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Site-Directed Mutagenesis of Proteinase 3C Results in a Poliovirus Deficient in Synthesis of Viral RNA Polymerase

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We used a synthetic double-stranded oligonucleotide to introduce amino acid substitutions into the proteinase 3C region of a poliovirus type 1 cDNA clone. The six different mutant viruses recovered exhibited a small-plaque phenotype when assayed on HeLa cells. Further investigation revealed that all the mutations (with the exception of one) yielded P3 region proteins that displayed altered mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A conservative Val → Ala change at amino acid 54 of the proteinase resulted in a virus that was deficient in the production of the mature viral RNA polymerase 3D. Although this mutant achieved less than one-half of the wild-type levels of RNA synthesis during the course of infection, it still grew to nearly wild-type titers.

Polioviruses, and the picornaviruses as a group, are distinguished by small nonenveloped virions which contain a single plus-stranded RNA genome of approximately 7.5 kilobases in length. Immediately after adsorption and uncoating, this RNA can associate with the cellular translational machinery to direct the synthesis of virus-specific proteins. For poliovirus, the initial translation product derives from a single large open reading frame between nucleotides 743 and 7370 of the genome that, were it not nascently and cotranslationally cleaved by two virus-encoded proteinases, would yield a giant polyprotein of 247 kilodaltons. Since all viral proteins synthesized during the course of infection arise from this initial precursor, the differential and temporal control of gene expression must reside at the level of protein processing.

The specific sites of proteolytic processing within the polyprotein have been determined by amino- and carboxy-terminal sequence analyses of cleaved products (22, 30). Most cleavages of the precursor occur between Gln-Gly pairs and are mediated by the viral proteinase 3C (12; Fig. 1). Only 9 of a possible 13 such Gln-Gly sites are ever cleaved during a poliovirus infection. Therefore, determinants other than the mere presence of these amino acids are necessary for the recognition of the site as a substrate for 3C. The nascent cleavage between regions P1 and P2 takes place at a Tyr-Gly pair and is carried out by a second viral proteinase, 2A (33). Processing of another Tyr-Gly site in the P3 region gives rise to 3C′ and 3D′, cleavage alternates of 3C and 3D. Eight other Tyr-Gly pairs, predicted by the nucleotide sequence of the genome, are apparently never used as cleavage sites.

To study the enzyme-substrate relationships of the Gln-Gly cleavages, we have taken a molecular genetic approach to the mutagenesis of the proteinase 3C. Classically, the genetic analysis of poliovirus has taken advantage of both naturally arising variants and chemically induced mutations. However, these strains often contain multiple mutations, which could only be tediously mapped to general regions of the genome by recombination. The elucidation of the complete nucleotide sequence of the viral RNA (16, 24) and the isolation of infectious cDNA clones of the type 1 poliovirus genome (21, 25, 31) have made possible the generation of viable mutant viruses that possess site-specific mutations within the coding (2, 3) and noncoding (26, 29, 32) regions of the genome.

Mutagenesis of 3C that yields viable virus has proved to be particularly difficult because of the highly conserved nature of this region of the genome and the pleiotropic effects that such mutations may have on the overall proteolytic processing and replication of the virus. In fact, no infectious virus mutants resulting from the site-specific mutagenesis of the proteinase have been generated to date. We have used a double-stranded oligonucleotide to introduce a number of conserved and nonconserved amino acid substitutions into 3C and report the isolation of six viable proteinase mutants. These mutants displayed a small-plaque phenotype and exhibited altered electrophoretic mobility of the P3 region polypeptides in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Of primary interest, a Val → Ala substitution at amino acid 54 of 3C resulted in a mutant that was deficient in RNA synthesis, presumably as a consequence of the low levels of 3C and 3D produced.

MATERIALS AND METHODS

Construction of mutant plasmids. The plasmid pSVP37-5 (Fig. 2) was digested to completion with *Bgl*II and *Cla*I. The large (10.5-kilobase) fragment was purified on a preparative 1% agarose gel. Synthetic deoxyoligonucleotides, corresponding to the region of the poliovirus genome between nucleotides 5584 and 5605, were purified separately on a 10-ml Sephadex G-50 column equilibrated with 10 mM TEAB [pH 7] (triethylammonium bicarbonate). Complementary pairs of oligonucleotides, each containing a degenerate nucleotide at position 5589, 5592, or 5598, were annealed for 5 min at 80°C, 5 min at 37°C, and 5 min at room temperature. An additional pair of complementary oligonucleotides contained the deletion of nucleotides 5597 to 5599, corresponding to the loss of the Val codon at amino acid position 54. A 200-fold molar excess of each double-stranded oligonucleotide was ligated with the 10.5-kilobase *Cla*I-*Bgl*II vector fragment and transformed into *Escherichia coli* C600 to obtain plasmids containing the full-length poliovirus cDNA bearing a single site mutation in 3C. Transformants were screened for the presence of the insert by the digestion of

