresh medium was added to each infection and they were then incubated at various temperatures for the periods specified in each experiment. At the end of each infection, cells were collected by centrifugation and washed once with cold medium before they were disrupted by several cycles of freeze-and-thaw. The titer of released virus was determined in a standard plaque assay. For study of the uncoating defect of 2C-31R1, infected cell cultures incubated at 32°C were divided into two parts, and at 15 min before each time point, one part was switched to 39°C.

Virus complementation analysis. For the complementation analysis of 2C-31R1 by type 2 poliovirus, the two stocks were used to coinfect HeLa cells at 37°C, with a multiplicity of infection of 10 for each virus. At 8 h postinfection (p.i.), the culture was terminated and a virus stock was prepared from the infected cell lysate. The virus stock was assayed at 32, 37, or 39°C to estimate the relative titer of each virus before it was used in the subsequent uncoating analysis at 32°C (see text).

DNA sequence analysis. Sequence analysis of the revertant cDNA clones was done by the dideoxynucleotide method of Sanger et al. (27) with double-stranded plasmid DNA as the template (10).

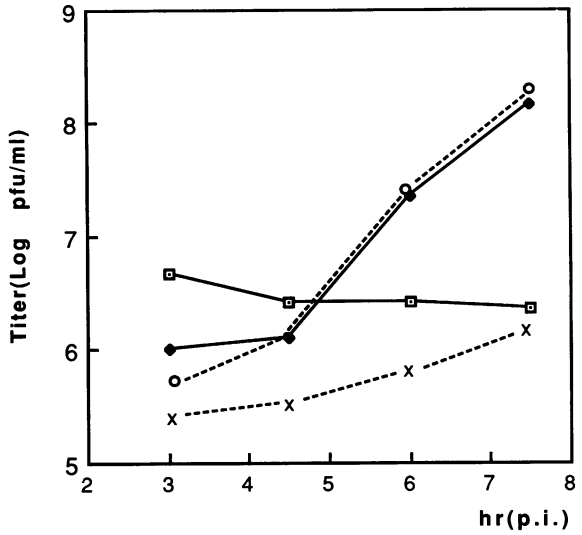

FIG. 1. Single-cycle growth of 2C-31R1 and wild-type virus at 32°C. Each point represents virus recovered from lysates of 2.5×10^6 HeLa cells infected with either virus with a multiplicity of infection of 10. Symbols: —, infections kept at 32°C throughout the entire cycle; ----, infections during which temperature was shifted from 32 to 39°C for 15 min before termination; ○, ◆, wild-type virus infection; ×, ⊡, 2C-31R1 infections.

RESULTS

Isolation of cold-sensitive 2C revertant defective in uncoating. Previously, we reported the isolation of a poliovirus 2C mutant, 2C-31, which was temperature sensitive and defective in viral RNA synthesis (18). When the virus was grown at 39°C, revertants could be obtained at a frequency of 10^{-4} per replication cycle. In contrast to 2C-31, most revertants grew well at 39°C and were not defective in viral RNA synthesis (data not shown). One of them, named 2C-31R1, was cold sensitive. A given stock of this virus gave 20- to 100-fold fewer plaques on HeLa cell monolayers at 32°C than at either 37 or 39°C. In addition, plaques generated at 32°C were much smaller than those of the wild-type virus (data not shown). When a single cycle of growth was analyzed, there was very little yield from 2C-31R1-infected cells even after 7.5 h of incubation at 32°C. However, at earlier time points, the titer of virus obtained from the infected cell lysates was actually higher than that at later points. In fact, the titer was several times higher than that of the wild-type control (Fig. 1, compare the yields at 3 h p.i., for example).

Because the overall growth of 2C-31R1 was much slower than that of the wild type as judged by the plaque size difference of the two at 32°C, and because infectious virus was recovered from the infected cell pellets after low-speed centrifugation and there was less than 10% of total input virus left in the supernatants (data not shown), it seemed possible that 2C-31R1 had a defect in virion uncoating such that the input virus in association with the infected cells remained intact and infectious several hours p.i.

