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An Assembly Defect as a Result of an Attenuating Mutation in the Capsid Proteins of the Poliovirus Type 3 Vaccine Strain

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The molecular basis of the temperature-sensitive (*ts*) phenotype of P3/Sabin, the type 3 vaccine strain of poliovirus, was investigated in light of the known correlation between *ts* and attenuation phenotypes. A phenylalanine at residue 91 of the capsid protein VP3 was a major determinant of both phenotypes, and attenuation and *ts* could be reverted by the same second-site mutations. The *ts* phenotype was due to a defect early in the assembly process that inhibited the formation of 14S pentamers, empty capsids, and virions. It was further shown that capsid proteins that were not incorporated into higher-order structures had short half-lives at the nonpermissive temperature.

The genetic basis of attenuation of the Sabin type 3 vaccine strain of poliovirus, and that of reversion to virulence, have been extensively studied (1, 4, 15, 32). The vaccine strain, P3/Sabin, differs from its neurovirulent parent, P3/Leon, at 10 nucleotide positions (28), but attenuation is due mainly to just two mutations, a C-U transition at nucleotide 472 in the 5′ noncoding region and a Ser-Phe substitution at VP3-091 in the capsid proteins (32). The latter change renders growth of the virus in HEp-2C cells sensitive to elevated temperatures (21), and suppression of this phenotype by second-site mutation results in loss of attenuation (15). This strict correlation between temperature-sensitive (*ts*) and attenuation phenotypes attributable to the amino acid change in VP3 suggested that an understanding of the molecular basis of the *ts* phenotype would give similar insights into the molecular basis of attenuation of the Sabin type 3 vaccine strain.

The crystal structures of P3/Sabin and P1/Mahoney have been determined to 0.24 and 0.29 nm (5, 9), and the positions of VP3-091 and phenotypic suppressor mutations suggested a structural basis for the *ts* phenotype. In P1/Mahoney, and presumably in P3/Leon, the side chain of the Ser at VP3-091 points into a pocket where the serine hydroxyl-group hydrogen bonds with a buried water molecule. In P3/Sabin, however, the large aromatic side chain of the Phe residue points outward, making unfavorable contacts with the solvent (5). This situation might predispose the virion to thermal transitions, resulting in burial of the Phe residue such that the virus particle is rendered unstable or defective in some way. Suppressor mutations have been found in several locations (5, 21), including protomer-protomer and pentamer-pentamer interfaces where they could act by stabilizing the interfaces against such transitions. Another mutation was found at a residue in the hydrophobic pocket analogous to the binding site in some rhinoviruses of antiviral compounds that stabilize these virions to heat (6).

These data are consistent with the idea that the *ts* phenotype of P3/Sabin is the result of thermolability of one or more capsid structures. Here we show that the capsid protein mutation does not affect virion stability itself but rather inhibits virion assembly, specifically at the protomer-to-pentamer step.

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MATERIALS AND METHODS

Cells and viruses. HEp-2C cells were grown in monolayers as described by Minor (19). Construction and recovery of the cDNA-derived recombinant SV3/L have been described previously (32). SV3/L differs from P3/Leon only at residue 91 of VP3, which is a Phe in SV3/L, like P3/Sabin, and a Ser in P3/Leon. Isolates of P3/Leon and P3/Sabin were those used for cDNA cloning and recombinant construction (29, 32).

Plaque assays. Cell sheets in six-well plates were inoculated with dilutions of virus for 30 min and then overlaid with 1% agar in minimal essential medium supplemented with 2% fetal calf serum. Plates were incubated in sealed plastic boxes submerged in precision water baths (16) at relevant temperatures for 3 days, and then cell sheets were stained with naphthalene black.

Virus purification and RNA extraction. Viruses were grown and purified on sucrose gradients before phenol extraction of RNA as described by Minor (19).

Antisera. Monoclonal anti-poliovirus receptor 280 (22) was a gift from P. Pipkin. At a dilution of 1/1,280, antibody 280 prevented poliovirus infection of HEp-2C cell sheets for at least 5 days (data not shown). Polyclonal antiserum 119 was raised in rabbits against a derivative of P3/Sabin. This serum selectively precipitated capsid proteins from sucrose gradient fractions containing 14S pentamers, empty capsids, and virions but not from those containing 5S promoters (data not shown).

