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Poliovirus Mutant That Contains a Cold-Sensitive Defect in Viral RNA Synthesis

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By manipulating an infectious cDNA clone of poliovirus, we have introduced a single-codon insertion into the 3A region of the viral genome which has been proposed to encode a functional precursor of the virion-linked protein VPg. The resulting mutant was cold sensitive in monkey kidney cells. Viral RNA synthesis was poor at 32.5°C, although no other function of the virus was obviously affected. The synthesis of both positive and negative strands was severely depressed. Temperature shift experiments suggest that a normal level of production of the affected function was required only during the early (exponential) phase of RNA synthesis. Analysis of viral polyprotein processing at the nonpermissive temperature revealed that some of the normal cleavages were not made, most likely as a consequence of the defect in RNA synthesis or as a result of the concomitant reduction in the level of virally encoded proteases.

The cycle of poliovirus replication was described almost 20 years ago (for a review, see reference 1), but the molecular details of viral RNA synthesis are still not well understood. A major obstacle to further progress has been the inability to reproduce many aspects of viral replication with purified factors in vitro (4). Besides the role of viral RNA polymerase (protein 3D), the role of other viral factors in RNA synthesis is not clear. Although its exact function is not known, the 22-amino acid protein VPg (protein 3B) or a larger VPg-containing precursor polypeptide has been proposed to participate in RNA synthesis (3, 17). This suggestion arose from the observation that VPg is linked to the 5′ end of both plus and minus strands of RNA (12, 13), as well as to relatively small nascent chains (13). Although free VPg and uridylylated forms of VPg have been observed in infected cells (8, 18), protein 3AB and two much less abundant larger polypeptides which contain VPg at their carboxy termini have also been observed (3, 19). It has been suggested that one of these molecules, most likely 3AB, acts as a VPg donor during the replication process (3, 17). Protein 3AB may be analogous to the 85,000-dalton adenovirus protein that acts as a primer for the synthesis of viral DNA before being truncated to a 50,000-dalton genome-linked protein (7, 11).

Given the limitations of the biochemical studies, genetic dissection of the viral genome could provide an insight into the role of individual polypeptides in the replication process. The recent generation of an infectious cDNA copy of the virus (14) now makes it possible to overcome some of the difficulties of classical genetic approaches and to introduce well-defined mutations into the viral genome for the first time. In this report, we describe the physiology of a poliovirus mutant, designated 3A-2, which contains a lesion in the 3A region of the genome. Studies on the mutant confirm that this region is involved in viral replication and suggest that 3A or a 3A-containing polypeptide is required in a much smaller quantity than is normally produced during the course of a wild-type infection.

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MATERIALS AND METHODS

Cells and virus stocks. Vero cells (African green monkey kidney cells) were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Hazleton).

The method used to produce the wild-type poliovirus type I (Mahoney strain) stock and the DNA procedures used to generate and express the 3A-2 plasmid have been described previously (6). A master stock of mutant virus, used in all experiments, was produced by expanding a plaque obtained from a transfection by conventional means on HeLa cells at 39.5°C. HeLa cells were selected for the growth of mutant stocks, because the virus obtained from transfections had a phenotype that was closer to that of the wild type at high temperature on this cell line than on monkey kidney cells.

Measurement of virus yield and viral macromolecular synthesis. The methods used to monitor progeny virus yield and viral RNA and protein syntheses at various times during a single cycle of infection have been described (6). Total viral RNA synthesis was measured as the incorporation of [^{14}C]uridine into trichloroacetic acid-precipitable material in the presence of actinomycin D. The synthesis of individual RNA strands was measured by isolating cytoplasmic RNA and performing RNA dot blots according to procedures described previously (9, 15). The *Bam*HI fragment containing base pairs 670 to 2099 of the poliovirus genome was cloned into a Riboprobe vector (Promega), and the blots were probed with transcripts of this fragment synthesized in vitro with SP6 and T7 promoters.

Viral protein synthesis was analyzed by pulse labeling infected cells with [^{35}S]methionine and fractionating cell extracts by gel electrophoresis. In experiments in which protein synthesis was monitored in cells incubated at 32.5°C, the pulses lasted for 20 min instead of 10. Immunoprecipitations were performed essentially as described previously (3), except that samples were not diluted. Cell extracts were first preabsorbed with normal rabbit serum and then incubated for 1 to 2 h on ice with an anti-2C antiserum that also contains some activity against 3D-containing polypeptides (6). In all cases, proteins were resolved on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels by the method of Laemmli (10).