We tested the possibility of an uncoating defect by shifting the infected cells at each time point during a growth curve to the permissive temperature of 39°C for 15 min just before the infection was terminated. If the high numbers of infectious particles recovered from cell lysates during the 2C-31R1 infection were due to a defect in uncoating, by shifting to the permissive temperature, the virions should begin to uncoat and the numbers of infectious particles recovered should be reduced. This treatment, while it had little effect on the yield of wild-type virus, dramatically reduced the number of infectious particles recovered from the 2C-31R1-infected HeLa cells up to 6 h p.i. (Fig. 1), indicating that indeed 2C-31R1 may have an uncoating defect at 32°C.

To further test this possibility, we temperature pulsed recently infected cells by a 30-min exposure to 39°C followed by a 7.5-h incubation at 32°C. If the inability of 2C-31R1 to grow at 32°C was due to a defect in virion uncoating, this brief treatment at the permissive temperature should facilitate the uncoating process and increase the final yield. The temperature shift at the beginning of the infection increased the yield of 2C-31R1 almost 20-fold, while it had little effect on the wild-type virus (Table 1). Thus, the cold sensitivity of 2C-31R1 was very likely due to a defect during virion uncoating.

The uncoating defect of 2C-31R1 is the result of secondary mutations in the 2C-coding sequence. To locate the genetic determinant(s) that caused the defect in uncoating of the 2C-31R1 virions, we partially cloned the 2C-31R1 genome by isolating a cDNA fragment that contained the sequence from nucleotide 1118 to nucleotide 6056. This fragment, when recombined with the rest of the wild-type sequence and

TABLE 1. Effect of temperature shifting at the beginning of infection[a]

Virus	Exptl conditions	Yield (PFU/ml)
Wild type	At 32°C	3×10^8
	Pulsed at 39°C	3.5×10^8
2C-31R1	At 32°C	3×10^6
	Pulsed at 39°C	5.5×10^7

[a] For infection 1, the temperature was kept at 32°C for the entire period of 8 h, and for infection 2, the temperature was first shifted to 39°C for 30 min and then kept at 32°C for the rest of the incubation period.

