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Cold-Adapted Poliovirus Mutants Bypass a Postentry Replication Block

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In the current model of poliovirus entry, the initial interaction of the native virion with its cellular receptor is followed by a transition to an altered form, which then acts as an intermediate in viral entry. While the native virion sediments at 160S in a sucrose gradient, the altered particle sediments at 135S, has lost the coat protein VP4, and has become more hydrophobic. Altered particles can be found both associated with cells and in the culture medium. It has been hypothesized that the cell-associated 135S particle releases the viral genome into the cell cytoplasm and that nonproductive transitions to the 135S form are responsible for the high particle-to-PFU ratio observed for polioviruses. At 25°C, a temperature at which the transition to 135S particles does not occur, the P1/Mahoney strain of poliovirus was unable to replicate, and cold-adapted (*ca*) mutants were selected from the population. These mutants have not gained the ability to convert to 135S particles at 25°C, and the block to wild-type (*wt*) infection at low temperatures is not at the level of cellular entry. The particle-to-PFU ratio of poliovirus does not change at 25°C in the absence of alteration. Three independent amino acid changes in the 2C coding region were identified in *ca* mutants, at positions 218 (Val to Ile), 241 (Arg to Ala), and 309 (Met to Val). Introduction of any of these mutations individually into *wt* poliovirus by site-directed mutagenesis confers the *ca* phenotype. All three serotypes of the Sabin vaccine strains and the P3/Leon strain of poliovirus also exhibit the *ca* phenotype. These results do not support a model of poliovirus entry into cells that includes an obligatory transition to the 135S particle.

Studies on the mechanisms by which viruses enter their host cells provide clues about how a virus capsid can be stable enough to survive harsh conditions in the environment yet able to release its contents (the virus genome) when it encounters a susceptible cell. The evolutionary diversity of viruses virtually guarantees that the initial interactions between different viruses and host cells will vary dramatically. While detailed descriptions of cell entry have been established for influenza virus, adenovirus, and Semliki Forest virus (reviewed in reference 18), this level of understanding remains the exception rather than the rule. Remarkable progress has been made in recent years toward understanding the process of picornavirus entry, yet there are still several important questions which remain unanswered.

Research on the entry of picornaviruses into cells has been facilitated by the identification of cellular receptors for poliovirus (34), several echovirus serotypes (3, 4) and the major group human rhinoviruses (19, 52), and the elucidation of the three-dimensional structures of poliovirus (14, 22), rhinovirus (49), foot-and-mouth disease virus (1), mengovirus (31), Theiler's virus (30), and coxsackievirus B3 (39). A low-resolution image of rhinovirus complexed with its cell receptor provides clues about the initial interactions of these viruses with cells (41). Among picornaviruses, the mechanism of entry of poliovirus has been the most extensively studied (reviewed in reference 43).

Successful poliovirus entry into cells is mediated by the poliovirus receptor (32, 34). After binding to the poliovirus receptor on the cell surface, the virus undergoes a major structural change which results in the formation of altered particles, which can be distinguished from the native virion by sedimentation in a sucrose gradient. While the intact native virion sediments at 160S, the altered particle sediments at 135S and has lost the capsid protein VP4 and extruded the hydrophobic N terminus of VP1. The timing of alteration coincides with virus genome entry (15, 17, 55). In a typical infection, not all particles undergo this transition, but quantitative conversion is possible under the proper conditions (54). Alteration and virus entry can be blocked by antiviral compounds such as arildone and disoxaril, and drug-resistant viral mutants are capable of undergoing alteration in the presence of the inhibitors (6, 16, 38, 56). Altered particles contain full-length RNA (10) and can initiate a productive infection when very high concentrations of particles are used (9). These results have been interpreted to mean that the altered particle is an essential intermediate in virus entry (reviewed in reference 15). A less drastic change in the virus's structure, called breathing, which occurs in the absence of cell receptor, has also been implicated in the uncoating process, though its role remains unknown (27).

After undergoing alteration, some particles remain cell associated, while others are shed into the surrounding medium (13, 57). This phenomenon has been suggested as an explanation for the low plaque-forming efficiency of picornaviruses, typically 0.1 to 2% (50). Since a single picornavirus RNA genome microinjected into a cell is capable of initiating a productive round of replication, the particle/PFU ratio appears to result from a defect at the level of entry (2). Productive infection may result from uncoating of the RNA at the cell surface (20) or from within an endosomal compartment (57).

Previous studies on poliovirus entry have used the generation of altered particles as a measure of uncoating. Since all virus particles can undergo alteration but only a minority can successfully enter the cell, simply assaying for virus alteration will not provide a reliable indicator of the location and timing of entry. We therefore attempted to analyze virus entry at

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25°C, a temperature at which virus alteration does not occur (55). In the course of these experiments, we isolated a novel class of cold-adapted (*ca*) mutants of poliovirus P1/Mahoney which have gained the ability to infect cells at 25°C. *ca* mutants do not undergo alteration to 135S particles at 25°C. Analysis of wild-type (*wt*) and mutant viruses demonstrates that *wt* virus fails to replicate at 25°C because of a postentry block in infection and that *ca* mutants bypass this defect with a mutation in the 2C gene, which encodes a nonstructural protein. We propose that the 135S particle is not an essential intermediate in the cellular entry of poliovirus. Poliovirus *ca* mutants provide a novel tool for studying the intracellular events in the poliovirus life cycle.

MATERIALS AND METHODS

Cells and viruses. HeLa S3 cells were grown in suspension culture in Joklik minimal essential medium supplemented with 5% bovine calf serum and 10 μg of gentamicin sulfate per ml. For monolayer growth, cells were transferred to Dulbecco's modified Eagle medium with 10% bovine calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (Gibco BRL).