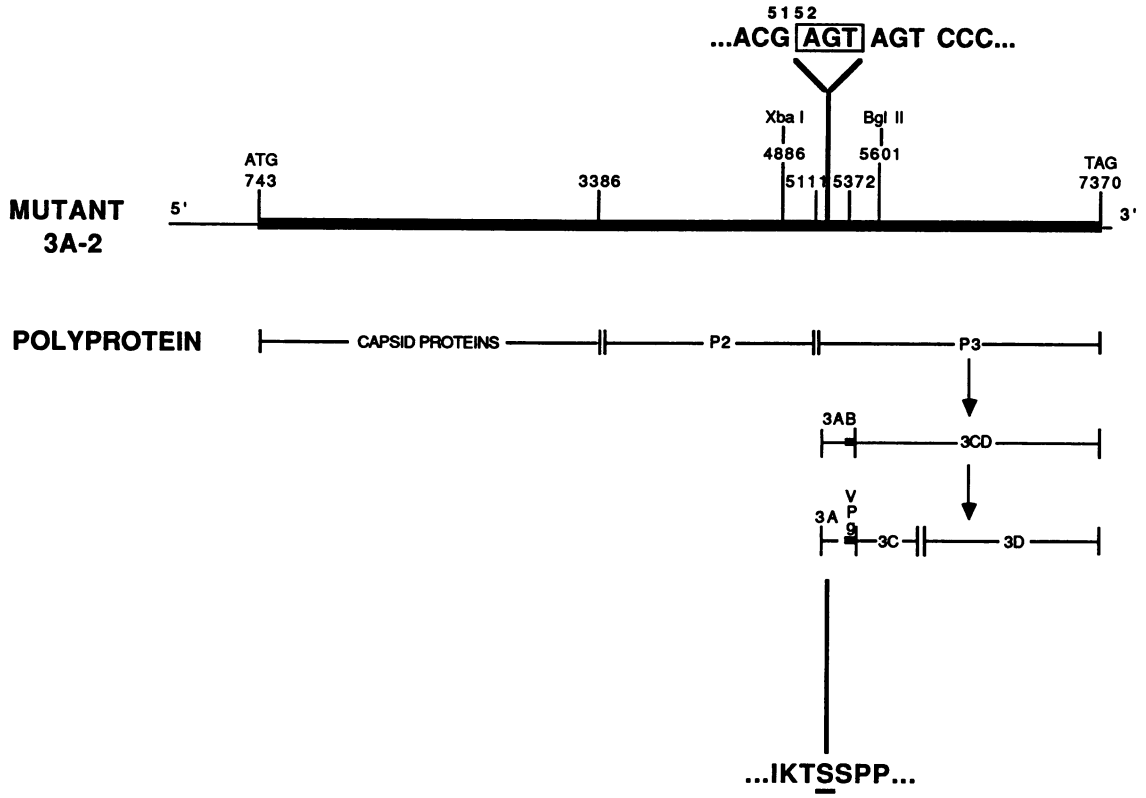

FIG. 1. Sequence of mutant 3A-2. Mutant 3A-2 was generated by using a mutagenesis scheme described previously (6). Restriction digest and sequence analyses revealed that three base pairs had been inserted into plasmid pSV2-polio at the *Hin*FI site at position 5152 of the poliovirus cDNA insert. Translation of the mutant RNA was predicted to result in the addition of an extra serine in the 3A region of the polyprotein as shown. The *Xba*I-*Bgl*II fragment (nucleotides 4886 to 5601) carries the mutant phenotype, and the expected sequence is indicated.

RESULTS

Generation of mutant 3A-2. Mutant 3A-2 is a single-codon-insertion mutant that was generated by a method described previously (6). Briefly, this method involves partial digestion of an infectious cDNA clone of poliovirus type I (Mahoney strain) with restriction enzymes that leave three-nucleotide protruding ends, filling in the ends with DNA polymerase I (Klenow fragment), and recircularization of the plasmid with DNA ligase. The position of the insertion was then mapped by digesting mutant plasmids to completion with the appropriate restriction enzyme and determining which recognition sequence had been lost. In the case of mutant 3A-2, the *Hin*FI site at position 5152 was observed to be missing (Fig. 1); hence the mutant was predicted to contain an extra amino acid in the 3A region of the genome. To confirm that the insertion was the only mutation in the plasmid, an *Xba*I-*Bgl*II fragment containing the insertion (Fig. 1) was first exchanged with the equivalent wild-type fragment and shown to confer the mutant phenotype. Then the mutant fragment was shown to have a nucleotide sequence which differed from the wild-type sequence only at the site of the insertion (Fig. 1).