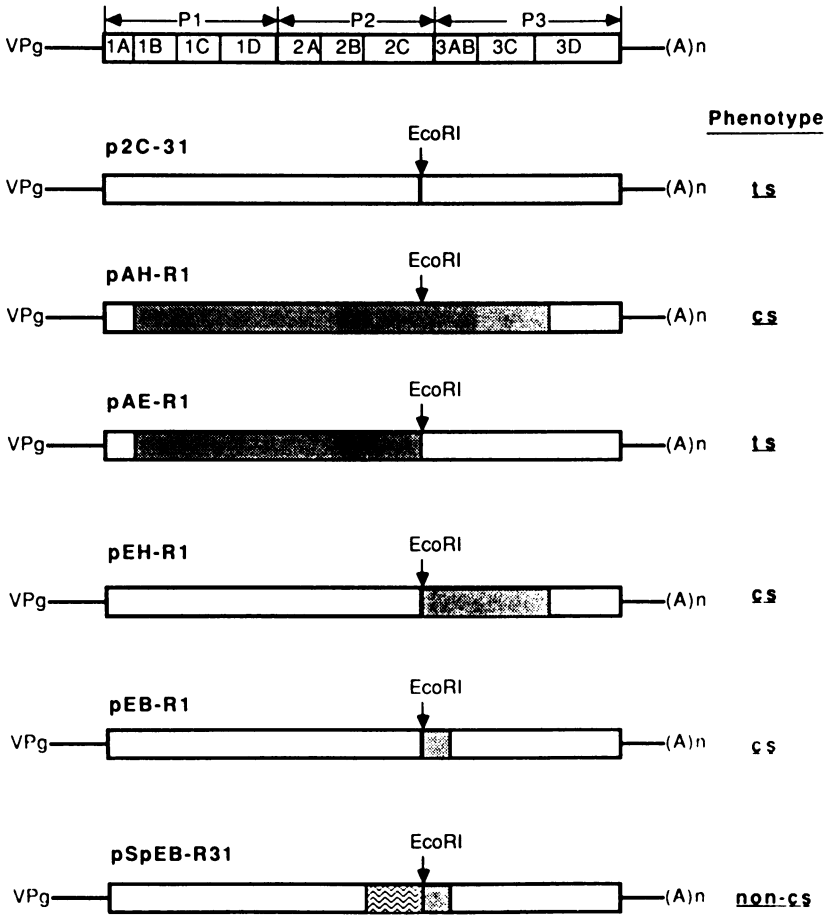

FIG. 2. Mix-and-match recombination analysis. Only the viral cDNA portions of the plasmid constructs are shown. The phenotypes of the viruses derived from HeLa cells transfected with 5 μg of DNA from each construct are indicated at the right of the figure. Regions in boxes represent the coding sequence of the polioviral genome. Empty boxes represent regions of the parental 2C-31 cDNA sequences, with the arrow indicating the location of the original linker insertion (an *Eco*RI linker [18]) in that mutant. Solid boxes represent 2C-31R1 sequences; the box with wavy lines represents 2C-31R3 sequence. The poliovirus genome and the relative locations of various viral proteins in the polyprotein precursor are indicated at the top. *ts*, Temperature sensitive; *cs*, cold sensitive.

transfected into HeLa cells, gave rise to virus indistinguishable from 2C-31R1 (Fig. 2, see the result for plasmid pAH-R1). Restriction enzyme mapping indicated that the original *Eco*RI linker insertion present in the parental 2C-31 genome (18) remained intact in the 2C-31R1 cDNA, indicating that a second-site mutation(s) was the cause of the uncoating defect. The inserted *Eco*RI linker represented a unique site in the viral genome which enabled us to locate the secondary mutation(s) by making plasmid constructs with fragments either upstream or downstream of the linker in a series of mix-and-match recombinations between the 2C-31 and 2C-31R1 sequences. As shown in Fig. 2, for pEB-R1, introduction of an *Eco*RI (4886)-*Bgl*I (5318) fragment of the 2C-31R1 cDNA containing 432 base pairs downstream from the linker into the 2C-31 genome gave rise to virus that was cold sensitive and was also shown to be defective in virion uncoating at 32°C (data not shown). The segment upstream of the R1 site gave rise to temperature-sensitive virus and therefore had no suppression activity (pAE-R1 in Fig. 2). Direct DNA sequence analysis of the *Eco*RI-*Bgl*I fragment revealed only two point mutations at nucleotides 5000 and 5007 that both resulted in conservative amino acid residue changes in the 2C polypeptide sequence (Fig. 3). These changes appeared to be responsible both for the reversion of 2C-31 mutation and for causing the uncoating defect.

The uncoating defect could be suppressed by additional mutation in 2C sequence. Another revertant, 2C-31R3, isolated from the same virus stock from which 2C-31R1 was obtained was not cold sensitive and uncoated well at 32°C (data not shown). When its cDNA sequence was analyzed, it was found that it contained the sequence identical to that of 2C-31R1 downstream of the *Eco*RI linker site and could confer the same cold-sensitive–uncoating defect phenotype when recombined with the rest of 2C-31 sequence (data not shown). Apparently, it contained a sequence(s) elsewhere that could suppress the determinant of the uncoating defect. Indeed, when an *Sph*I (4154)-*Eco*RI (4886) cDNA fragment of 2C-31R3 was recombined with the *Eco*RI-*Bgl*I fragment from 2C-31R1 cDNA, the cold-sensitive phenotype was suppressed and the virus obtained had the same phenotype as that of 2C-31R3 (Fig. 2, see the result of pSpEB-R31). DNA sequence analysis indicated that a single point mutation at nucleotide 4542 of the 2C-coding sequence resulting in an asparagine-to-serine residue change (Fig. 3) was sufficient to revert the uncoating defect caused by mutations in the 2C-31R1 genome.