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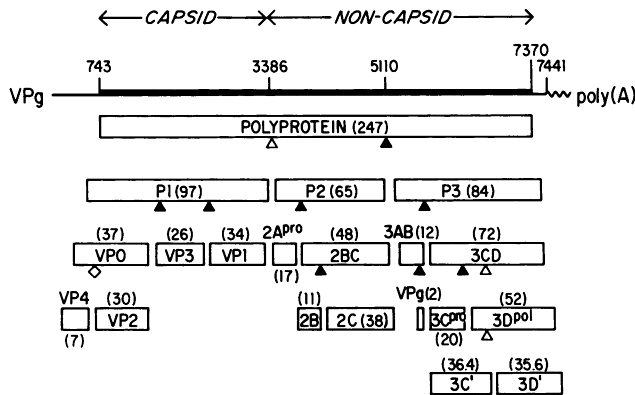


FIG. 1. Protein processing of polypeptides encoded by poliovirus RNA. The horizontal line represents the poliovirus genomic RNA. The portion of the RNA between nucleotides 743 and 7370 (bold line) denotes the polyprotein coding region. The open rectangles beneath the RNA represent the virus-specific polypeptides that resulted from processing of the precursor polyprotein. Symbols: ▲, Gln-Gly cleavage sites; △, Tyr-Gly cleavage sites; ◇, Asn-Ser cleavage site of the capsid precursor, VP0. The molecular weights ($\times 10^3$) of the polypeptides are provided in parentheses.

rapid plasmid preparations (4) with *Bgl*II. The nucleotide sequence of the resulting mutant plasmids was verified by limited chemical degradation of CsCl-pure plasmid DNA (19).

Transfections. Subconfluent monolayers of COS-1 cells were transfected with 0.1 to 5 μg of CsCl-purified plasmid DNAs by the calcium phosphate precipitation technique (10) with a 3-min glycerol boost (9, 23). For 60-mm plates, 0.1 to 5.0 μg of mutant plasmid DNA was transfected with sufficient salmon sperm DNA carrier to total 10 μg of DNA per plate. After transfection (4 to 6 h), monolayers were overlaid with either semisolid medium consisting of Dulbecco modified Eagle medium, 6% fetal calf serum, and 0.45% agarose or liquid medium consisting of Dulbecco modified Eagle medium with 10% fetal calf serum.

Virus stocks and plaque assays. Mutant virus stocks were prepared by picking well-isolated plaques under semisolid agar from COS-1 monolayers on day 4 posttransfection. The plaques were then expanded by two serial passages through HeLa cell monolayers. When no plaques were observed in the initial transfection, the transfection was repeated with a liquid rather than with a semisolid overlay. On day 4 posttransfection, the liquid supernatants were harvested and used to infect HeLa monolayers, which were then overlaid with semisolid agar. Plaque assays of mutant virus stocks were carried out in 60-mm plates of HeLa cell monolayers under semisolid medium. Cells were incubated at 33 or 37°C for 2 to 3 days and stained with crystal violet (14).

Sequencing of mutant viral RNA. Mutant viral RNA was prepared by the method of Harris et al. (13) with the following modifications. HeLa S3 cells (100 ml, with 5×10^6 cells per ml) in suspension culture were infected with mutant virus at a multiplicity of infection of 1 to 10. Infections were incubated from 8 to 9 h, after which the cells were pelleted, suspended in hypotonic buffer (10 mM Tris hydrochloride [pH 7.35], 10 mM NaCl, 1.5 mM $MgCl_2$), and subjected to five cycles of freezing and thawing to release virus. Cell debris and nuclei were pelleted by centrifugation. The supernatant was adjusted to a final concentration of 1% SDS and 2 mM EDTA and centrifuged overnight in a 60 Ti rotor at $60,000 \times g$ (24°C) to pellet virus. The virus pellet was then suspended in 0.1 buffer (0.1 M NaCl, 10 mM Tris hydrochloride [pH 7.5], 5 mM EDTA, 0.5% SDS) and layered onto a 15 to 30% sucrose gradient and centrifuged in a SW27 rotor for 2.5 h at $90,000 \times g$. Fractions representing peak A_{260} were pooled, phenol-chloroform extracted, and ethanol precipitated. In some cases the viral RNA was further purified on a second 15 to 30% sucrose gradient by centrifugation for 4.5 h at $200,000 \times g$ in an SW41 rotor. Sequencing of the viral RNA was performed by the annealing of a deoxyoligonucleotide 20-mer primer complementary to nucleotides 5735 to 5754 and by extension with [α-^{32}P]dATP, dideoxynucleotides, and reverse transcriptase (6, 11, 28).