Poliovirus strains P1/Mahoney, P2/Lansing, P3/Leon, P2/Sabin, and P3/Sabin were derived by transfecting HeLa cell monolayers with viral RNA transcribed from cloned genomic cDNAs (37, 44, 45, 51). An infectious P1/Sabin cDNA was a gift from A. Macadam, National Institute for Biological Standards and Control, South Mimms, England. Virus stocks were prepared by infecting HeLa cell monolayers with plaque-purified virus isolates and harvesting cells and supernatant 24 h later. Cells were then lysed by three consecutive freeze-thaw cycles, and cellular debris was removed by centrifugation at 5,000 rpm and 4°C in a Sorvall RT6000 tabletop centrifuge. Stocks were then stored in 50- to 500-μl aliquots at −70°C. Viral titers were determined by plaque assays on HeLa cell monolayers as described previously (46).

Alteration assays. Alteration assays were performed substantially as described previously (8). Virus was grown in the presence of [^{35}S]methionine (Amersham), harvested as for a viral stock (see above), and then pelleted at 40,000 rpm and 10°C in an SW41 rotor (Beckman). The pellet was resuspended and then centrifuged through a 7.5 to 45% sucrose gradient for 75 min at 10°C and 40,000 rpm in an SW41 rotor. Gradients were fractionated, samples of each fraction were counted in a scintillation counter, and the peak fractions were pooled and dialyzed against phosphate-buffered saline (PBS). The purified virus was adjusted to 5 mg of bovine serum albumin (fraction V; Sigma) per ml and stored at −70°C. Then 4×10^8 PFU of labeled virus was incubated with 10^7 HeLa S3 cells overnight in 0.5 ml of PBS at 4°C. Cells were pelleted by centrifugation for 5 min at 7,000 rpm and 4°C in an Eppendorf 5415C microcentrifuge, washed twice with PBS at 4°C, and then resuspended in a total volume of 250 μl of PBS equilibrated to either 25 or 37°C (see Results). After the indicated time, 20 μl of 10× Nonidet P-40 cell lysis buffer (5% Nonidet P-40 and 200 mM HEPES in 1× PBS) was added, and the cell lysate was clarified by centrifugation for 5 min at 4°C and 15,000 rpm in an Eppendorf microcentrifuge. The supernatant was loaded onto a 15 to 30% sucrose gradient and centrifuged in an SW41 rotor at 40,000 rpm for 75 min at 10°C. Fractions were collected from the gradient and counted as described above, and the results were plotted. Gradient markers were made by heating labeled P1/Mahoney poliovirus at 56°C for 10 min, cooling on ice for 20 min, and then adding an equal quantity of unheated labeled virus.

Temperature shift and transfection growth curves. For temperature shift growth curves, HeLa S3 cells in monolayers were infected at a multiplicity of infection (MOI) of 10 with P1/Mahoney *wt* or *ca* viruses, which were allowed to adsorb to the cells for 45 min at 25°C; the cells were then washed twice with PBS and overlaid with Dulbecco's modified Eagle medium which had been equilibrated to 25°C. Plates were then placed at 37°C for 0, 1, 2, or 4 h before being shifted to 25°C. After the shift, samples of the cell supernatant were taken at different times, and viral titers were determined by plaque assays.

For transfection growth curves, HeLa S3 cells in monolayers were transfected at 25°C with viral RNA transcribed in vitro from genomic cDNA (25). Cells were then incubated at 25°C, and samples of the supernatant were taken and processed as described above.

Identification of *ca* mutations. Viral cDNA was prepared by a modification of a method described previously (7). HeLa S3 cells in monolayer culture on 15-cm-diameter dishes were infected with stocks of plaque-purified *ca* mutants. Cells and medium were collected after 24 h, subjected to three cycles of freezing and thawing, and then clarified by centrifugation at 5,000 rpm and 4°C in a Sorvall RT6000 tabletop centrifuge; 0.5 ml of 0.5 M EDTA (pH 8.0) was added to the resulting supernatant, which was then loaded on top of a 0.5-ml cushion of 30% sucrose in NTE buffer (100 mM NaCl, 50 mM Tris-HCl [pH 8.0], 10 mM EDTA). This mixture was centrifuged at 40,000 rpm for 2 h at 4°C, and the viral pellet was resuspended in 0.5 ml of NTE buffer and 0.5% sodium dodecyl sulfate. Proteinase K was added to a final concentration of 50 μg/ml, and the sample was incubated at 37°C for 1 h. After phenol extraction, the viral RNA was precipitated with 3 M sodium acetate (pH 6.0) and 2 volumes of 95% ethanol and then centrifuged at 15,000 rpm in an Eppendorf microcentrifuge at 4°C for 15 min. The resulting pellet was washed with 70% ethanol and resuspended in diethylpyrocarbonate-treated distilled H_2O.

The cDNA synthesis reaction mixture was incubated for 30 min at 37°C in a 50-μl volume containing 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM each dGTP, dATP, dTTP, and dCTP, 80 U of RNAsin (Promega Corp.), 400 U of SuperScript II reverse transcriptase (Gibco), and 100 ng of oligo(dT). The cDNA was then used as a template for PCR with Vent DNA polymerase (New England Biolabs) according to the manufacturer's protocol. The primers used were as follows: for r1 recombinant construction, a positive-sense primer complementary to nucleotides 332 to 352 and a negative-sense primer complementary to nucleotides 3409 to 3428; for r2 recombinant construction, a positive-sense primer complementary to nucleotides 3409 to 3428 and a negative-sense primer complementary to nucleotides 5602 to 5621; and for r3 recombinant construction, a positive-sense primer complementary to nucleotides 5581 to 5600 and a negative-sense primer complementary to nucleotides 7052 to 7071. The PCR product in each case was cloned into the vector pT7MSacII (28) which had been digested with *Age*I and *Sac*II (for r1), *Sac*II and *Bgl*II (for r2), or *Bgl*II and *Pvu*II (for r3) before being treated with shrimp alkaline phosphatase (United States Biochemical). Clones carrying the desired inserts were identified by restriction endonuclease cleavage and dideoxynucleotide sequencing with a Sequenase kit (United States Biochemical) by the manufacturer's protocol.