Growth of 3A-2 in single cycle of infection. When monolayers of Vero cells were transfected with either mutant or wild-type DNA and incubated at 39.5°C, approximately the same numbers of plaques were observed on each plate. When the cells were incubated at 32.5°C, however, virtually no mutant plaques were seen, even after 1 week. The virus isolated from transfections was also cold sensitive for plaque formation on Vero cells. On Vero cell monolayers, an expanded (master) 3A-2 virus stock yielded plaques at 39.5°C that were large but noticeably smaller than wild-type plaques produced in parallel (Fig. 2A and B). However, when monolayers were inoculated with the amount of virus which yielded approximately 100 plaques at high temperature and then incubated at 32.5°C until wild-type plaques were relatively large, only a few pinpoint lesions were observed (Fig. 2C and D). When 10 or 100 times as much mutant virus was added, a few larger plaques, presumably produced by variants which existed in the original population or which arose during the plaque assay, were observed (Fig. 2E and F). The titer of moderate-size and large plaques was several hundredfold below that observed at 39.5°C.

At 39.5°C, mutant progeny accumulated slightly more slowly than did wild-type virions, but by the end of the mutant growth phase (5 to 6 h), 3A-2-infected cells contained only two to three times fewer progeny than did wild-type-infected cells. This discrepancy and the slight lag in growth could explain the difference in the sizes of wild-type and mutant plaques. If we consider the slower metabolism of cells at the lower temperature, the kinetics of progeny virus formation in wild-type-infected cells is very similar to that observed at high temperature. With mutant-infected cells, however, several hundredfold less virus was produced by midcycle (5 to 6 h) than by the equivalent time at high temperature (3 to 3.5 h). More than 90% of the virus had a mutant phenotype. By the end of the cycle (8 h), the discrepancy dropped to less than 50-fold, but most of the

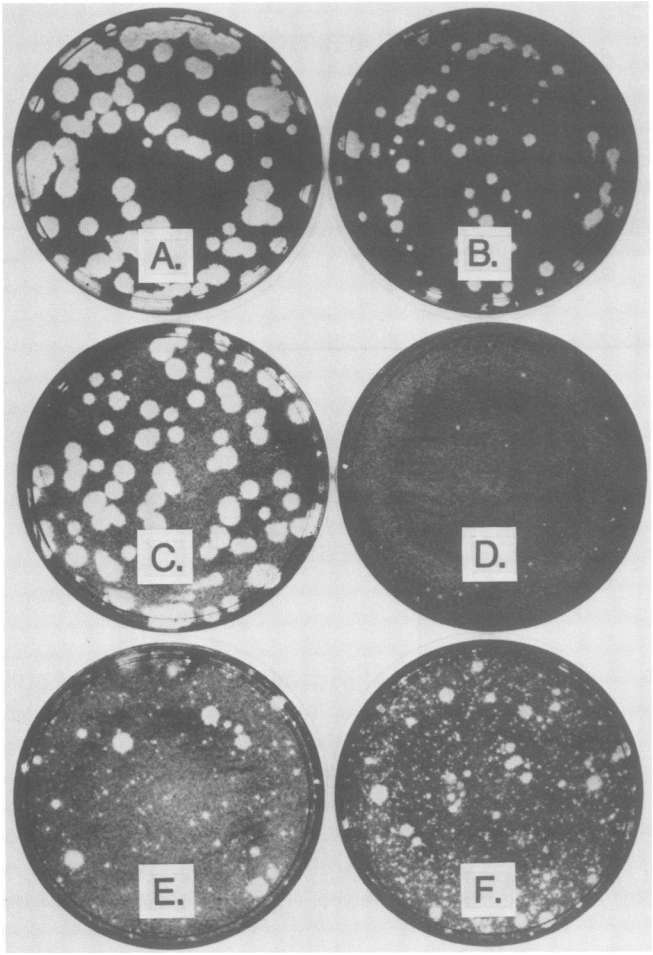

FIG. 2. Phenotype of mutant 3A-2. In the plaque assay, Vero cells were inoculated with wild-type or mutant virus from master stocks and incubated at 39.5°C (A and B) or 32.5°C (C through F) for approximately 40 or 60 h, respectively, and then stained. (A and C) Wild type; (B and D to F) mutant 3A-2. Cells shown in panel E were inoculated with 10 times as much virus as those shown in panels B and D, and cells shown in panel F were inoculated with 100 times as much virus.