Shift-up one-step growth. Replica cell sheets in 25-cm^2 flasks were transfected with equal amounts of viral RNA (31) at room temperature for 30 min, and then 5 ml of minimal essential medium containing 1% fetal calf serum and 1% anti-poliovirus receptor monoclonal antibody (MAb) 280 (22) was added to each flask. Flasks were submerged in water baths at 35°C and then shifted to 40°C baths one at a time at hourly intervals until 10 h posttransfection. Flasks were harvested at 10 or 24 h posttransfection by rapid freezing in CO_2-ethanol baths and were frozen and thawed twice more before virus titration.

Infection at 40°C. Cell sheets in 25-cm^2 flasks were incubated in a water bath at 40°C for 15 min before inoculation at 40°C with SV3/L or P3/Leon at a multiplicity of infection of 0.1. At 1 h postinfection (p.i.), inocula were removed and cell sheets were washed three times at 40°C and then incubated at 40°C in minimal essential medium containing 1% fetal calf serum and 1% MAb 280. At 2 h p.i., one flask per virus was shifted to 35°C and another was harvested by rapid freezing. One flask was left at 40°C. An additional control flask was inoculated at 35°C and incubated at 35°C throughout the experiment. At 10 h p.i., flasks were harvested by rapid freezing and then frozen and thawed twice more before virus titration.

In vivo labeling of viral proteins. Cell sheets in 25-cm^2 flasks were inoculated at a multiplicity of infection of 10 for 1 h at 35°C and then incubated in minimal essential medium containing 1% fetal calf serum. After 2 h, medium was removed and replaced by methionine-free medium. After a further 2-h incubation at 35°C, flasks were submerged in water baths at 40°C or left at 35°C for 15 min before labeling with [^{35}S]methionine (10 μCi/ml; >1,000 Ci/mol; Amersham). For labeling experiments, flasks were incubated for a further 45 or 90 min at the relevant temperature, medium was removed, still at the relevant temperature, and then cell sheets were rapidly frozen in CO_2-ethanol. For pulse-chase experiments, labeling was done for 10 min before the addition of excess unlabeled methionine. Cell sheets were harvested at various times thereafter as described above.

Cells were lysed at 4°C by the addition of 0.5 ml of phosphate-buffered saline containing 0.5% Nonidet P-40 (NP-40), 0.25% deoxycholate, 5 mM EDTA, 1% aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM *N*-ethylmaleimide (protease inhibitors were added just before use). After removal of nuclei by centrifugation, lysates were stored at −70°C before further analysis.

Sucrose gradients. Cell lysates were fractionated on 10-ml 5 to 20% or 15 to 30% sucrose gradients prepared in lysis buffer by using an SW41 rotor (Beckman). Gradients were centrifuged at 35,000 rpm for 16 h (5 to 20%) or at 18,000 rpm for 12 h (15 to 30%); then 0.5-ml fractions were collected after bottom puncture. Pellets from 5 to 20% gradients were resuspended in 0.5 ml of lysis buffer. Aliquots of 5 μl were sometimes removed for scintillation counting.

Immunoprecipitation. Aliquots of 100 μl of cell lysates or sucrose gradient fractions were incubated with 5 μl of polyclonal antiserum 119 overnight at 4°C. Samples were then incubated with 100 μl of 10% protein G (Sigma) in sodium dodecyl sulfate (SDS)–NP-40 wash (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 0.5% SDS, 1% NP-40, 1% aprotinin) at 4°C for 2 h and then centrifuged at 6,000 × *g* for 2 min. Pellets were washed once each with 0.5 ml of SDS–NP-40 wash, 0.5 M NaCl wash (0.5 M NaCl, 50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1 μg of ovalbumin per ml, 0.05% NP-40, 1% aprotinin), and 0.05% NP-40 wash (125 mM NaCl, 50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 0.05% NP-40, 1% aprotinin) and then resuspended in 50 to 100 μl of gel loading buffer. Samples were boiled for 1 min and centrifuged at 12,000 × *g* for 5 min, and supernatants were either analyzed directly by SDS-polyacrylamide gel electrophoresis (PAGE) (13) or stored frozen.

RESULTS

Temperature sensitivity. The *ts* phenotype of P3/Sabin and related viruses is conveniently measured as a reduction in PFU in plaque assays at nonpermissive temperatures. At 40°C, the titer of P3/Leon is reduced, relative to that at 35°C, by less than 1 $\log_{10}$ unit, whereas that of P3/Sabin is reduced by >5 $\log_{10}$ units (15). Genetic analysis of this phenotype (21) showed that the Ser-Phe change at VP3-091 was responsible for a 3- to 4-$\log_{10}$-unit reduction at 40°C, the residual phenotype being due to one or more differences between P3/Sabin and P3/Leon that have only marginal effects on the *ts* phenotype of a virus with a Ser at VP3-091.