cDNAs of r1, r2, and r3 were transcribed in vitro and transfected into HeLa S3 cells in monolayer culture, and virus stocks were harvested as described above. A minimum of nine clones of each recombinant, representing three different plaque-purified isolates from the original *ca* stock, were tested for growth at 25°C. The cloned regions of the r2 chimeras were sequenced in their entirety by using a Sequenase kit and the manufacturer's protocol.

Site-directed mutagenesis. Mutagenic oligonucleotides were designed to insert the GC4844CG (*ca*2), C4775T (*ca*-3), and T5048C (*ca*-4) mutations and synthesized by the Columbia University DNA Facility. These oligonucleotides were then used for PCR-based site-directed mutagenesis as described previously (21). In the first PCR, full-length P1/Mahoney cDNA was amplified with a nonmutagenic positive-sense primer annealing to nucleotides 3410 to 3429 and a mutagenic negative-sense primer annealing to nucleotides 4833 to 4854 (*ca*-2), 4764 to 4785 (*ca*-3), or 5035 to 5056 (*ca*-4). In the second PCR, full-length P1/Mahoney cDNA was amplified with a nonmutagenic negative-sense primer annealing to nucleotides 5602 to 5619 and a mutagenic positive-sense primer annealing to nucleotides 4833 to 4854 (*ca*-2), 4764 to 4785 (*ca*-3), or 5035 to 5056 (*ca*-4). In the third PCR, the products of the first and second reactions for each mutation were used as the template with a nonmutagenic positive-sense primer annealing to nucleotides 3410 to 3429 and a nonmutagenic negative sense primer annealing to nucleotides 5602 to 5619. All reactions were performed with Vent DNA polymerase (New England Biolabs) by the manufacturer's protocol. The final PCR products were digested with *Sac*II and *Bgl*II and cloned into the full-length viral cDNA in the pT7MSac vector as described above. The nucleotide sequence of the entire exchanged fragment was determined by dideoxynucleotide sequencing with a Sequenase kit by the manufacturer's protocol. cDNAs containing only the introduced mutation were used in further studies.

Mutagenized cDNAs were transcribed in vitro, RNAs were transfected into HeLa S3 cells, and viral stocks were produced and tested for cold adaptation as described above.

Assay of viral titers at 25°C. Fluorescent focus assays were performed by infecting HeLa S3 cells in monolayer culture in 35-mm-diameter dishes with dilutions of the viral stock to be tested. The virus was allowed to adsorb for 45 min at room temperature before being overlaid with Dulbecco's modified Eagle medium with 10% bovine calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml and incubated at either 25 or 37°C for the indicated time. Cells were then washed twice with PBS, fixed with 90% methanol, washed twice more with PBS, and stored at 4°C if necessary. Virus replication was detected by adsorbing a rabbit anti-P1/Mahoney antibody (33) to the dishes for 30 min at 25°C, washing twice with PBS, then adding goat anti-rabbit antiserum (Boehringer Mannheim catalog no. 605 210), incubating for 30 min at 25°C, washing twice with PBS, and viewing under a Leitz Laborlux D fluorescence microscope. Fluorescent cells per field were counted, and the number of fluorescence-forming units per milliliter was calculated.

For temperature shift plaque assays, HeLa S3 cells in monolayer culture on 35-mm-diameter dishes were infected as for a fluorescence focus assay, but plates were instead overlaid with Dulbecco's modified Eagle medium containing 5% bovine calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 3% methylcellulose. Dishes were then incubated at 25°C for 48 h to allow the virus to infect the cells and begin replication. Infected cells were then moved to 37°C for 72 h to allow plaque formation. The overlay was removed, and cells were fixed with methanol and then stained with crystal violet to visualize plaques.

RESULTS

***ca* mutants arise at 25°C.** Because the formation of 135S altered particles does not occur at temperatures below 33°C (20, 55), we determined whether poliovirus could replicate at lower temperatures. Cells were infected with poliovirus P1/