titer consisted of variants which gave large plaques at all temperatures. Presumably, the predominance of these "revertant" viruses was due to their ability to grow much more rapidly than did the mutant at low temperature. A preliminary analysis of cDNA produced from two revertant virus strains indicated that they harbor both the original insertion and a second-site mutation (in at least one case within 3A) that suppresses the mutant phenotype (data not shown). The presence of a second-site mutation is consistent with the observation that the revertant plaques are slightly smaller than those produced by the wild-type virus. Given the high frequency with which revertants arise at low temperature, it is likely that a single mutation is sufficient to correct the initial defect.

RNA synthesis in 3A-2-infected cells. In Vero cells, mutant 3A-2 synthesized RNA effectively at the permissive temperature, though not quite as well as did the wild-type virus (Fig. 3A). The slight impairment of RNA synthesis correlated well with and probably explains the slightly lower yield of infectious virus in the mutant-infected cells. Another indication that mutant RNA synthesis was nearly normal was the fact that the same proportions of full-length single-stranded and double-stranded RNAs were found in both mutant- and wild-type-infected cells (data not shown). At the nonpermissive temperature, very little viral RNA was produced in 3A-2-infected cells; after 8 h postinfection, a small amount of RNA, probably produced predominantly by revertant virus, could be detected (Fig. 3A).

To determine whether the 3A-2 defect selectively affected the synthesis of plus- or minus-strand RNA, cytoplasmic RNA was isolated from Vero cells that had been infected with wild-type or mutant virus and then incubated for 3.25 and 4.5 h at 32.5°C. RNA was not isolated at later times because of the substantial accumulation of revertant virus after 6 h. The level of each strand was assessed by an RNA dot blot. In these experiments, the fraction of plus-strand RNA derived from residual noninfectious input virus was measured by isolating RNA from cells treated after virus adsorption with guanidine, a reagent which totally inhibits viral replication but does not alter cellular metabolism at millimolar concentrations (1). Significant plus-strand synthesis could be observed in wild-type-infected cells by 3.25 h postinfection. In contrast, almost no new plus strands had been made by that time in mutant-infected cells; most of the RNA was derived from input virus (Fig. 3B). By 4.5 h postinfection, at least 30-fold more plus strand and at least 20-fold more minus strand had been synthesized in wild-type cells compared with mutant-infected cells (Fig. 3B and C). These results indicate that the synthesis of both RNA strands is affected to approximately the same extent.

The inhibition of host cell transcription was effective, although slightly delayed, in mutant-infected Vero cells at the nonpermissive temperature (data not shown). Hence the lack of viral RNA synthesis does not appear to be related to a defect in this inhibitory function.

Temperature shift experiments were performed to examine the reversibility of the 3A-2 defect. When Vero cells were incubated at 32.5°C for 5 h and then shifted to 39.5°C, efficient RNA synthesis was observed after the shift (Fig. 4). Ultimately, the same amount of viral RNA was produced in these cells as that in cells which had been incubated continuously at high temperature. The linear phase of RNA production began within 1 h of the shift; the efficient inhibition of host cell translation (see below) and transcription and low-level RNA synthesis during the incubation at 32.5°C (Fig. 3) probably accounts for this rapid progression to the second stage of replication.

When cells were incubated at high temperature until the approximate onset of the linear phase of replication (3 h) and then shifted to the nonpermissive temperature, RNA synthesis continued normally, as if the shift had not been performed (Fig. 4). Analysis of the RNA produced after the shift by agarose gel electrophoresis indicated that the product contained normal proportions of full-length single-stranded and double-stranded RNAs (data not shown). Hence it is possible that the 3A-2 defect is in a function which acts only early in the life cycle of the virus or that the protein made at 39.5°C is stable at low temperature and continues to function after the shift. Because a large amplification of viral RNA molecules occurred before the shift, enough templates were available to engage in a high level of viral protein synthesis after the shift (data not shown). For this reason, it might also be argued that even if a small fraction of the relevant protein is active at low temperature or even if the defective protein has a low level of residual activity at 32.5°C, enough total protein was produced to support continued RNA synthesis.