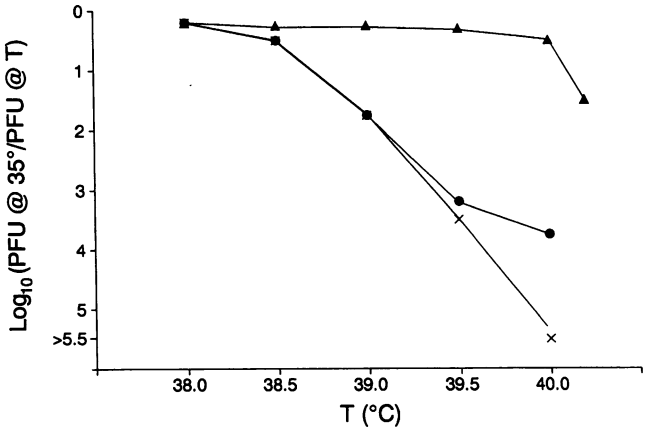

FIG. 1. Temperature sensitivity of P3/Sabin (×), SV3/L (●), and P3/Leon (▲).

Further investigation of the effect of temperature on these viruses showed that the *ts* phenotype of P3/Sabin (defined as a reduction in titer of >1 $\log_{10}$ unit) was expressed at temperatures above 38.7°C (Fig. 1). Titers of P3/Leon were hardly affected by temperatures up to 40.1°C. Data for the recombinant SV3/L, which only differs from P3/Leon at VP3-091, confirmed that the *ts* phenotype of P3/Sabin was mainly due to the Phe at VP3-091. To study the effect of this mutation on its own, subsequent studies, other than thermolability assays, compared the phenotypes of P3/Leon and SV3/L.

Thermolability. Preparations of P3/Leon and P3/Sabin, either as tissue culture S10 supernatants or purified on CsCl gradients, were incubated at 40, 43, 45, and 50°C for periods ranging from 15 min to 24 h with or without additional fetal calf serum and divalent cations and then titrated. While the thermolabilities of preparations varied with each treatment, there was no significant difference between the results for P3/Leon and P3/Sabin in each experiment. Results of some of the assays are shown in Table 1. The effect of sample volume, illustrated by comparison of assays a and b, was reproducible.

Temperature shift assays. The stage of the viral growth cycle affected by the presence of a Phe at VP3-091 was investigated by temperature shift one-step growth assays. In one series of one-step growth experiments, cell sheets were transfected with viral RNA at room temperature and then incubated in the presence of the anti-poliovirus receptor MAb 280 (22) to prevent a second round of infection. Cell sheets were incubated at 35°C, shifted to 40°C at various times, and then harvested at 10 or 24 h posttransfection by rapid freezing before virus titration. Figure 2 illustrates the effect of the time of shift to 40°C on the one-step growth titer at 10 h posttransfection expressed relative to titers obtained after incubation at 35°C. That these were in fact one-step growth titers was confirmed by results for cell sheets incu-

TABLE 1. Examples of thermolability assays

Assay[a]	Incubation temp (°C) and time	Log_{10} titer reduction	
		P3/Sabin	P3/Leon
a	40, 4 h	2.2	1.8
	40, 24 h	3.0	2.8
b	40, 3.5 h	0.1	0.5
	40, 20 h	0.95	0.8
c	40, 30 min	0.1	0.5
	43, 30 min	0.2	0.6
	45, 30 min	0.6	0.4
	50, 30 min	4.8	5.2

[a] In assay a, aliquots of 0.1 ml of tissue culture supernatants were incubated in 1.5-ml Eppendorf tubes. In assay b, aliquots of 1 ml of tissue culture supernatants were incubated in 1.5-ml tubes. In assay c, aliquots of 1 ml of tissue culture supernatants diluted 100 times in phosphate-buffered saline were incubated in 1.5-ml tubes.

bated for a total of 24 h (in the presence of MAb 280), which were similar to those obtained at 10 h (data not shown).

Incubation at 40°C, except after 8 h at 35°C, reduced the one-step growth titers of SV3/L to a greater extent than those of P3/Leon. Furthermore, incubation of SV3/L-infected cell sheets at 35°C for up to 4 h posttransfection had little or no alleviating effect on the reduction in final titer obtained. Titers obtained from cell sheets shifted to 40°C at later times probably reflected virion production before the shift. Thus, the *ts* defect appeared to act late in the infectious cycle and could not be suppressed by incubation at the permissive temperature during the early period of viral infection.