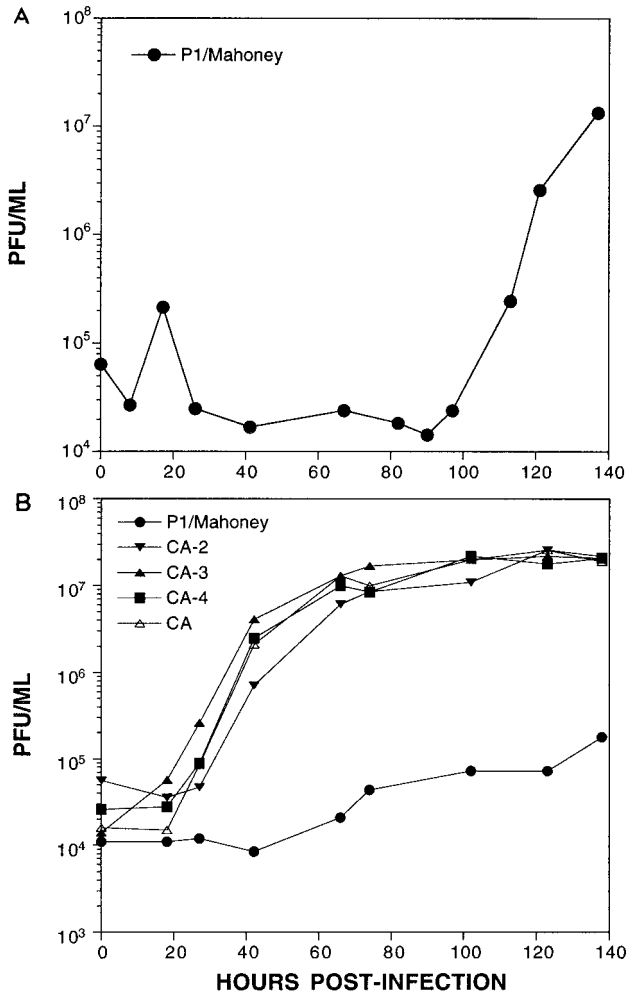

FIG. 1. Growth curve analysis at 25°C. (A) HeLa cells were infected at an MOI of 10 with *wt* P1/Mahoney and incubated at 25°C. At the indicated times postinfection, samples of cell supernatant were removed and the viral titer was determined by plaque assay. (B) HeLa cells were infected at an MOI of 10 with *wt* (●), *ca* from the final time point in panel A (△), or triple-plaque-purified *ca* stock *ca*-2 (▼), *ca*-3 (▲), or *ca*-4 (■) and incubated at 25°C. Viral titers at the indicated times were determined by plaque assay.

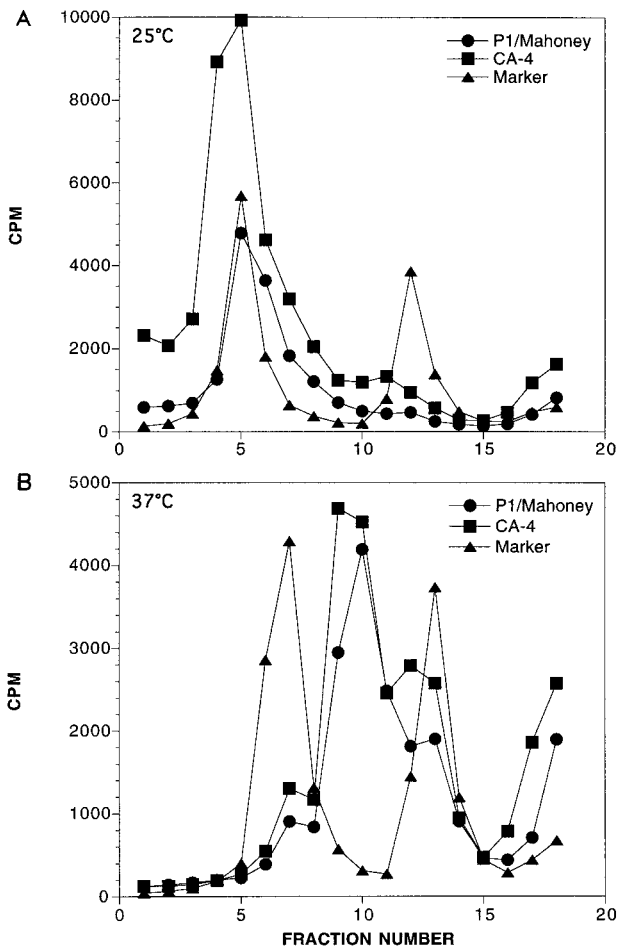

FIG. 2. Sucrose gradient assay for conversion to 135S particles. (A) ^{35}S-labeled *wt* or *ca*-4 virus was adsorbed to HeLa cells in suspension at 4°C, and excess virions were washed off. Cells were incubated at 25°C for 24 h, and then cells and medium were treated with detergent and loaded onto a sucrose gradient. Markers for 160S and 80S viral particles were prepared as described in Materials and Methods. After centrifugation, gradients were fractionated and the fractions were assayed in a liquid scintillation counter. (B) ^{35}S-labeled *wt* or *ca*-4 P1/Mahoney was adsorbed to HeLa cells in suspension at 4°C, and excess virions were washed off. Cells were incubated at 37°C for 2 h, and alteration was assayed as described above. Sedimentation is from right to left.

Mahoney at an MOI of 10 and incubated at 25°C. The resulting growth curve showed no virus replication until 120 h postinfection (Fig. 1A), which suggested the emergence of a mutant population. The cell supernatant from the final time point in this experiment was used in a second round of infection at 25°C, and the resulting growth curve (Fig. 1B) demonstrates the presence of mutants adapted for growth at the lower temperature. The cell supernatant from the final time point of Fig. 1B was subjected to triple plaque purification at 37°C. Growth curve analysis at 25°C of three representative plaque-purified stocks indicates that the *ca* phenotype has been maintained (Fig. 1B). The plaque sizes of *ca* mutants were similar at 37 and 39.5°C, and viral yields at the two temperatures were similar, indicating that *ca* mutants are not temperature sensitive (11). *ca* mutants grow as well as *wt* virus in both high- and low-MOI growth curve experiments at 37°C (11). These results indicate that *ca* mutants have retained the ability to grow at physiological temperatures.

***ca* mutants do not undergo transition to 135S particles at 25°C.** One explanation for the ability of *ca* mutants to replicate at 25°C is that they might undergo the transition to 135S particles at this temperature. To test this hypothesis, alteration assays were performed on *wt* and *ca* viruses at 37 and 25°C for times ranging from 30 min to 36 h (the latter point at which new *ca* virus is produced at 25°C). The results indicate that both *wt* and *ca* viruses undergo alteration at 37°C but not at the lower temperature (Fig. 2), suggesting that virus entry occurs at 25°C in the absence of the transition to 135S particles.