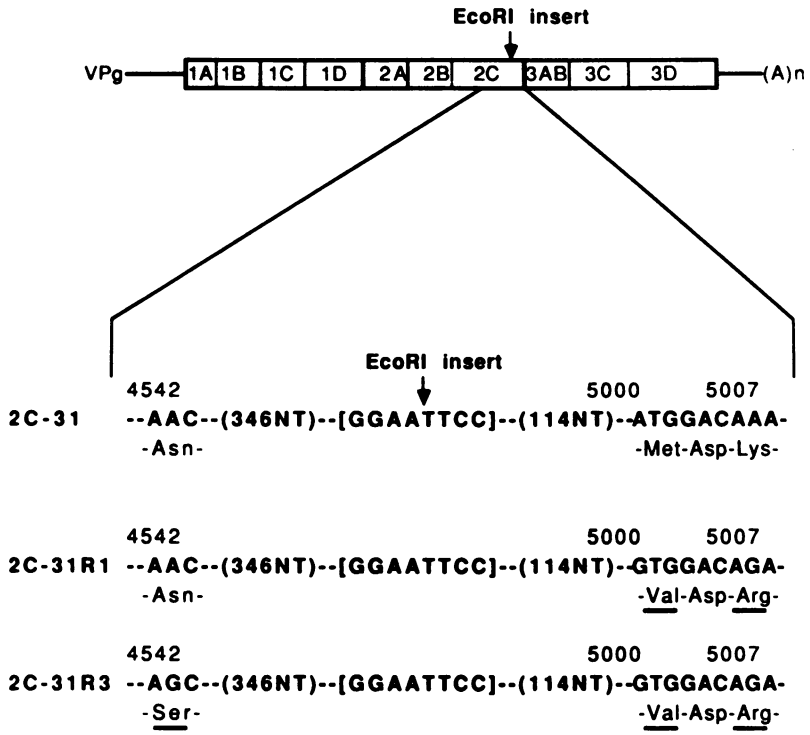

FIG. 3. Secondary mutations found in the revertant genomes. The arrow indicates the location of an *Eco*RI linker insertion in the parental 2C-31 genome. Numbers above the sequence indicate the locations of secondary point mutations found in either 2C-31R1 or 2C-31R3 cDNA, and the resulting amino acid residue changes are underlined.

Complementation of 2C-31R1 by type 2 poliovirus. To determine whether the uncoating defect of 2C-31R1 could be complemented by another poliovirus, we coinfected HeLa cells with 2C-31R1 and poliovirus type 2 under conditions permissive for both viruses and then harvested the virus from the coinfection and analyzed the uncoating of the virus in a subsequent infection done at 32°C. We reasoned that if the uncoating defect of 2C-31R1 could be corrected by the presence of type 2 poliovirus during the first round of infection, then there should be no significant amount of 2C-31R1 blocked at the uncoating stage during the subsequent infection at 32°C. On the other hand, if the defect resided in the RNA and not in the 2C protein and therefore could not be complemented, or if the defect had a dominant negative effect, then there should be a large amount of either 2C-31R1 alone or 2C-31R1 and type 2 blocked at uncoating. We chose type 2 poliovirus as the partner for the following reasons. First, it grows at about the same rate as 2C-31R1 at 37°C, a temperature that is relatively permissive for both viruses and at which the first round of coinfection could be carried out. This is important because the ideal helper virus should replicate well during coinfection to provide enough 2C protein but not well enough to outgrow 2C-31R1. Second, type 2 poliovirus has a partial temperature sensitivity such that at 39°C it forms a reduced number of plaques with a distinctive small-plaque morphology. Incubation at different temperatures thus provided a convenient way to distinguish the progeny carrying the type 2 genome from that with 2C-31R1 in the mixed virus stock harvested from the coinfection (see below). Third, type 2 recombines poorly with type 1, minimizing the confounding effects of recombination.

When equal numbers of type 2 and 2C-31R1 were used to coinfect HeLa cells, the virus harvested from the culture 7 h p.i. at 37°C contained about 60% 2C-31R1 and 40% type 2 poliovirus. This calculation was based on the observation that both the cold-sensitive 2C-31R1 and the temperature-sensitive type 2 poliovirus had a titer reduction of more than 20-fold when assayed at either 32 or 39°C, respectively (Table 2, lines 1 and 2). Therefore, in the mixed yield from coinfection, the titer at 39°C will represent mainly 2C-31R1 and the titer at 32°C will represent mainly type 2 poliovirus. The mixed yield had 2.3×10^8 PFU at 32°C (type 2) and 4×10^8 PFU at 39°C (2C-31R1). This was confirmed by noting that in the assay for the mixed virus, almost all plaques formed at 32°C had a morphology similar to that of type 2 virus, while all plaques formed at 39°C were similar to those of 2C-31R1 (data not shown).