These results did not rule out an effect of the Phe at VP3-091 on early stages of infection as well. Therefore, cells were infected at the nonpermissive temperature, the inoculum was removed, the antireceptor MAb was added, and the cell sheets were shifted down to 35°C at 2 h p.i. At this time, no infectious virus could be recovered from infected cell sheets (Table 2). Comparison of one-step growth titers for P3/Leon and SV3/L showed that, while infection and incubation for 2 h at 40°C reduced virus yield relative to infection at 35°C, the effect was similar for both viruses (Table 2). Thus, the Phe at VP3-091 did not seem to affect virus adsorption at the nonpermissive temperature. Furthermore,

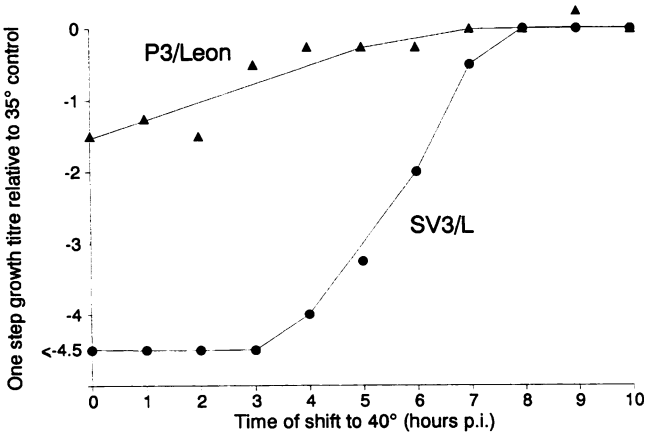

FIG. 2. Effect of time of shift to 40°C on one-step growth titers, expressed relative to one-step titers reached after incubation at 35°C (see Materials and Methods).

TABLE 2. Effect of inoculation at 40°C on one-step growth titers

Inoculation temp (°C)	Incubation temp (°C) and time (h)	Titer/ml (log_{10})	
		P3/Leon	SV3/L
35	35, 10	7.55	7.3
40	40, 10	6.05	2.8
40	40, 2, then 35, 8	6.8	6.55
40	40, 2	≤2.3	≤2.3

uncoating of SV3/L virions had occurred at 40°C, at least to the stage at which particles were no longer infectious. This is consistent with other studies which have shown that in HeLa cells at 39.5°C, wild-type poliovirus RNA release is complete by 60 min p.i. (12). Taken together, these experiments showed that the Phe at VP3-091 caused a defect in virion production at the nonpermissive temperature only at a late stage in infection. In view of the structural data on locations of VP3-091 and suppressor mutations, the effect of the Phe at VP3-091 on capsid protein synthesis and assembly at the nonpermissive temperature was investigated.

Protein synthesis and assembly. Results of temperature shift experiments showed that the process affected by temperature in SV3/L-infected cells had started by 4 h p.i. but that temperature shift at any subsequent time up to 7 h p.i. resulted in reductions in virion production. Thus, [^{35}S]methionine labeling of proteins at 5 h p.i. was expected to illustrate any differences in protein synthesis by SV3/L and P3/Leon relevant to the *ts* phenotype of the former. To study protein synthesis in cells at both 35 and 40°C at comparable stages of infection, infected cell sheets were all incubated at 35°C until 15 min before the addition of [^{35}S]methionine. Shift-up for this period allowed cultures to reach the incubation temperature before the addition of label.

Figure 3 shows the results of such an experiment in which proteins were labeled from 5 to 6.5 h p.i. The Phe at VP3-091 had no significant effect on labeling or processing of capsid proteins at 35°C, but differences were apparent between extracts from cells infected with SV3/L and P3/Leon when labeled at 40°C. The total amount of SV3/L capsid proteins observed at the end of the 40°C labeling period was reduced relative to that of P3/Leon capsid proteins (e.g., compare intensities of VP3 bands). There was no similar reduction for proteins other than VP0, -1, -2, and -3 (VP4 was not observed since it contains no methionine). In fact, in Fig. 3,

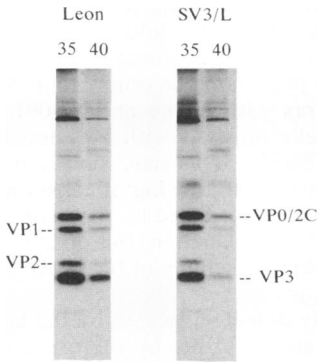

FIG. 3. Effect of temperature on capsid protein levels. Cell sheets were infected with P3/Leon or SV3/L, and then proteins were labeled at 35 or 40°C from 5 to 6.5 h p.i. before cell lysis. Aliquots of 50 μl of each lysate were analyzed by SDS-PAGE.