TABLE 1. Temperature shift plaque assay

Temp (°C)[a]	PFU/ml	
	P1/Mahoney *wt*	P1/Mahoney *ca*
37	4.6×10^8	2.4×10^8
25→37	4.8×10^7	2.3×10^7

[a] Infected cells were incubated at 37°C for 72 h or at 25°C for 48 h and then shifted to 37°C for 72 h.

TABLE 2. Fluorescent focus assay

Virus assayed	Temp (°C)	Time (h) post-infection	Apparent titer (FFU/ml)
P1/Mahoney *wt*	37	6	6.0×10^8
	37	8	1.8×10^9
ca grown at 37°C	37	6	4.0×10^8
	37	8	1.6×10^9
ca grown at 25°C	37	6	1.2×10^8
	37	8	4.2×10^8
P1/Mahoney *wt*	25	24	$<10^5$
	25	36	$<10^5$
ca grown at 37°C	25	24	1.0×10^8
	25	36	5.8×10^8
ca grown at 25°C	25	24	$<10^5$
	25	36	1.6×10^8

Nonproductive transitions to 135S particles might be responsible for the high particle/PFU ratio observed for poliovirus (50). According to this model, *ca* mutants would have a lower particle/PFU ratio during infection at 25°C than at 37°C, since they undergo normal levels of alteration at the higher temperature but do not alter detectably at the lower one. The absence of alteration should result in an increase in the apparent titer of the *ca* virus during an infection at 25°C compared to 37°C. To explore this possibility, we performed a temperature shift plaque assay in which cells were infected with *wt* or *ca* virus stock prepared at 37°C. Infected cells were incubated at 25°C under a methylcellulose overlay for 48 h and then shifted to 37°C for 72 h before staining to count plaques. Under these conditions, the virus would be able to adsorb and enter cells in the absence of alteration to 135S particles, and the progeny of each successful entry event would then form a plaque at the higher temperature. No increase in apparent titer at 25°C was observed (Table 1), indicating that the particle/PFU ratio did not decrease at this temperature. A fluorescent focus assay performed on cells infected with *wt* or *ca* virus at either 25 or 37°C confirmed this result (Table 2).

The *ca* mutation acts at a postentry step in the virus life cycle. Two approaches were used to explore the nature of the defect in *wt* poliovirus replication at 25°C. A temperature shift growth curve analysis was used to determine the timing of the block to replication at the low temperature. Cells were infected at a high MOI with either *wt* or *ca* virus, incubated for various times at 37°C, and then shifted to 25°C. The results indicate that 1 h at the permissive temperature (37°C) is not sufficient to allow *wt* virus to replicate at 25°C (Fig. 3). After 2 or 4 h at 37°C, *wt* virus could grow at 25°C, indicating that the last block to *wt* replication at 25°C occurs between 1 and 2 h after virus binding, though the defect is not completely overcome until at least 2 h postbinding, consistent with a block in RNA replication. To confirm that the cold-sensitive step was not at the level of cell entry, this step was bypassed by transfecting *wt* or *ca* RNA into cells at 25°C. Under these conditions, *wt* virus is still unable to grow at the lower temperature, while *ca* viral RNA clearly initiates a productive infection (Fig. 4).

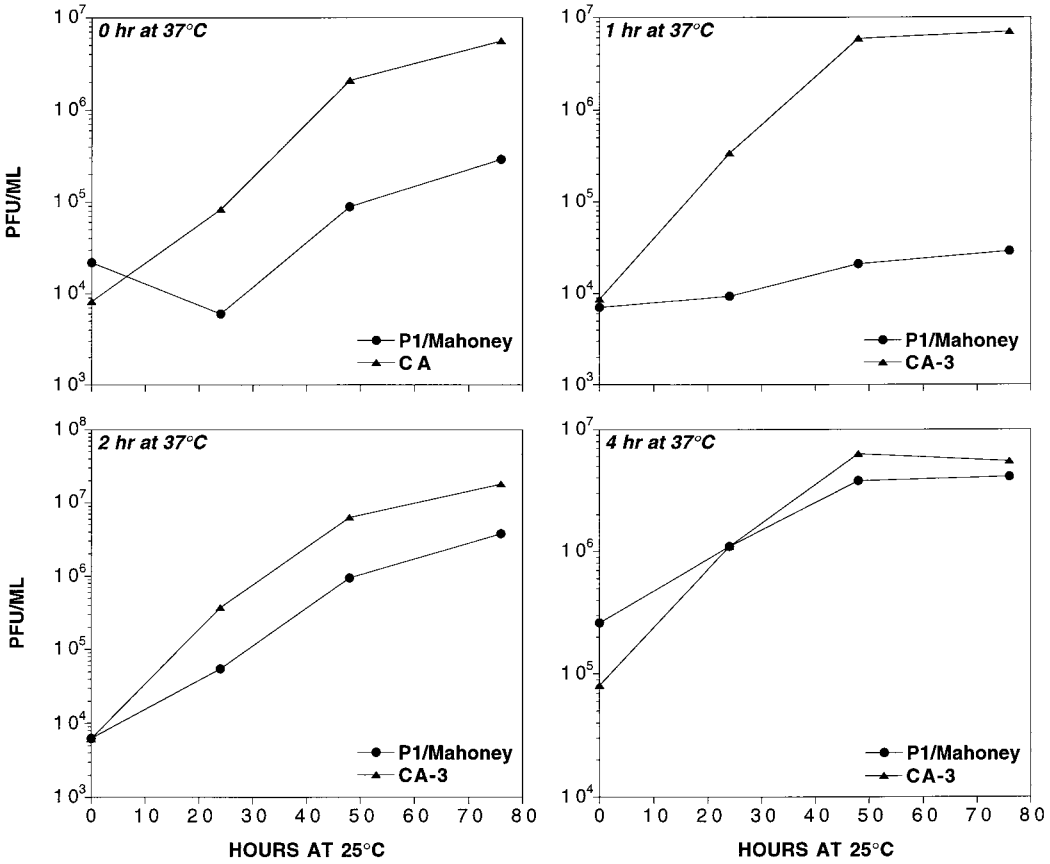

FIG. 3. Temperature shift growth curve analysis. *wt* (●) or *ca*-3 (▲) virus was adsorbed to HeLa cells in monolayer cultures at 25°C, and the unbound virions were washed off. Cells were then incubated at 37°C for 0, 1, 2, or 4 h before being shifted to 25°C. At different times after the shift, samples of cell supernatant were removed and viral titers were determined by plaque assay.