Labeling of infected cells with [^{35}S]methionine. Nearly confluent HeLa cell monolayers were infected with mutant virus or transfection-derived wild-type virus (type 1 Mahoney) at a multiplicity of 25. Virus was allowed to adsorb at room temperature for 30 min, after which the cells were overlaid with methionine-free minimal essential medium plus 10% fetal calf serum. Infected monolayers were pulsed for 1-h intervals with 50 μCi of [^{35}S]methionine

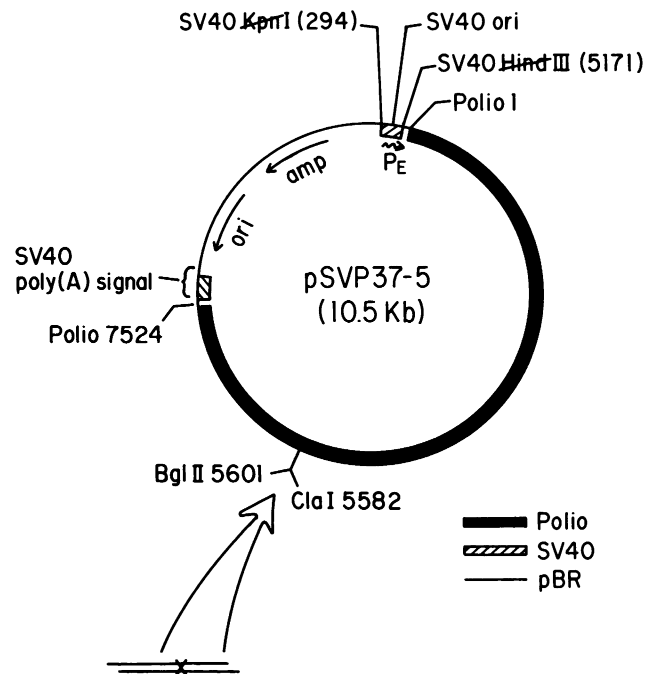


FIG. 2. pSVP37-5 is a eucaryotic expression vector containing the full-length poliovirus type 1 cDNA. The poliovirus sequence has been inserted into the blunted *Cla*I site of pBR322 such that the site is not regenerated. The 363-base-pair fragment of simian virus 40 which contains the early promoter, transcriptional enhancers, and origin of replication has been cloned into the pBR322 *Eco*RI site. The lines drawn through these restriction sites indicate that they were not regenerated in the cloning procedure. The details of the steps involved in the isolation of viable mutant poliovirus are provided in Materials and Methods.

beginning at 1 h postinfection. At the end of the labeling period, the cells were washed twice with phosphate-buffered saline and suspended in Laemmli sample buffer. For pulse-chase labeling experiments, 5 ml of HeLa suspension cultures (5×10^6 cells per ml) were infected at a multiplicity of 25 in methionine-free minimal essential medium. After a 30-min period at room temperature with occasional agitation of the infection mixture, the cells were pelleted by centrifugation and suspended in the same volume of methionine-free MEM with 10% fetal calf serum–glutamine–25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.25). The infected cell suspensions were incubated at 37°C with constant stirring for 4 h. At this time the cells were given a 10-min pulse of 60 μCi of [^{35}S]methionine, which was followed by a chase of unlabeled L-methionine at a final concentration of 0.1 mM. Immediately after the chase, samples representing 10^6 cells were harvested at 15-min intervals (up to 75 min postchase), washed twice with phosphate-buffered saline, and suspended in Laemmli sample buffer. Lysates were boiled and analyzed on 12.5% SDS-PAGE gels (17). Gels were fluorographed and exposed to XAR film (5).

Immunoprecipitation of infected cell extracts. Lysates from infected cells pulse-labeled with [^{35}S]methionine between 4 and 5 h postinfection were boiled and diluted 10-fold with ice-cold extraction buffer (50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 100 U of Trasylol per ml) to a final volume of 400 μl. The diluted lysates were preadsorbed with 20 μl of a 10% suspension of *Staphylococcus aureus* and immunoprecipitated with antisera previously prepared to polypeptide 2C or 3C (12) for 1 h on ice. The immune complexes were recovered by the addition of 40 μl of *S. aureus* and washed three times with SNNTE (50 mM Tris hydrochloride [pH 7.4], 500 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 5% sucrose) and one time with NTE (10 mM Tris hydrochloride [pH 7.4], 50 mM NaCl, 1 mM EDTA). The immunoprecipitates were suspended in Laemmli sample buffer, boiled, and centrifuged to separate the *S. aureus* cells. The supernatants were electrophoresed on 12.5% SDS-PAGE gels, which were then fluorographed and exposed to XAR film.