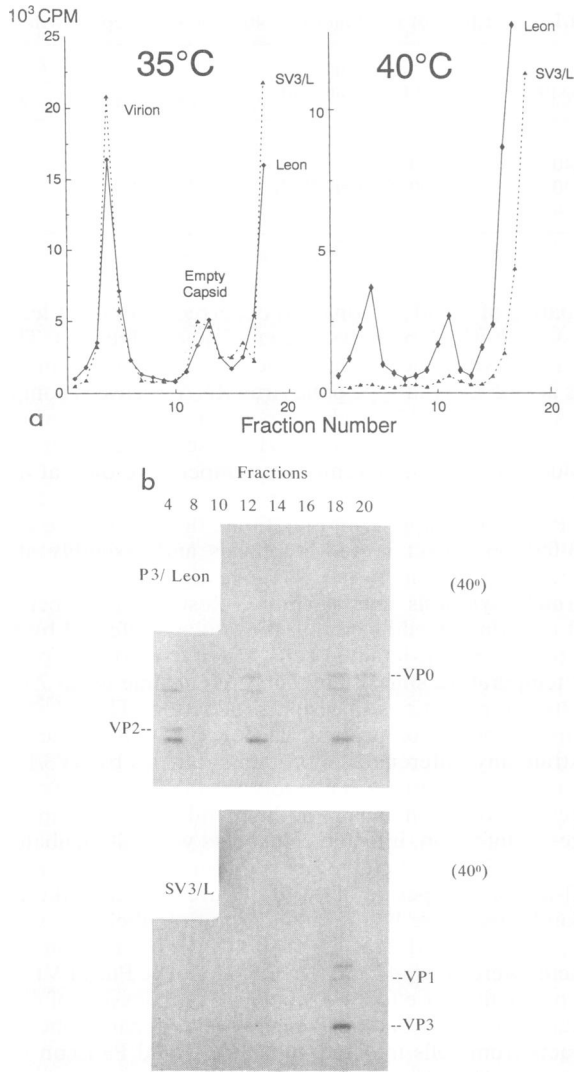

FIG. 4. Sucrose gradient analysis of capsid structures synthesized at 35 and 40°C. Lysates shown in Fig. 3 were sedimented through 15 to 30% sucrose gradients, and 0.5-ml fractions were collected. Aliquots of 5 μl were scintillation counted (a), and aliquots of 50 μl were analyzed by SDS-PAGE (b).

all proteins other than capsid proteins are equally or more intensely labeled in SV3/L-infected cells than in P3/Leon-infected cells at 40°C. This reduction could be due to a defect in synthesis or processing or simply to a shorter half-life for capsid structures with a Phe at VP3-091. Also, VP2 was observed in cells infected with P3/Leon but not in those infected with SV3/L. This may simply have reflected the differential intensity of labeling of capsid proteins. Alternatively, since VP2 is produced by the maturation cleavage of VP0 upon RNA encapsidation, this observation could be the result of a defect in assembly of SV3/L at the nonpermissive temperature.

The assembly defect suggested by the lack of VP0 cleavage was investigated further by sucrose gradient centrifugation. Cell extracts shown in Fig. 3 were fractionated on 15 to 30% sucrose gradients, radioactivity in the fractions was assayed, and the samples were analyzed by SDS-PAGE (Fig. 4). Gradient profiles for P3/Leon and SV3/L at 35°C were almost identical. After labeling at 40°C, P3/Leon virion production was reduced by about 75% and empty capsid production was reduced by about 50%, whereas for SV3/L, virion production was not detectable (>99% reduction) and empty capsid production was reduced by at least 90% (Fig. 4a). Analysis of these gradient fractions by SDS-PAGE (Fig. 4b) showed that SV3/L capsid proteins were present in fractions at the top of the gradient, containing protomers and pentamers, so the reduction in total SV3/L capsid proteins observed relative to P3/Leon was chiefly due to reduced levels of higher-order structures. The band in fraction 20 with the same mobility as VP0 is probably 2C.