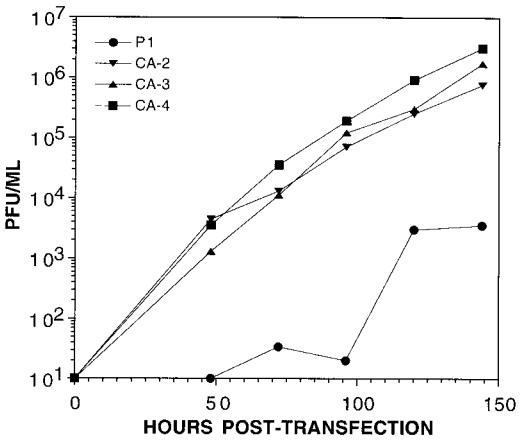

FIG. 4. Growth of poliovirus at 25°C after transfection with viral RNA transcripts. cDNA clones of *wt* (P1), *ca*-2, *ca*-3, or *ca*-4 were transcribed in vitro, and the resulting RNA was used to transfect HeLa cells in monolayer cultures which were incubated at 25°C. Samples of cell supernatants were removed at different times postinfection, and viral titers were determined by plaque assay.

The *ca* mutation maps to the 2C gene. To determine which region of the viral genome confers the *ca* phenotype, we constructed three recombinant viruses in which different sections of *wt* P1/Mahoney were substituted with the corresponding regions from clonal *ca* isolates. Only the r2 recombinants, which contain approximately the middle one-third of the *ca* virus genome, are cold adapted, as judged by total virus yield and growth curve analysis at 25°C (Fig. 5A). All nine r2 recombinants, containing the central region of the genome from three independent plaque-purified *ca* isolates, were cold adapted.

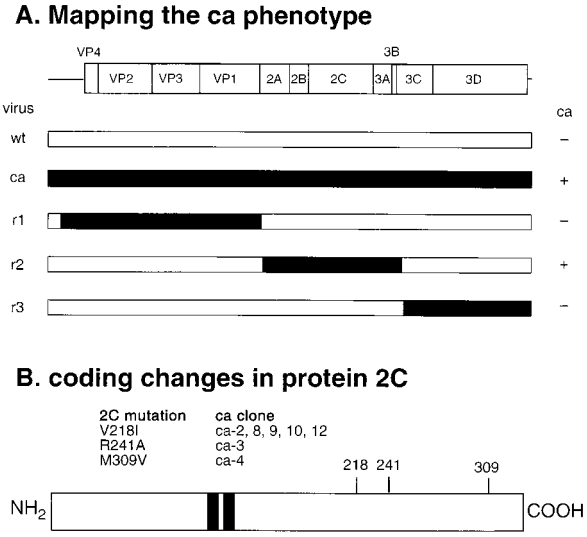

FIG. 5. Mapping of the *ca* mutations. (A) Genomic RNAs of viral recombinants between *wt* and *ca* viruses are shown schematically, with P1/Mahoney sequences in open boxes and *ca* virus sequences in black boxes. At the top is a genetic map of poliovirus genomic RNA. Recombinants r1, r2, and r3 were produced by using cDNA derived from all three *ca* viruses (*ca*-2, *ca*-3, and *ca*-4). The viruses were tested for cold adaptation by comparing viral yields at 0 and 96 h postinfection at 25°C. +, increase in titer of over 3 orders of magnitude; −, increase of less than 2 orders of magnitude. The *ca* phenotype of recombinant r2 viruses was confirmed by performing one-step growth curve analysis at 25°C. (B) Locations of amino acid changes in the 2C coding region. The nucleotide sequences of cDNAs derived from seven different plaque-purified *ca* virus clones were determined in the region corresponding to the portion of r2 derived from *ca*. A single amino acid change in the 2C coding region was found in all seven *ca* viruses, representing three different coding mutations. The coding changes in the *ca* viruses are shown as amino acid locations above a schematic representation of the 2C protein (amino acid 1 is the amino terminus of 2C). The nucleotide triphosphate-binding motif (black boxes) in 2C is shown (35).

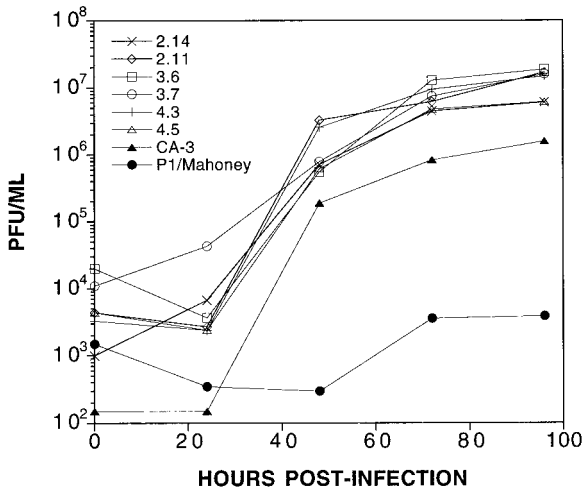

FIG. 6. Site-directed mutants exhibit the *ca* phenotype. HeLa cells in monolayer culture were infected at an MOI of 10 with independently derived site-directed mutants representing each *ca* coding change. Viruses 2.14 and 2.11 contain the V218I mutation, viruses 3.6 and 3.7 contain the R241A mutation, and viruses 4.3 and 4.5 contain the M309V mutation. Plaque-purified *ca*-3 and *wt* P1/Mahoney were included as controls. The infected cells were incubated at 25°C, samples of the cell supernatant were removed at different times postinfection, and the viral titers were determined by plaque assay.