Protein synthesis in mutant-infected cells. Sodium dodecyl

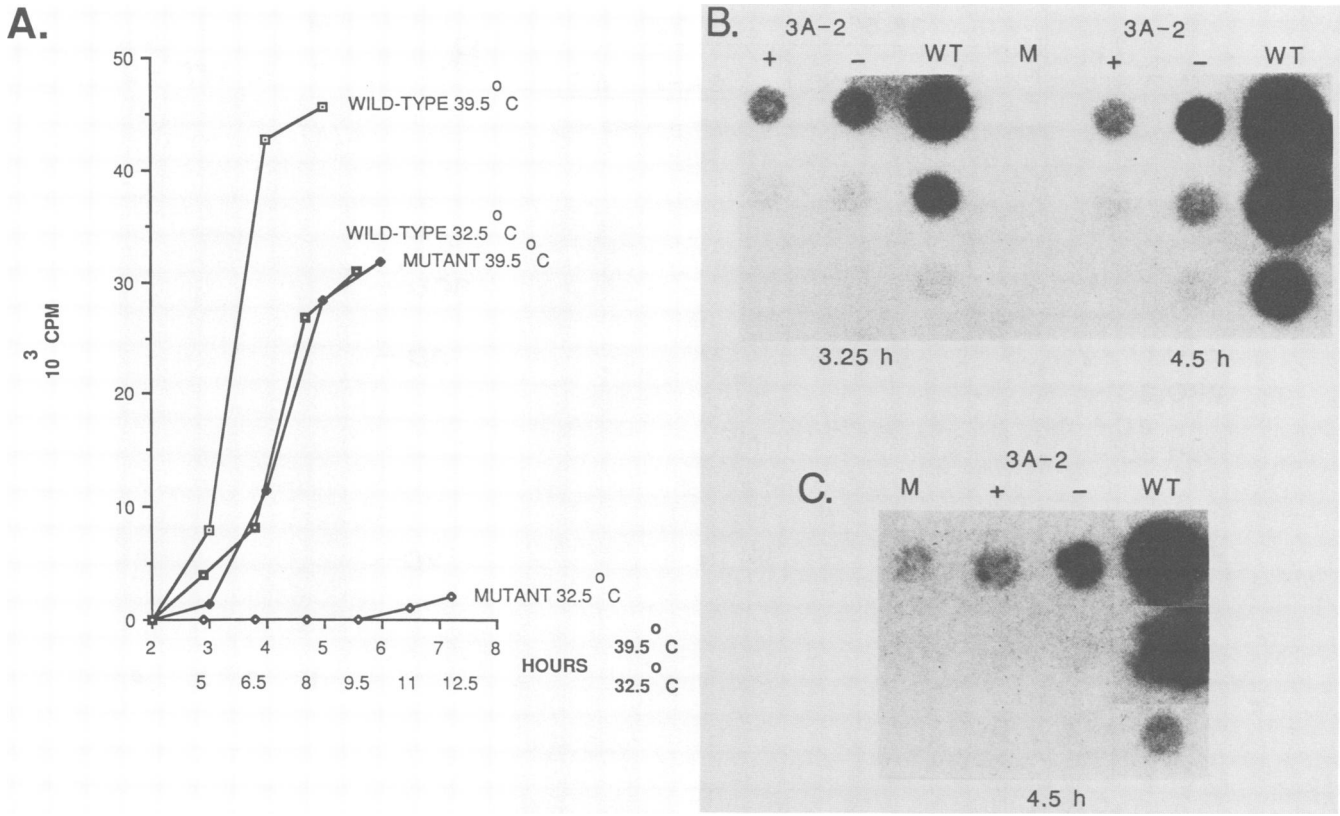

FIG. 3. Time course of RNA synthesis in wild-type- and mutant 3A-2-infected cells at 39.5 and 32.5°C. (A) Vero cells were infected at an MOI of 10 and then incubated at 39.5 or 32.5°C. RNA synthesis was measured at different times postinfection as described in the text. Symbols: ⊡ or □, wild-type RNA synthesis at 39.5 or 32.5°C, respectively; ◆ or ◇, 3A-2 RNA synthesis at 39.5 or 32.5°C, respectively. To compensate for the slower metabolism of cells at low temperature, the 32.5°C incubation times have been divided by 1.7 and then aligned to the scale used for the 39.5°C incubation times. (B and C) Vero cells were infected at an MOI of 5 and incubated at 32.5°C for 3.25 or 4.5 h. Cytoplasmic RNAs obtained from 2×10^6, 4×10^5, and 8×10^4 cells were loaded into the wells. The probe detected plus-strand (B) or minus-strand (C) poliovirus RNA. M, Mock infected; 3A-2 –, mutant infected; 3A-2 +, mutant infected and treated with 1 mM guanidine after virus adsorption; WT, wild type infected.