Fractionation of similar extracts on 5 to 20% sucrose gradients allowed more precise identification of the assembly defect. Figure 5 shows SDS-PAGE analysis of such gradients (the bottom of the gradient is on the right in this figure). SV3/L capsid proteins synthesized at 40°C were found in fractions 14 to 16, fraction 6, and the pellet. By reference to published data (e.g., reference 17), the peaks corresponded to 5S protomers and 14S pentamers, respectively, and the pellet contained empty capsids and virions. P3/Leon capsid proteins were found mostly in 14S pentamers (fraction 6) and empty capsids and virions (pellet) (Fig. 5a). Comparison of the amounts of VP3 in each peak by densitometry showed that the approximate proportions of protomer/pentamer were 2:1 for SV3/L as opposed to 1:3 for P3/Leon. Thus, at 40°C, SV3/L was defective in the protomer-to-pentamer assembly step. A similar analysis of 35°C extracts (Fig. 5b) showed that even at this permissive temperature, the ratio of protomers to pentamers in SV3/L-infected cells was higher than that in P3/Leon-infected cells (approximately 1:1 as opposed to 1:3).

In the 14S pentamer peaks (fraction 6), the levels of VP3 were high relative to those of the other capsid proteins, even given that VP3 contains 13 methionine residues while VP0 and VP1 each contain 5. This effect was similar for both SV3/L and P3/Leon. It is possible that VP0 and VP1 are more susceptible than VP3 to mechanical or proteolytic breakdown during extraction or fractionation. Extra bands are visible below VP1 in fraction 6 of each gradient in which pentamers are present. It may be relevant that the N termini of VP0 and VP1, but not VP3, are disordered in empty capsids and, presumably, in pentameric subunits (10).

These experiments showed that the block was early in the assembly process. However, the amount of label accumulating in protomeric capsid precursors did not correspond to the total amount of label found in P3/Leon capsid proteins. Thus, either there was a further defect in synthesis and/or processing or capsid precursors were synthesized normally but, in the absence of assembly, were degraded.

To distinguish between these possibilities, proteins were pulse-labeled at 5.5 h p.i. (for 10 min) 15 min after a shift to 40°C and then chased with an excess of unlabeled methionine for periods from 0 to 30 min.

Figure 6 shows the results of one such experiment. It is apparent that the kinetics of label incorporation into P1 and subsequent processing into VP0, -1, and -3 were similar for both viruses over the labeling period and the first 15 min of the chase. Thus, there did not appear to be any defect in synthesis or primary cleavage of the P1 protein of SV3/L at 40°C. The 30-min time points showed loss of label from capsid proteins of both viruses but to a greater degree for SV3/L. This difference was not only due to virion production by P3/Leon (as shown by the appearance of VP2) since VP0 was also longer lived in P3/Leon-infected extracts.