Nucleotide sequence analysis of cDNA from seven plaque-purified *ca* isolates revealed three independent mutations near the C terminus of the 2C coding region (Fig. 5B). These mutations were introduced individually into *wt* viral cDNA by site-directed mutagenesis, and viruses were derived by transfection. Growth curve analysis at 25°C done at high MOI revealed that each of the site-directed mutations is capable of conferring the *ca* phenotype (Fig. 6).

The Sabin vaccine strains contain novel *ca* mutations. Growth curve analysis was used to determine whether other poliovirus strains can replicate at 25°C. All three serotypes of the Sabin live attenuated vaccine strains exhibit growth kinetics nearly identical to those of *ca* mutants (Fig. 7). In addition, *wt* P3/Leon is cold adapted, while P2/Lansing is not. Previously constructed viral recombinants between P1/Sabin and P1/Mahoney viruses (5) were used to localize the mutations responsible for the *ca* phenotype of P1/Sabin. Recombinants containing either the central region or the C-terminal region of the polyprotein from P1/Sabin were cold adapted (Fig. 8). There are no amino acid differences between the 2C proteins of P1/Sabin and P1/Mahoney viruses (40, 45). These results indicate that mutations in proteins other than 2C, including 2A, 2B, and $3D^{pol}$, can confer the *ca* phenotype.

DISCUSSION

The current model for poliovirus entry proposes that virus binding is followed by an essential transition to the 135S particle, which is an intermediate in virus genome entry (15, 17, 53). While the proposal that genome entry occurs from the hydrophobic 135S particle has been widely accepted, several observations are inconsistent with this model. First, the capsid protein VP4, which is absent from the 135S particle, appears to

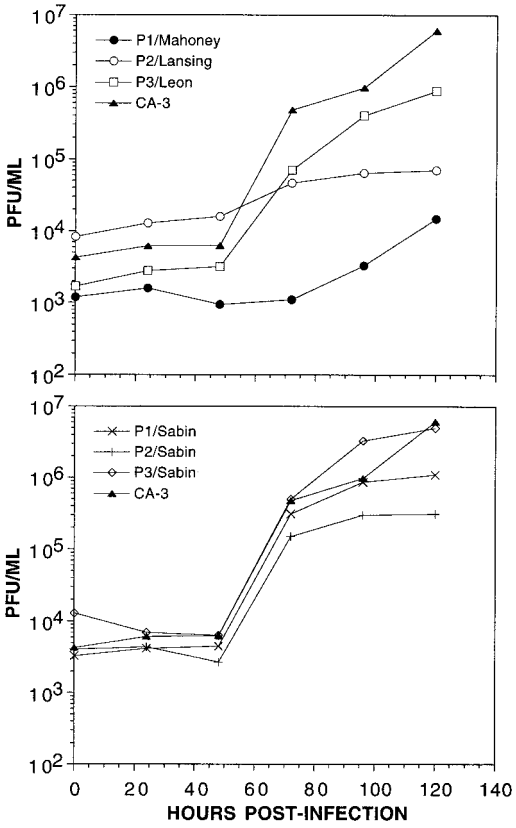

FIG. 7. Other strains of poliovirus exhibit the *ca* phenotype. HeLa cell monolayer cultures were infected at an MOI of 10 with the indicated viruses, and the infected cells were incubated at 25°C. At different times postinfection, samples of the cell supernatants were removed and the viral titers were determined by plaque assay.

be essential for virus entry (36). Second, while 135S particles can deliver RNA to cells and initiate an infection, the infectivity of these particles is only 0.1 to 0.001% of that of native particles (9). Third, by changing the conditions used in an alteration assay, it is possible to observe a productive infection in the absence of any detectable levels of 135S particles (24), suggesting that alteration might be an artifact. Fourth, since all virus particles are capable of undergoing alteration but only a fraction undergo productive entry, alteration is an unreliable indicator of virus entry (54).

Results presented here offer further evidence that virus alteration may not be required for poliovirus cell entry. *ca* mutants of poliovirus P1/Mahoney are capable of growing to high titers at 25°C but do not convert to 135S particles at that temperature, even when assayed over long time periods. The mutations in *ca* viruses appear to act at a postentry stage of virus infection. Because no capsid mutations conferring the *ca* phenotype have been identified to date, it seems unlikely that a function of the capsid (such as alteration) is under selective pressure at 25°C.

It might be argued that the limit of detection of the alteration assay used in these studies is not sufficient to detect very small amounts of 135S particles. Because the particle/PFU ratio of poliovirus is so high, conversion of only a very small proportion (0.1 to 2%) of the population to 135S particles would suffice to initiate productive entry and infection. While this issue cannot be resolved without directly examining virus genome entry, it seems unlikely for three reasons. First, from results of similar assays for alteration, it was previously concluded that the transition to 135S particles does not occur at temperatures lower than 33°C (55). Hypothesizing that a low, undetectable level of alteration occurs at 25°C leaves the current model of virus entry intact, but such a justification would be tautological. Second, the current model for poliovirus entry predicts that the severe reduction in 135S particle formation at the lower temperature would create a selective pressure on the virus to alter at 25°C. The poliovirus capsid is clearly capable of tolerating mutations which permit greater flexibility and faster 135S particle formation under different conditions (38), but such capsid mutations were not observed in *ca* viruses. Third, the maximum level of 135S particle formation which could occur without being detected in our alteration assays is approximately 0.5% of the input virus infectivity, a level which would effectively reduce the input MOI in 25°C growth curves from 10 to less than 0.1. Such a reduction would not be consistent with the one-step growth kinetics seen in these experiments.