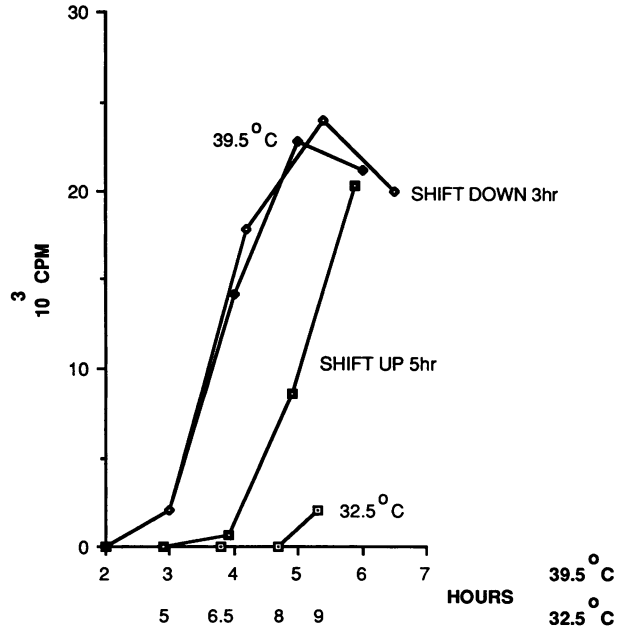

FIG. 4. RNA synthesis in 3A-2-infected Vero cells after temperature shift. Vero cells were infected at an MOI of 5 and then incubated at 39.5 or 32.5°C. After 3 h, half of the dishes incubated at high temperature were shifted to low temperature; after 5 h, half of the dishes incubated at low temperature were shifted to high temperature. The remainder of the dishes were incubated at the original temperature for the duration of the experiment. RNA synthesis was measured as described in the legend to Fig. 3A. Symbols: ◆, RNA synthesis at 39.5°C; ◇, RNA synthesis after a shift down at 3 h postinfection; ⊡, RNA synthesis at 32.5°C; □, RNA synthesis after a shift up at 5 h postinfection. Temperature compensation is as described in the legend to Fig. 3.

sulfate-polyacrylamide gel electrophoresis of extracts of Vero cells pulse labeled with [^{35}S]methionine showed that the patterns of proteins produced by wild-type- and 3A-2-infected cells at 39.5°C were the same (Fig. 5A, lanes 3 to 6). The cleavage of polyprotein appeared to be normal in the mutant-infected cells at this temperature. It should be noted that when poliovirus-infected Vero cells were pulse labeled after host translation was inhibited, cell extracts reproducibly contained a number of radioactive polypeptides that were not observed in extracts prepared from other cell types (Fig. 5A, compare the HeLa cell extract in lane 1 and the Vero cell extracts in the other lanes). These polypeptides are probably degradation products of viral proteins generated by