Characterization of antiserum 119 with sucrose gradient

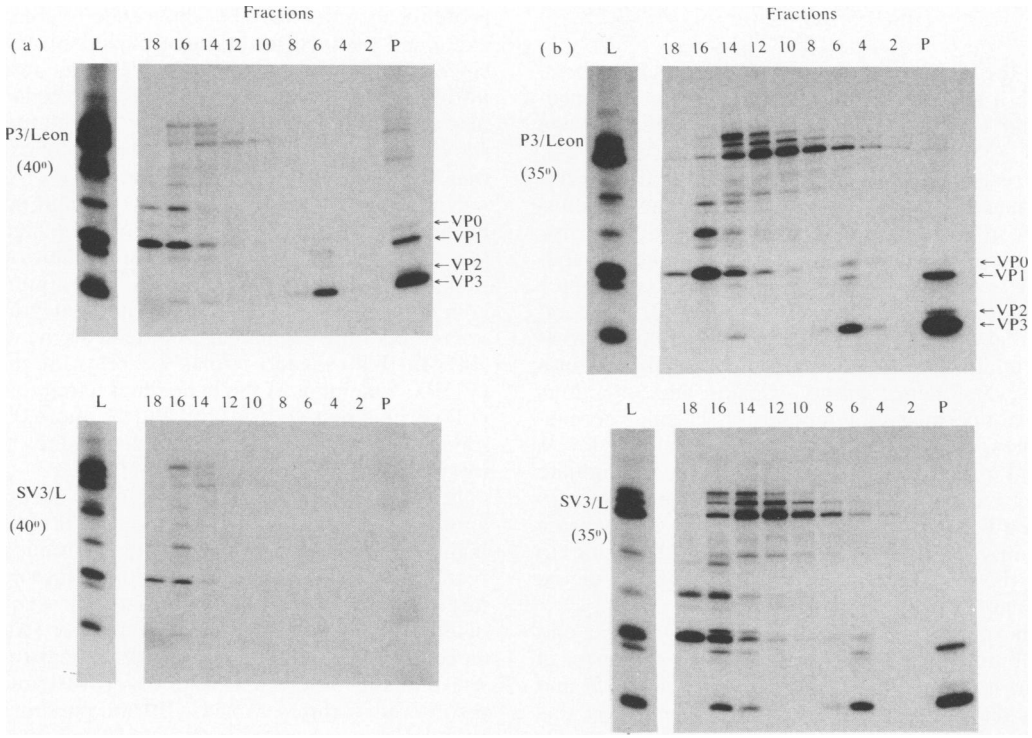

FIG. 5. Sucrose gradient analysis of subviral particles. Cell sheets were infected with P3/Leon or SV3/L, and then proteins were labeled from 5 to 5.75 h p.i. at 40°C (a) or 35°C (b). Cells were lysed in 0.5 ml of lysis buffer, and 0.2-ml aliquots of lysates were sedimented through 5 to 20% gradients. Fractions of 0.5 ml were collected. Pellets (lanes P) were resuspended in 0.5 ml of lysis buffer. Aliquots of 50 μl of cell lysates (lanes L), gradient fractions, and resuspended pellets (lanes P) were analyzed by SDS-PAGE. Autoradiograms were exposed for 7 days (a) and 5 days (b) except for lysate (lanes L) tracks (3 days [a] and 1 day [b]).

fractions showed that this antiserum selectively precipitated pentameric or larger structures (and P1, inefficiently) (data not shown). Immunoprecipitation of capsid proteins from lysates shown in Fig. 6 with antiserum 119 showed that no such structures were detectable in SV3/L-infected cells labeled at 40°C, whereas the capsid proteins were immunoprecipitated from P3/Leon-infected cells (Fig. 7). While the levels of capsid proteins after 15 min of chase were similar in both cases (Fig. 6), the structures in which these proteins were found differed. This result was consistent with the sucrose gradient analysis of intracellular structures in that the majority of SV3/L capsid proteins labeled at 40°C were in protomers, whereas those of P3/Leon were mainly found in pentameric or larger structures (Fig. 4 and 5). Thus, the faster rate of degradation of SV3/L capsid proteins at 40°C correlated with the block in synthesis of pentamers, empty capsids, and virions.

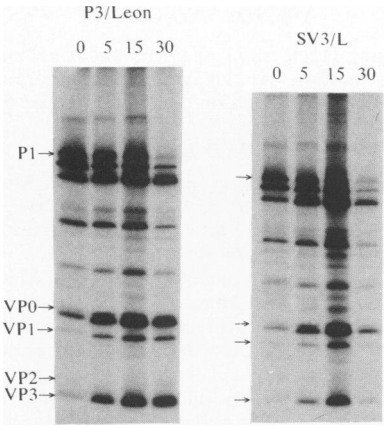

FIG. 6. Pulse-chase labeling. Cell sheets infected with P3/Leon or SV3/L were pulsed at 5.5 h p.i. for 10 min and then incubated with 100-fold excess of unlabeled methionine for 0, 5, 15, or 30 min before lysis. Aliquots of 50 μl of cell lysates were analyzed by SDS-PAGE.

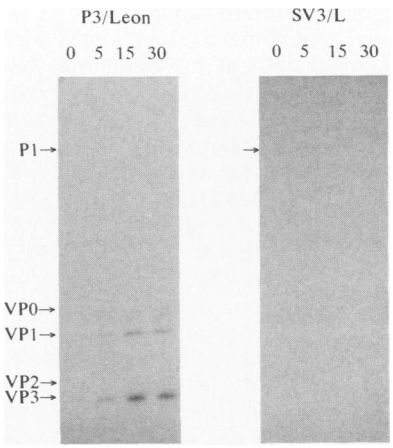

FIG. 7. Immunoprecipitation of subviral particles. Aliquots of 50 μl of lysates shown in Fig. 6 were precipitated with antiserum 119 and analyzed by SDS-PAGE.

DISCUSSION

Mutations in the capsid proteins of polioviruses have been associated with a number of different phenotypes and processes including thermolability (18), polyprotein cleavage (33), assembly (3, 17), uncoating (12), host range (24), antiviral drug resistance (8), and antibody reactivity (20). The results presented here show that the Ser-Phe substitution at VP3-091 in P3/Leon that renders viral growth sensitive to elevated temperatures does not increase thermolability or result in *ts* defects in early events such as receptor binding and uncoating or cleavage of P1 into VP0, VP1, and VP3. The primary effect of the Ser-Phe substitution at VP3-091 is to inhibit assembly of protomeric subunits into 14S pentamers, and hence empty capsids and infectious virions, at the nonpermissive temperature. Even at permissive temperatures, the Phe residue altered the relative levels of these capsid protein structures (Fig. 5b). Second-site mutations that revert both the *ts* and the attenuation phenotypes associated with this substitution (15) also compensate for this assembly defect (14a). Thus, it is tempting to speculate that the assembly defect is also the basis of the attenuation phenotype of SV3/L.