The lower apparent titer of *ca* viruses at 25°C compared to 37°C in the temperature shift and fluorescent focus assays might be interpreted as evidence that a decrease in the production of 135S particles has reduced viral infectivity. This explanation seems unlikely, as selective pressure should result in the emergence of mutants that alter at 25°C. It seems more likely that the lower virus titer at 25°C is a consequence of the slower metabolism of the infected cells and their inability to efficiently support later steps in the virus life cycle. *ca* mutants are better able to bypass this block than *wt* virus but are not able to overcome it completely. In support of this idea, the results of temperature shift growth curves and transfection experiments indicate a postentry block in *wt* poliovirus repli-

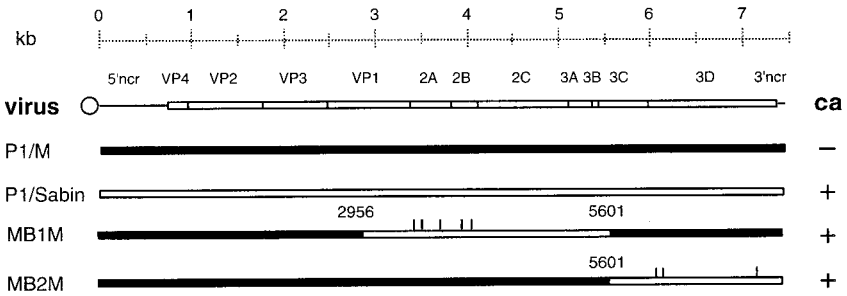

FIG. 8. Mapping of the *ca* phenotype of poliovirus P1/Sabin. Genomic RNAs of viral recombinants between P1/Mahoney and P1/Sabin viruses are shown schematically, with P1/Mahoney sequences in black boxes and P1/Sabin virus sequences in open boxes. At the top is a genetic map of poliovirus genomic RNA. The viruses were tested for cold adaptation by performing one-step growth curve analysis at 25°C in HeLa cells. +, increase in titer of over 3 orders of magnitude; −, increase of less than 2 orders of magnitude. Amino acid differences between P1/Sabin and P1/Mahoney are shown as short vertical bars. ncr, noncoding region.

cation at 25°C. In fluorescent focus assays, the *ca* virus stock prepared at 25°C appears to have a lower growth rate than *ca* virus stock prepared at 37°C in the subsequent infection at 25°C (Table 2). Since the 2C gene product has been suggested to play a role in viral assembly as well as replication (26), it is possible that the mutant virus does not fully overcome the low-temperature block to 2C function, resulting in the production of partially defective particles and slower subsequent growth.

The hypothesis that alteration to 135S particles is not necessary for poliovirus cell entry raises two important questions. First, if this transition is not essential for virus entry, why would it be conserved? Second, since antiviral compounds which inhibit 135S particle formation also inhibit poliovirus infectivity (6, 16, 38, 56), how does an entry model which does not involve alteration explain the action of these compounds? Alteration to 135S particles has been observed only under artificial conditions, with high concentrations of either cultured cells (29) or solubilized receptor protein (8, 23, 55), and so it is not known whether 135S particle formation has been subjected to selective pressure in vivo. Second, picornaviruses are small, highly constrained physical entities, and if alteration does occur in normal infections, it may be a by-product of the structural flexibility required to permit cell entry. The 135S particle might be a nonproductive end product, and a less drastic receptor-induced conformational change might produce the true intermediate in RNA uncoating. In support of this model, a more subtle transition in the virus capsid, known as breathing, may be important for virus entry (27). Antiviral drugs may inhibit virus infectivity not because they block alteration but because they increase the overall rigidity of the virus capsid, preventing more subtle transitions necessary for uncoating. Finally, studies of poliovirus entry have used assays of viral infectivity or other postentry events rather than examining virus genome entry directly, largely because of the confounding factors introduced by 135S particle formation at 37°C.

It has been suggested that the high particle/PFU ratio of poliovirus is the result of the release of 135S particles from cells early in infection (50). The results of temperature shift plaque assays and fluorescent focus assays at 25°C do not support this hypothesis.

The observation that the Sabin vaccine strains exhibit a *ca* phenotype in the absence of coding changes in the 2C gene raises the possibility that the block to *wt* virus replication at 25°C involves a complex of viral proteins which includes the 2C gene product. Further experiments are being performed to identify additional *ca* mutants and to determine whether these viruses, and the Sabin strains, are capable of undergoing alteration to 135S particles at 25°C.

Identification of the cold-sensitive block in poliovirus replication will provide information about the intracellular events in poliovirus-infected cells. The function of the 2C gene is incompletely understood, but it is the target of the antiviral compound guanidine, which inhibits viral RNA synthesis (42), has nucleotide phosphatase and RNA binding functions, and is probably involved in RNA replication and virus assembly (12, 26, 35, 47, 48). The 2C protein may be involved in a cold-sensitive step in viral RNA synthesis. Alternatively, 2C may be involved in determining the structure of the mature virion during assembly, such that virions produced at 25°C are incapable of initiating a productive second round of infection. Our results indicate that mutations in other viral proteins can also confer the *ca* phenotype, suggesting that a multiprotein complex is involved in cold adaptation. Experiments are in progress to distinguish among these possibilities.

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