When this virus stock was used in the second round of infection carried out at 32°C, only about 2 to 3% of the total input virus could be recovered as infectious particles from the cell lysate 3 h p.i., a value similar to that observed for type 2 or the wild-type (type 1) virus (Fig. 1), while over 30% of the total input virus could be recovered from cells infected with 2C-31R1 alone (Table 3). This clearly indicated that the

TABLE 2. Yield of virus from single-cycle infections at 37°C[a]

Virus	Titer (PFU/ml) at:		
	32°C	37°C	39°C
2C-31R1	2×10^7	5×10^8	5×10^8
Type 2	4×10^8	2.2×10^8	1×10^7
2C-31R1 + type 2	2.3×10^8	5×10^8	4×10^8

[a] Titers of viruses recovered from lysates of 2×10^6 HeLa cells were determined by the standard plaque assay at different temperatures.

TABLE 3. Complementation of 2C-31R1 by type 2 poliovirus[a]

Virus	Titer (PFU/ml)[b] at:	
	32°C	39°C
2C-31R1		8.6×10^6 (8.2×10^5)
Type 2	8×10^5 (1.8×10^5)	
2C-31R1 + type 2	2×10^5 (2×10^4)	6×10^5 (6×10^4)

[a] About 5×10^6 HeLa cells were infected with each virus preparation (Table 2), with a multiplicity of infection of 10.

[b] Numbers represent virus recovered from one-half of the culture that was kept either at 32°C or shifted to 39°C for 15 min (in parentheses) before terminating the infection.

TABLE 4. Physical properties of 2C-31R1 virions[a]

Virus	Titer (PFU/ml)			
	Temp		pH	
	22°C	43°C	7.0	5.0
Wild type	6.5×10^7	1.5×10^7	6.0×10^7	6.6×10^7
2C-31R1	1.0×10^8	3.6×10^7	9.6×10^7	6.6×10^7

[a] About 10^8 virions were kept either at different temperatures for 15 min or at a different pH for 1 h. The titer was then determined by a standard plaque assay.

2C-31R1 progeny from the coinfection with type 2 poliovirus were able to uncoat in the subsequent infection at 32°C. Therefore, the genetic determinant of the uncoating defect could be complemented and did not reside in the RNA of 2C-31R1 but rather in the 2C protein itself.

The capsid proteins of 2C-31R1 are properly processed and myristoylated. When the 2C-31R1 virions were labeled with [^{35}S]methionine, the cleavage pattern of various virion proteins appeared normal on sodium dodecyl sulfate-polyacrylamide gels (Fig. 4A). It is known that poliovirus capsid protein VP4 and its precursor, VP0, are myristoylated (11). The same modification was found for the 2C-31R1 capsid (Fig. 4B).

Physical properties of 2C-31R1 virions. Poliovirus virions are relatively heat stable and resistant to mild acid treatment. When the purified 2C-31R1 virions were subjected to mild heat (43°C, 15 min) or acid (pH 5, 1 h) treatment, they showed a degree of resistance similar to that of the wild type (Table 4).

DISCUSSION

The previously described poliovirus mutant 2C-31 is defective in viral RNA synthesis (18). It is surprising, therefore, that revertant 2C-31R1 became defective in virion uncoating. 2C-31R1 contained secondary mutations in the 2C-coding sequence that resulted in two conservative amino acid residue changes in the polypeptide sequence downstream of the original mutation. These changes in the 2C sequence apparently resulted in the defect in virion uncoating. Because protein 2C was not found in the virions and because the defect of uncoating could be complemented, the results imply that protein 2C, in addition to its function in viral RNA synthesis, plays a role in determining virion structure. How 2C might be involved in this process is not clear.

Previous data suggested that protein 2C is tightly associated with the membrane-bound viral replication complex in poliovirus-infected cells (4, 5, 29) and that it may be involved in the initiation of viral RNA synthesis (8, 9, 18, 30). Because only the newly made viral RNAs are incorporated into virions (6), it has been postulated that viral RNA replication and virion formation are coupled processes which occur in association with membranes (6, 7). One of the functions of 2C, therefore, could be to facilitate virion assembly around newly made viral RNA. This could be either to help the virion building blocks to recognize the potential packaging signal associated with the viral RNA or to provide a proper environment for the virion assembly. It was found that the assembly of the 14S subunits into 74S empty capsids in vitro could be facilitated by membrane fractions isolated from poliovirus-infected cells (23), a finding which has been interpreted as supporting the hypothesis that there is a membrane-associated virus-specific assembly factor.