Labeling of infected cells with [^{3}H]uridine. HeLa suspension cultures (5 ml, with 5×10^6 cells per ml) in serum-free minimal essential medium were infected with wild-type or mutant virus at a multiplicity of 25 PFU per cell. After incubation for 30 min at room temperature with occasional agitation, the cells were centrifuged and suspended in an equal volume of minimal essential medium containing 8% newborn calf serum and 25 mM HEPES (pH 7.25) and incubated at 37°C with constant stirring. After infection (2 h), actinomycin D (5 μg/ml) was added to inhibit host cell DNA-directed RNA synthesis. At 2 h 15 min postinfection, cells were labeled with 50 μCi of [^{3}H]uridine. Immediately after labeling, 200-μl samples of the infected cells were harvested at specific times and were washed with 1 ml of phosphate-buffered saline, suspended in 1 ml of H_2O, vortexed vigorously to break up cells, and frozen at −20°C. The extracts were thawed and precipitated on ice with the addition of 250 μl of 50% trichloroacetic acid. Precipitates were collected onto glass fiber filters, and the radioactivity of the samples was determined by liquid scintillation.

RESULTS

Construction of amino acid substitutions in plasmid pSVP37-5. The general scheme for the isolation of infectious poliovirus cDNA clones bearing single amino acid substitutions in 3C is provided in Fig. 2. The region of the proteinase targeted for mutagenesis lies between unique *Cla*I and *Bgl*II sites at nucleotides 5582 and 5601, respectively. On the basis of amino acid sequence comparison among a number of the picornaviruses, this region of 3C contains a highly conserved Gly residue at position 51, which may form a hinge in the secondary structure of the protein (1). Also present are a number of unconserved residues which, upon mutagenesis, might be more likely to yield viable virus. The *Cla*I-*Bgl*II site is contained within the amino half of 3C, which is overall less conserved than the carboxy half, where the putative active site of the enzyme resides (1).

Four pairs of complementary oligonucleotides were synthesized that, when annealed, formed mini-restriction fragments with sticky ends that could be cloned into the *Cla*I-*Bgl*II site of the plasmid pSVP37-5. Three of the pairs were designed such that a random nucleotide was introduced at position 5589, 5592, or 5598, corresponding to position 2 of codons 51, 52, or 54, respectively, in 3C. It is conceivable that each oligonucleotide pair could give rise to three different mutations in addition to regenerating the wild type. The potential amino acid substitutions that this strategy would yield are summarized in Fig. 3. A fourth double-stranded oligonucleotide contained a deletion of the Val codon at position 54.

Plasmid pSVP37-5 is a derivative of the infectious cDNA clone, pEV104, which has been previously described (31). In this construct, the poliovirus cDNA was cloned into the *Cla*I site of a pBR322 derivative that had been blunted with the Klenow fragment of DNA polymerase I, leaving the *Cla*I site in the poliovirus sequence as a unique site. Synthesis of viral RNA proceeded under the direction of the simian virus 40 early promoter and transcriptional enhancers. The plasmid also contained the simian virus 40 origin of replication, which allowed for the amplification of the plasmid upon transfection into COS-1 cells. pSVP37-5 was grown up in a *dam*$^-$ *E. coli* strain so that sequences immediately outside the *Cla*I recognition site that interfered with digestion would be unmethylated. The vector was digested completely with *Cla*I and *Bgl*II and then ligated with a given pair of complementary oligonucleotides. Transformants were screened as described in Materials and Methods. Plasmids were sequenced across the substituted region to screen for the presence of a specific mutation. The sequencing data demonstrated that we were able to generate all the predicted mutations and regenerate wild-type cDNA with this method (data not shown).

Transfection of COS-1 cells with mutant plasmid cDNA and recovery of mutant virus. To determine whether the mutant cDNA clones would yield infectious virus, we transfected the plasmid DNA onto COS-1 monolayers. After 4 to 5 days at 33 or 37°C, representative plaques were picked and expanded through two serial passages in HeLa monolayers. In cases where no plaques were observed within 1 week, the transfection was repeated and the cells were maintained under liquid medium. The supernatants were harvested 5 days after transfection and analyzed for virus growth on HeLa monolayers. The results of the initial transfections are briefly summarized in column 2 of Table 1. In cases where viable virus was recovered, the mutant virus was assigned a name. The nomenclature for designation of mutant viruses derived from poliovirus cDNA (as suggested by Racaniello and Meriam [26]) is explained as follows: all viable mutants from our laboratory are given the prefix Se1 (1 indicates type 1 Mahoney), 3C designates the location of the mutation, and