The location of residue 91 of VP3 in protomeric and pentameric subunits is not known, but the presence of trapped solvent molecules in this region of virions (5) and empty capsids (8a) suggests that the helix containing this residue, or the G-H loop of VP3 that folds across it, undergoes a structural transition during the assembly process. The relative ease with which empty capsids and 14S pentamers are interconvertible in vitro argues against major structural rearrangements at this stage. Furthermore, the locations of VP3-091 in empty capsids and virions of P1/Mahoney are analogous (8a). Thus, it is possible that a structural transition occurs during assembly of protomers into pentameric subunits. The environment of the aromatic ring of VP3-091 Phe in P3/Sabin is energetically unfavorable (5). It is possible that this side chain is less exposed to solvent in protomers than in pentamers. This could result in a shift in equilibrium between these two forms toward protomers, similar to that observed here, which could be accentuated by increases in temperature. Another possibility is that the outward orientation of the Phe at VP3-091, owing to its bulk, requires contortion of the helix containing this residue that may interfere with protomer-protomer interaction. Thus, 14S pentamers of P3/Sabin, unlike the virions themselves, may be thermolabile.

The low levels of pentamers and empty capsids observed in SV3/L-infected cells at the nonpermissive temperature (Fig. 4 and 5) suggest that the Phe at VP3-091 acts by altering the equilibrium between capsid precursors rather than by causing an irreversible block. This may allow suppressor mutations to act later in the assembly process by speeding up subsequent assembly steps (14a).

A further aspect of the *ts* phenotype of SV3/L is the short half-life of capsid proteins at 40°C. The rate of synthesis of P1 and its cleavage into VP0, -3, and -1 appeared similar in cells infected with either P3/Leon or SV3/L at 40°C, but the rate of degradation was greater for SV3/L capsid proteins. This may be a direct result of the residue at VP3-091 in some unknown way. Alternatively, if lower-order structures such as protomers and/or pentamers were degraded at a greater rate than empty capsids and virions, the observed difference in stability of SV3/L and P3/Leon capsid proteins would be an indirect result of the residue at VP3-091 through its influence on the predominant structure in which capsid proteins accumulate. This difference is greater than can be explained by incorporation of capsid proteins into virions since in P3/Leon-infected cells, VP0 was observed to have a longer half-life than in SV3/L-infected cells (Fig. 6). Thus, dissociable empty capsids may be relatively stable and provide a higher effective concentration of capsid precursors than would otherwise be possible.

Neither the exact mechanism by which poliovirus capsid protein sequences are degraded nor the signals present on the various structures that determine their rates of degradation are known at present. It is of interest that many components of the ubiquitin-dependent degradation pathway are temperature regulated, as indeed are heat shock proteins (14). In this respect, it may be relevant that synthesis of GRP78, a member of the heat shock family, is not shut off in poliovirus-infected cells (26). Furthermore, GroEL, a bacterial hsp60 homolog, has been implicated in the virion assembly pathway of bacteriophage λ (7).

It is plausible that surfaces of lower-order structures that subsequently take part in protein-protein interactions in higher-order structures contain hydrophobic regions that are recognized as misfolded by cellular factors and are thus targeted for degradation. Alternatively, sequences that are disordered and accessible in lower-order structures may act as similar signals. Sequences in VP2 near the VP0 cleavage site and the N-terminal regions of VP1 and VP0 that are disordered in empty capsids (10) and presumably pentameric subunits may represent such signals. In protomeric subunits, the N terminus of VP3 would also be improperly folded (8a).

Nucleotide differences that influence attenuation phenotypes of Sabin vaccine strains of poliovirus have been identified in the 5′ noncoding region (2, 11, 16, 23, 32), P1 (2, 25, 32), and $3D^{POL}$ (2). The mutations in the 5′ noncoding region may affect translation efficiency (30), probably by RNA secondary structure perturbation (27). The mutation in $3D^{POL}$ has not been studied in detail but presumably affects either replication or P1 processing. The attenuating mutation at VP3-091 in P3/Sabin has been shown here to affect virion assembly. Other mutations in the capsid proteins have also been found to influence attenuation (15, 21) and assembly (14a) of P3/Sabin derivatives. As well as shedding light on one of the mechanisms of attenuation of poliovaccines, these studies may contribute to our understanding of the dynamics of poliovirus assembly and the sequences involved in its regulation.

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