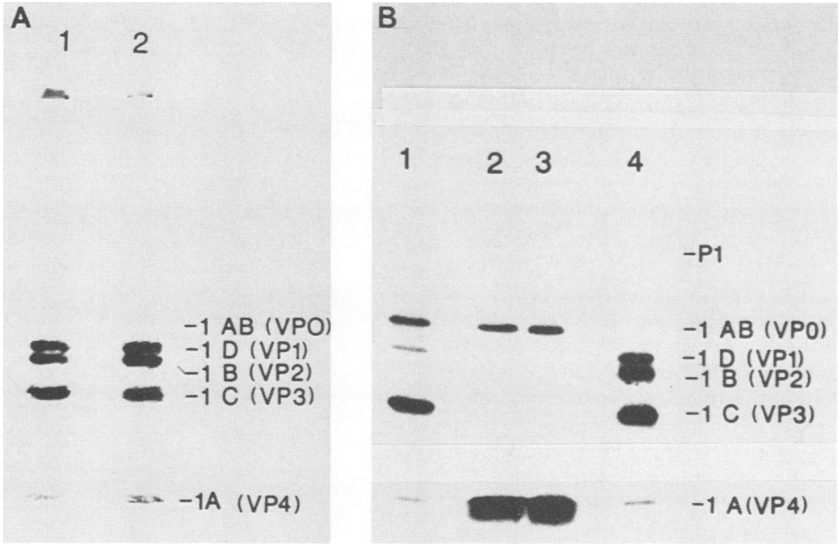

FIG. 4. Cleavage and myristoylation of the capsid proteins. (A) [^{35}S]methionine-labeled virions were purified from a 10 to 30% sucrose gradient and analyzed by electrophoresis through a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Lane 1, Wild type; lane 2, 2C-31R1. (B) Lysates of [^{3}H]myristic acid-labeled HeLa cells that were infected with either wild-type (lane 2) or 2C-31R1 (lane 3) virus were analyzed through a similar gel as in panel A. [^{35}S]methionine-labeled wild-type virions (lane 4) or infected cell lysate (lane 1) were included as controls.

Alternatively, 2C may provide some modification(s) for the capsid proteins that may be required for the maturation of the virions. The only known modification of the picornaviral capsid is the myristoylation of VP4 and its precursor, VP0 (11). While the role of this myristoylation in either virion assembly or uncoating is unknown, the same myristoylation pattern was found for the 2C-31R1 capsids (Fig. 4B) and therefore a defect in myristoylation does not appear to account for the uncoating defect of 2C-31R1 virions.

The nature of the uncoating defect of the 2C-31R1 virions is not clear. Because almost all the virus became tightly associated with cells within the first hour of infection and there was little virus left in the culture medium that could be recovered as infectious particles (data not shown), the potential alteration(s) that affects virion uncoating apparently does not affect the binding of virions to the cellular receptors. Poliovirions are known to be relatively heat stable and resistant to mild acidity, and the 2C-31R1 virions appear to have retained these properties (Table 4).

It is not clear whether the reversion of the temperature-sensitive phenotype was caused by one or both of the secondary point mutations found in the 2C-31R1 genome or how these mutations contribute to the uncoating defect. Site-directed mutagenesis with synthetic oligonucleotides containing one or both mutations is under way to address these questions.

Although 2C-31R3 was isolated from the same parental 2C-31 stock from which 2C-31R1 was obtained, it is likely that 2C-31R3 is a secondary revertant derived from 2C-31R1. The 2C-31 stock had been through a few passages at 32°C, which might have facilitated the generation of secondary revertants from 2C-31R1.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant AI 22346 from the National Institutes of Health.