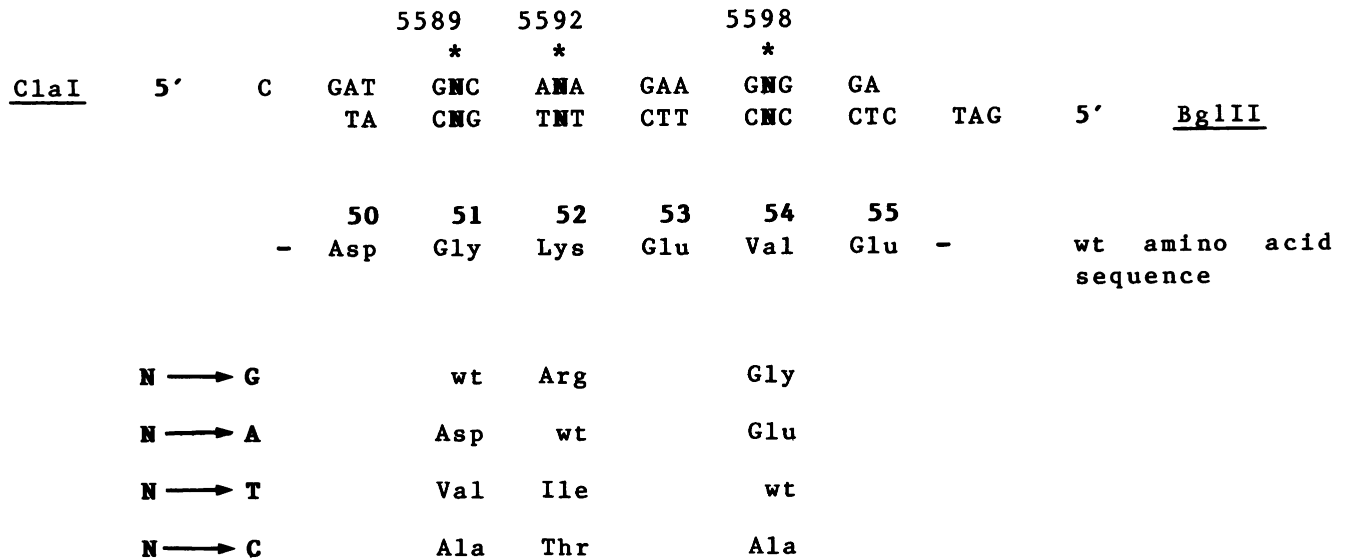


FIG. 3. Design of oligonucleotides for *ClaI-BglII* fragment mutagenesis. Three different pairs of complementary oligonucleotides were designed such that when annealed they would form a mini-restriction fragment that could be substituted for the corresponding sequences in the poliovirus cDNA. The numbers above the nucleotide sequence indicate the position relative to the poliovirus genome in which a random nucleotide was inserted during the synthesis of the oligonucleotides. The wild-type (wt) amino acid sequence corresponding to this region is shown below the nucleotide sequence. The numbers represent the position of the amino acid residue within the context of the proteinase 3C. The bottom portion of the figure shows the amino acid changes that would result from the insertion of each of the four nucleotides at a given position in the oligonucleotide.

a number identifies the mutation. The Gly → Val and Gly → Ala substitutions at amino acid 51 proved to be noninfectious within our limits of detection. The Val → Gly mutation appeared to be infectious at extremely low levels, and we have as yet been unable to isolate virus after repeated transfections.

It was necessary to verify that the integrity of the introduced mutations had been maintained in the virus arising from transfection. Mutant virus stocks derived from single plaque isolates were used to infect large-volume HeLa suspension cultures. Viral RNA was purified from these infections and sequenced across the mutagenized region with a complementary oligonucleotide primer, dideoxynucleotides, and reverse transcriptase. The sequencing data revealed that all the viable mutants had, in fact, maintained the predicted sequences for at least the 200 nucleotides analyzed (data not shown).

Initial plaque assays revealed that all the mutant viruses grew to titers of approximately 1 log per milliliter less than that typically observed for transfection-derived wild-type stocks. In addition, all mutants displayed a small-plaque phenotype, an example of which is shown in Fig. 4 for Se1-3C-02. However, none of the mutant viruses expressed temperature sensitivity for growth at 37°C (unpublished data).

[^{35}S]methionine pulse-chase labeling of HeLa cells infected with Se1-3C mutants. To investigate the effect of the 3C mutations on proteolytic processing during infection, we conducted pulse-chase labeling of virus proteins in infected HeLa suspension cultures. In all cases the source of the virus used for infection was a second passage stock derived

TABLE 1. Summary of poliovirus 3C mutations

Mutation	Virus	Electrophoretic mobility of 3C and related proteins[a]
Val 54 → Glu	Sel-3C-01	+ +, Retarded
Val 54 → Ala	Sel-3C-02	+, Retarded
Val 54 → Gly	ND[b]	ND[b]
Val 54 → Δ	Nonviable	
Gly 51 → Asp	Sel-3C-04	+ + +, Retarded
Gly 51 → Val	Nonviable	
Gly 51 → Ala	Nonviable	
Lys 52 → Arg	Sel-3C-05	+, Accelerated
Lys 52 → Thr	Sel-3C-06	+ +, Retarded
Lys 52 → Ile	Sel-3C-07	No change

[a] With respect to wild-type poliovirus. Relative mobility shift: +, slight; + +, moderate; + + +, large.
[b] ND, Not determined.