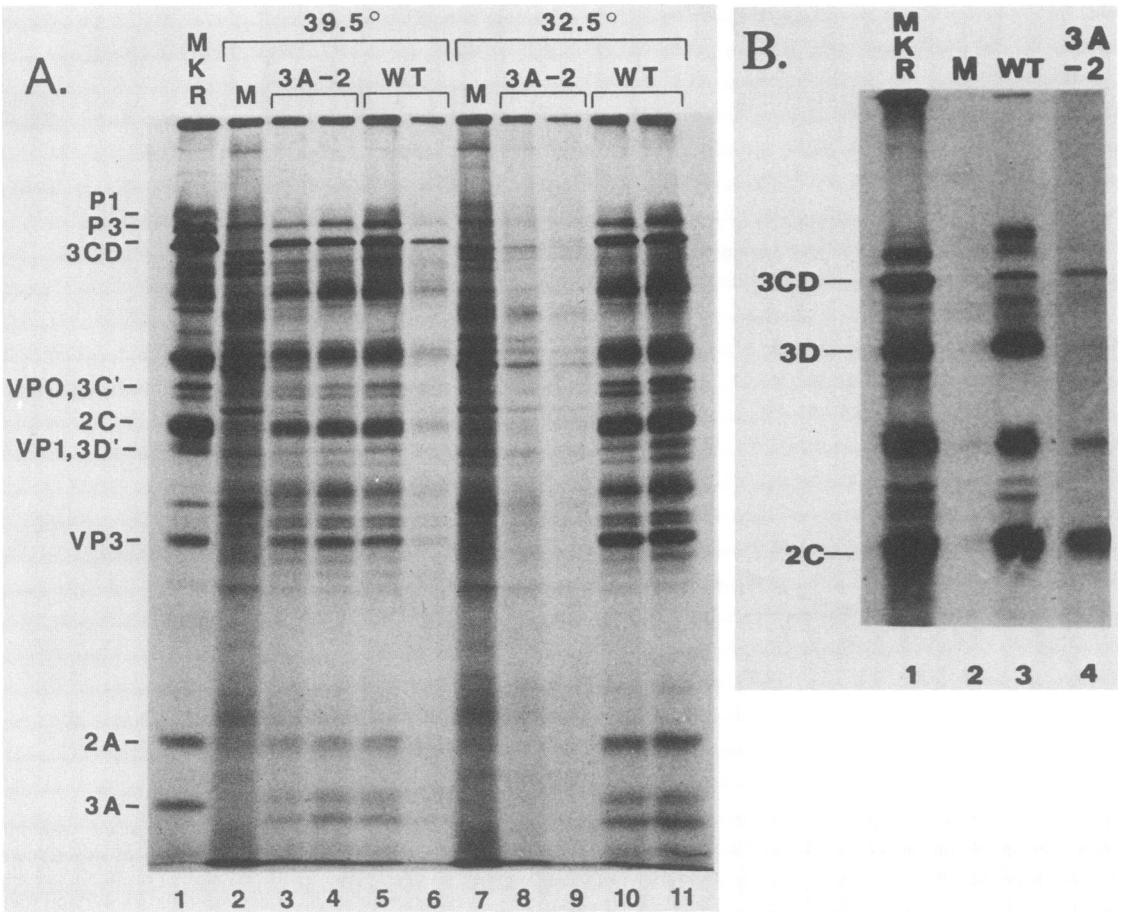

FIG. 5. Patterns of protein synthesis in mock-, mutant 3A-2-, and wild-type-infected Vero cells at high or low temperature. (A) Vero cells were infected at an MOI of 10 and incubated at 39.5 or 32.5°C. Cells were pulse labeled with [^{35}S]methionine at various times, and cell extracts were prepared. Portions of each extract (20 μl) were resolved by gel electrophoresis. Lane 1, Markers (MKR; pulse labeled wild-type-infected HeLa cell extract); lanes 2 to 6, extracts from mock- (M), mutant- (3A-2), and wild-type- (WT) infected cells incubated at 39.5°C and labeled at 2.75 (lanes 2, 3, and 5) or 3.75 (lanes 4 and 6) h postinfection; lanes 7 to 11, extracts from mock-, mutant-, and wild-type-infected cells incubated at 32.5°C and labeled at 4.5 (lanes 7, 8, and 10) or 6 (lanes 9 and 11) h postinfection. (B) Vero cells were infected at an MOI of 10 and incubated at 32.5°C for 4.5 h. The cells were labeled and processed as described for panel A. An anti-2C antiserum was used to immunoprecipitate proteins from 60 μl of mock- and wild-type-infected cell extracts and 180 μl of mutant-infected cell extracts. As noted elsewhere (5), this antiserum also contains some activity against 3D-containing polypeptides. The lanes are marked as described for panel A. The marker lane contained 20 μl of a pulse-labeled wild-type-infected Vero cell extract. Lane 4 was exposed for over 30 times longer than the rest of the gel.

an enzyme active in the extracts (D. Knipe, personal communication).

Assays which measured [^{35}S]methionine incorporation into trichloroacetic acid-precipitable material indicated that at 32.5°C a parallel decline in the rate of protein synthesis occurred in mutant- and wild-type-infected cells in the first 4.5 h after infection. In the mutant-infected cells, however, the decline was sometimes slightly slower (data not shown). Electrophoretic analysis of the proteins produced at 4.5 and 6 h postinfection showed that whereas mainly poliovirus proteins were synthesized in wild-type-infected cells (Fig. 5A, lanes 10 and 11), there was primarily residual host cell protein synthesis in mutant-infected cells (Fig. 5A, compare lane 7 with lanes 8 and 9). This result was not unexpected, given the very small pool of viral RNA molecules present in mutant-infected cells.