LITERATURE CITED

1. **Argos, P., G. Kamer, M. Nicklin, and E. Wimmer.** 1984. Similarly 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**:725–727.
2. **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.
3. **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.
4. **Bienz, K., D. Egger, Y. Rasser, and W. Bossart.** 1983. Intracellular distribution of poliovirus proteins and the induction of virus-specific cytoplasmic structures. Virology **131**:39–48.
5. **Butterworth, B. E., E. Shimshick, and F. H. Yin.** 1976. Association of the polioviral RNA polymerase complex with phospholipid membranes. J. Virol. **19**:457–466.
6. **Caliguiri, L. A., and R. W. Compans.** 1973. The formation of poliovirus particles in association with the RNA replication complexes. J. Gen. Virol. **21**:99–108.
7. **Caliguiri, L. A., and A. G. Mosser.** 1971. Proteins associated with the poliovirus RNA replication complex. Virology **46**: 375–386.
8. **Caliguiri, L. A., and I. Tamm.** 1968. Action of guanidine on the replication of poliovirus RNA. Virology **35**:408–417.
9. **Caliguiri, L. A., and I. Tamm.** 1973. Guanidine and 2-(β-hydroxybenzyl)benzimidazole (HBB): selective inhibitors of picornavirus multiplication, p. 254–294. *In* W. Carter (ed.), Selective inhibitors of viral functions. CRC Press, Inc., Boca Raton, Fla.
10. **Chen, E. Y., and P. H. Seeburg.** 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA **4**:165–170.
11. **Chow, M., J. Newman, D. Filman, J. M. Hogle, D. Rowlands, and F. Brown.** 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature (London) **327**: 482–486.
12. **Crowell, R. L., and B. J. Landau.** 1979. Receptors as determinants of cellular tropism in picornavirus infections, p. 1–33. *In* A. G. Bearn and P. W. Choppin (ed.), Receptors and human diseases. Josiah Macy Jr. Foundation, New York.
13. **Crowell, R. L., and B. J. Landau.** 1983. Receptors in the initiation of picornavirus infections. Compr. Virol. **18**:1–24.
14. **Franssen, H., J. Leunissen, R. Goldbach, G. Lomonossoff, and D. Zimmern.** 1984. Homologous sequences in non-structural proteins from cowpea mosaic virus and picornaviruses. EMBO J. **3**:855–861.
15. **Guttman, N., and D. Baltimore.** 1977. A plasma membrane component able to bind and alter virions of poliovirus type 1: studies on cell-free alteration using a simplified assay. Virology **82**:25–36.
16. **Jacobson, M. F., J. Asso, and D. Baltimore.** 1970. Further evidence on the formation of poliovirus proteins. J. Mol. Biol. **49**:657–669.
17. **Jacobson, M. F., and D. Baltimore.** 1968. Polypeptide cleavages in the formation of poliovirus proteins. Proc. Natl. Acad. Sci. USA **61**:77–84.
18. **Li, J.-P., and D. Baltimore.** 1988. Isolation of poliovirus 2C mutants defective in viral RNA synthesis. J. Virol. **62**:4016–4021.
19. **Lonberg-Holm, K., L. B. Gosser, and J. C. Kauer.** 1975. Early alteration of poliovirus in infected cells and its specific inhibition. J. Gen. Virol. **27**:329–342.
20. **Madshus, I. H., S. Olsenes, and K. Sandvig.** 1984. Mechanism of entry into the cytosol of poliovirus type 1: requirement for low pH. J. Cell Biol. **98**:1194–1200.
21. **Mendelsohn, C. L., E. Wimmer, and V. R. Racaniello.** 1989. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell **56**:855–865.
22. **Pallansch, M. A., O. M. Kew, B. L. Semler, D. R. Omilianowsky, C. W. Anderson, E. Wimmer, and R. R. Rueckert.** 1984. Protein processing map of poliovirus. J. Virol. **49**:873–880.
23. **Perlin, M., and B. A. Phillips.** 1973. *In vitro* assembly of polioviruses. III. Assembly of 14S particles into empty capsids by poliovirus-infected HeLa cell membranes. Virology **53**:107–114.
24. **Pincus, S., D. Diamond, E. Emini, and E. Wimmer.** 1986. Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. J. Virol. **57**:638–646.
25. **Racaniello, V. R.** 1984. Poliovirus type II produced from cloned cDNA is infectious in mice. Virus Res. **1**:669–675.
26. **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.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
28. **Summers, D. F., and J. V. Maizel.** 1968. Evidence for large precursor proteins in poliovirus synthesis. Proc. Natl. Acad. Sci. USA **59**:966–971.
29. **Takegami, T., B. L. Semler, C. W. Anderson, and E. Wimmer.** 1983. Membrane fractions active in poliovirus RNA replication contain VPg precursor polypeptides. Virology **128**:22–47.
30. **Tershak, D. R.** 1982. Inhibition of poliovirus polymerase by guanidine in vitro. J. Virol. **41**:313–318.