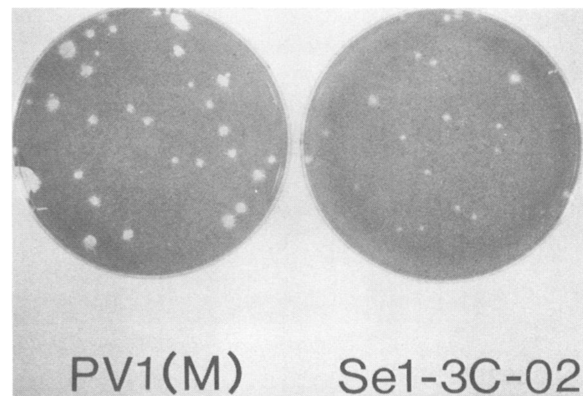
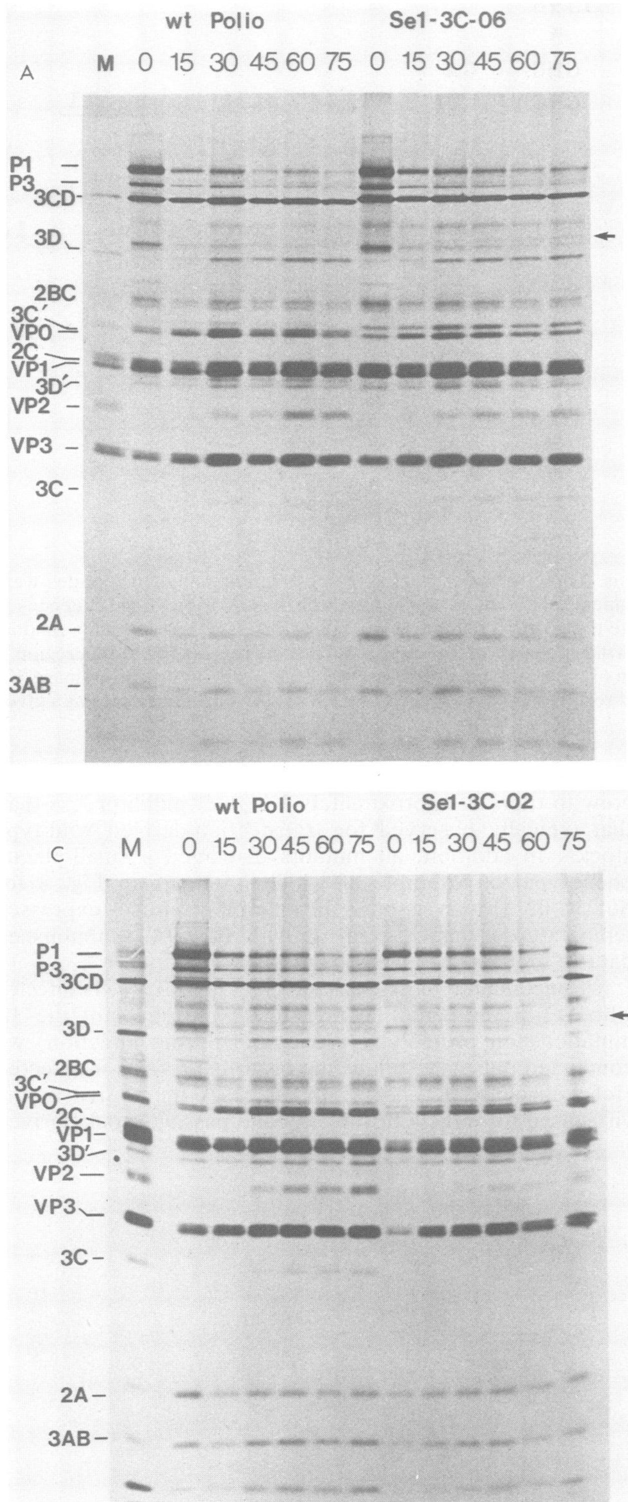


FIG. 4. Comparison of plaque phenotypes between poliovirus type 1 (Mahoney) [PV1(M)] and Se1-3C-02. HeLa cell monolayers were infected with second-passage stocks of virus as described for plaque assays in Materials and Methods. The two largest plaques in the Se1-3C-02-infected plate may represent second-site mutations yielding revertants to wild-type phenotype.