An extremely low level of poliovirus protein synthesis (clearly less than 1/50 of the normal level) could be detected in 3A-2-infected cells at 4.5 h postinfection by immunoprecipitation (Fig. 5B, lane 4). The pattern of immunoprecipitated proteins indicated that most of the cleavages in the P2 and P3 regions of the polyprotein were made normally (Fig. 5B, compare lanes 3 and 4). In particular, the mutation in 3A did not appear to prevent the cleavage of 3CD from P3. To prove that this cleavage was not affected by the low level of revertants present in the 3A-2-infected cells at 4.5 h postinfection, we examined protein synthesis in cells infected at a multiplicity of infection (MOI) of 100 and treated with 1 mM guanidine after virus adsorption. In the absence of replication, viral protein synthesis was readily detectable and very similar in mutant- and wild-type-infected cells. Although only a few percentage of cells was likely to contain revertant RNA molecules under these conditions, 3CD was still cleaved from P3 as effectively in mutant- as in wild-type-infected cells (data not shown).

The absence of protein 3D in mutant-infected cells (Fig. 5B, lane 4), however, suggested that the cleavage of 3CD into 3C and 3D was impaired. A similar absence of 3D has also been noted in cells infected by the temperature-sensitive mutant 3NC-202 and has been shown to result from the

reduced processing of 3CD (P. Sarnow, unpublished results). The processing problem observed in that case is probably not due to improper folding of the polyprotein, because the only lesion is in the 3′ noncoding region of the genome (16). The lack of 3CD cleavage has been attributed to the inability of the mutant to replicate and to the reduced level of virally encoded proteases at the nonpermissive temperature (P. Sarnow, unpublished results). Given these observations, the unusually low level of 3D in 3A-2-infected cells at 32.5°C is more likely due to an alteration of polyprotein processing common to highly replication-deficient mutants than to a specific defect in protein folding that prevents excision of 3D from its precursor. The results presented here, considered with the studies on mutant 3NC-202, suggest that the 3A-2 mutation does not suppress RNA synthesis by perturbing polyprotein processing. Hence the mutation probably disrupts the function of 3A or one of its precursors rather than a distant segment of the polyprotein.

DISCUSSION

We have begun to examine a poliovirus mutant that contains a small insertion in the 3A region of the genome. The primary consequence of the mutation is a severe depression of both positive- and negative-strand RNA synthesis at low temperature. Studies on mutant 3A-2 strongly suggest that at least one 3A-containing polypeptide is involved in replication. Presumably, because very few viral RNA molecules were synthesized at low temperature, the yield of infectious virus was very low. Experiments in which the temperature was shifted up indicate that productive infection is not inhibited by maintaining mutant 3A-2 at the nonpermissive temperature, at least for several hours. Experiments in which the temperature was shifted down suggest either that production of the function impaired by the mutation during the exponential phase of replication is sufficient to generate a full yield of RNA or that continuing production of the function is also required during the linear phase, but at a level far below that produced during a wild-type infection. In either case, the implication of these results is that an excess of the affected function is produced by wild-type virus. Analysis of the viral proteins present in mutant-infected cells also suggests that certain cleavages of the polyprotein may depend on active RNA synthesis.

It is conceivable that a function involved in poliovirus replication would be required primarily early in the virus life cycle; a 10^3-fold amplification of RNA molecules occurs during the exponential phase of synthesis, whereas only a 5- to 10-fold increase occurs during the linear phase (2). Although all of the poliovirus proteins are produced throughout the life cycle, there is some precedent for early and late functions. The protein which mediates the suppression of host cell translation must function early and may have no function at all late in infection. In addition, the encapsidation of viral RNA molecules seems to occur predominantly late, after the onset of the linear phase of replication (2).

There is also direct evidence that much more protein is synthesized during the course of poliovirus infection than is required for a full round of RNA synthesis (6). The results of complementation experiments support the hypothesis that the wild-type virus overproduces the specific function disrupted by the 3A-2 mutation; a mutant that produces only about 10% as much RNA and protein as the wild-type virus can still complement 3A-2 to a moderate extent (5). The complementation data nevertheless suggest that the mutation in 3A-2 affects a protein which is required in greater than very small quantities; there is a correlation between the amount of protein a mutant produces and the extent to which it can rescue 3A-2.

Finally, the generation of 3A-2 demonstrates that highly cold-sensitive mutants of poliovirus can be obtained. Only a small number of very unstable cold-sensitive mutants, compared with scores of stable temperature-sensitive mutants, have been isolated by classical methods (20). The isolation of a stable cold-sensitive mutant by insertion mutagenesis might be an indication that different types of mutations tend to give rise to different classes of mutants.

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