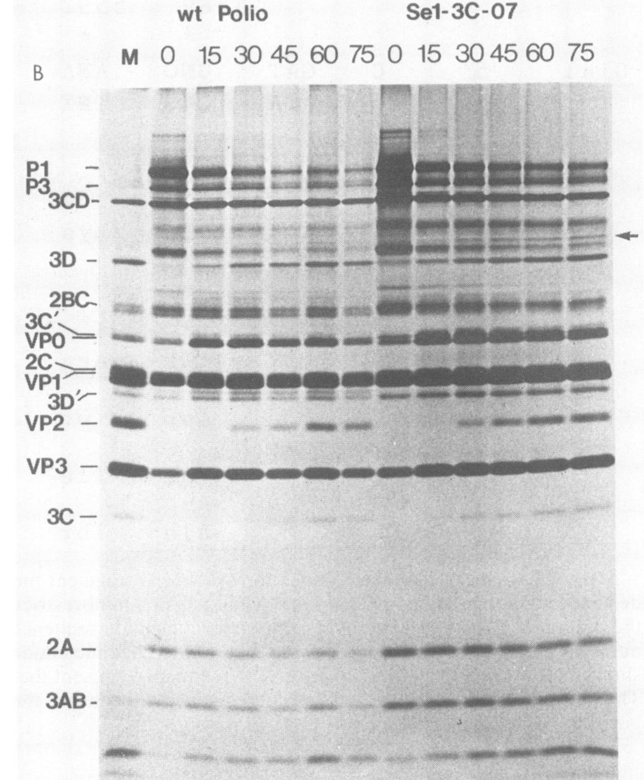


FIG. 5. Pulse-chase analysis of protein processing by poliovirus proteinase mutants. Labeling of infected cells with [^{35}S]methionine was carried out as described in Materials and Methods. Cells were infected at a multiplicity of 25 with wild-type transfection-derived poliovirus (wt) or Se1-3C-06 (A), Se1-3C-07 (B), and Se1-3C-02 (C). Numbers above the lanes indicate time in minutes after the chase with unlabeled L-methionine. M, Marker lane of [^{35}S]methionine-labeled poliovirus proteins (labeled from 3 to 6 h after infection of HeLa cells). Shown are autoradiograms of SDS-PAGE gels obtained after electrophoresis of labeled infected cell extracts and fluorography.

from a single plaque isolate. Details of the procedure are given in Materials and Methods. Briefly, infected cell cultures were incubated in methionine-free medium for 4 h at 37°C and then pulsed for 10 min with [^{35}S]methionine. After the labeling period, the cells were chased with a 1,000-fold molar excess of unlabeled L-methionine. Beginning immediately after the chase, samples of the infected cells were harvested at 15-min intervals and analyzed by SDS-PAGE.

The results of pulse-chase labeling studies with mutants Se1-3C-06, Se1-3C-07, and Se1-3C-02 are shown in Fig. 5. The large precursor proteins (P1 and P3) of Se1-3C-06 (Fig. 5A) appear to have been processed into mature viral proteins in a temporal fashion similar to what is observed for wild-type virus. Se1-3C-07 was somewhat delayed in the overall rate of proteolytic processing, as evidenced by the persistence of high-molecular-weight precursors P1 and P3 (Fig. 5B). In both mutants the presence of a band with an apparent M_r of 60,000 was observed in much higher levels than in the wild-type extracts (indicated by arrow in Fig. 5). The protein was later confirmed by immunoprecipitation with antiserum prepared to a Trp-3D fusion protein (antiserum gift of Oliver Richards) to be the virus-specific polypeptide 4a (data not shown). This protein has been previously described as an unstable precursor to the viral polymerase 3D found in the membrane-bound replication complex (8). We also observed in HeLa cells infected with Se1-3C-06 the presence of a presumably virus-specific protein migrating slightly above the capsid precursor VP0 and the altered gel mobility of what appeared to be 3C. The identification of these proteins will be described in the next section. The pulse-chase analyses of

the remainder of the mutants, with the exception of Se1-3C-02, showed that they were not significantly different from wild-type virus in the time course of proteolytic processing. All mutants did display the increased stability of 4a and the presence of aberrantly migrating virus-specific proteins. Although some 4a is visible in the wild-type virus lanes in Fig. 5C, when samples were normalized for total counts and immunoprecipitated, it could be demonstrated that Se1-3C-02 does produce more of the protein.

The pulse-chase labeling of Se1-3C-02 provided an unexpected result with respect to protein processing. Although the rate of processing was only slightly delayed in comparison with that of the wild type, the efficiency with which the mutant carried out the 3C-3D cleavage was dramatically decreased (Fig. 5C). A densitometer tracing comparing the 45-min pulse-chase time point for wild-type and Se1-3C-02 (Fig. 6) viruses clearly reveals that the mutant produced markedly less RNA polymerase 3D than did the wild type and produced nearly undetectable amounts of 3C.

Immunoprecipitation of [^{35}S]methionine pulse-labeled mutant virus proteins. The pulse-chase experiments described in the previous section revealed the presence of what appeared to be aberrantly migrating virus-specific proteins in the labeled mutant extracts. To identify the nature of these polypeptides, we performed immunoprecipitations of extracts from infected HeLa cells that had been labeled with [^{35}S]methionine for 1 h beginning 4 h postinfection. We suspected that the band migrating above VP0 (Fig. 5A) was a mutant form of the alternative P3 region cleavage product 3C′. This was confirmed by immunoprecipitation with antiserum to 3C which reacts with the proteinase itself and related polypeptides (Fig. 7). Immunoprecipitation with anti-3C showed that, with the exception of Se1-3C-07, the mutant forms of 3C, 3C′, and their precursor 3CD exhibited altered electrophoretic mobility when compared with the mobility of their wild-type poliovirus polypeptide counterparts. The immunoprecipitations with anti-2C (Fig. 7A) demonstrated that the altered electrophoretic mobility of viral proteins is specific for 3C-containing polypeptides, since the P2 region polypeptides specified by the mutant viruses were not changed in their electrophoretic properties.

A summary of the observed altered electrophoretic mobility phenotypes is provided in column 3 of Table 1. Amino acid substitutions have been previously shown to alter the electrophoretic mobility of SDS-protein complexes in a manner dependent on the intrinsic hydrophobicity or charge of the sustituted residue (α-crystallin A chain [7]). However, for the poliovirus proteinase mutants, the degree to which the mobility of the proteins is accelerated or retarded during SDS-PAGE does not appear to correlate to the relative pK_a or hydrophobicity of the sustituted amino acid residue. Rather, the effect is more likely due to a major change in the configuration of the protein as a result of the amino acid substitution. Supporting this explanation is the fact that in the immunoprecipitations with anti-3C, the electrophoretic mobility of the mutant forms of P3 and the polypeptide 3ABC′ remained unchanged from that of the wild type.

RNA synthesis by proteinase mutant Se1-3C-02. On the basis of its reduced levels of RNA polymerase production, we predicted that Se1-3C-02 would also be deficient in RNA synthesis. To verify this, we labeled infected cells with [^{3}H]uridine in the presence of actinomycin D. The kinetics of viral RNA synthesis for all the proteinase mutants were determined by harvesting the labeled, infected cells at specific time points and measuring trichloroacetic acid-precipitable counts. The results of these experiments (Fig. 8) show that Se1-3C-02 did indeed synthesize RNA at a slower rate than the wild type did and only achieved a maximal level of less than one-half that observed for wild-type poliovirus. Preliminary experiments indicated that Se1-3C-02 was temperature sensitive for RNA synthesis. At 39°C, the mutant synthesized approximately 30% of the maximal RNA level observed at 37°C. On the basis of RNA dot blot analysis, it appears that the mutation in Se1-3C-02 did not cause a differential defect in positive versus negative strand synthesis during the course of infection (data not shown). Two other mutants, Se1-3C-01 and Se1-3C-04, also displayed reduced levels of viral RNA. Repeated experiments indicated that the remainder of the mutants did not vary significantly from the wild-type virus in either the kinetics or total yield of viral RNA production.

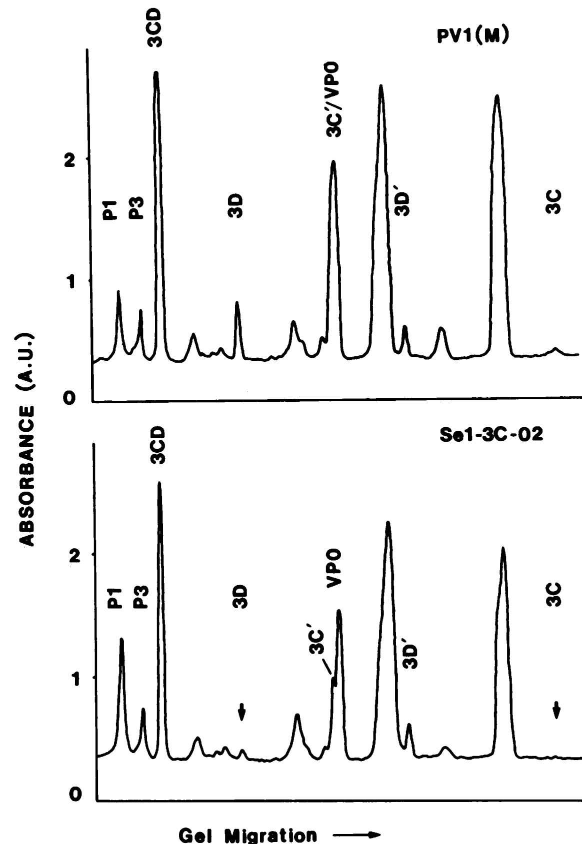


FIG. 6. Densitometry comparing 45-min time point lanes for wild-type and Se1-3C-02 viruses from Fig. 5C. Densitometry analysis of the autoradiogram was performed on an LKB ULTROSCAN II laser densitometer. The tracings are oriented such that the top of the gel is at the left of the figure. A.U., Arbitrary units.

DISCUSSION

We have generated site-specific mutations in the proteinase region of the poliovirus genome. These mutations were obtained by using a double-stranded synthetic oligonucleotide to introduce conservative and nonconservative amino acid substitutions into an infectious cDNA clone. When individual plasmids bearing specific mutations were transfected onto monkey cells, six different viable mutants of poliovirus were produced. It was surprising that changing a Gly (at amino acid residue 51) that is highly conserved

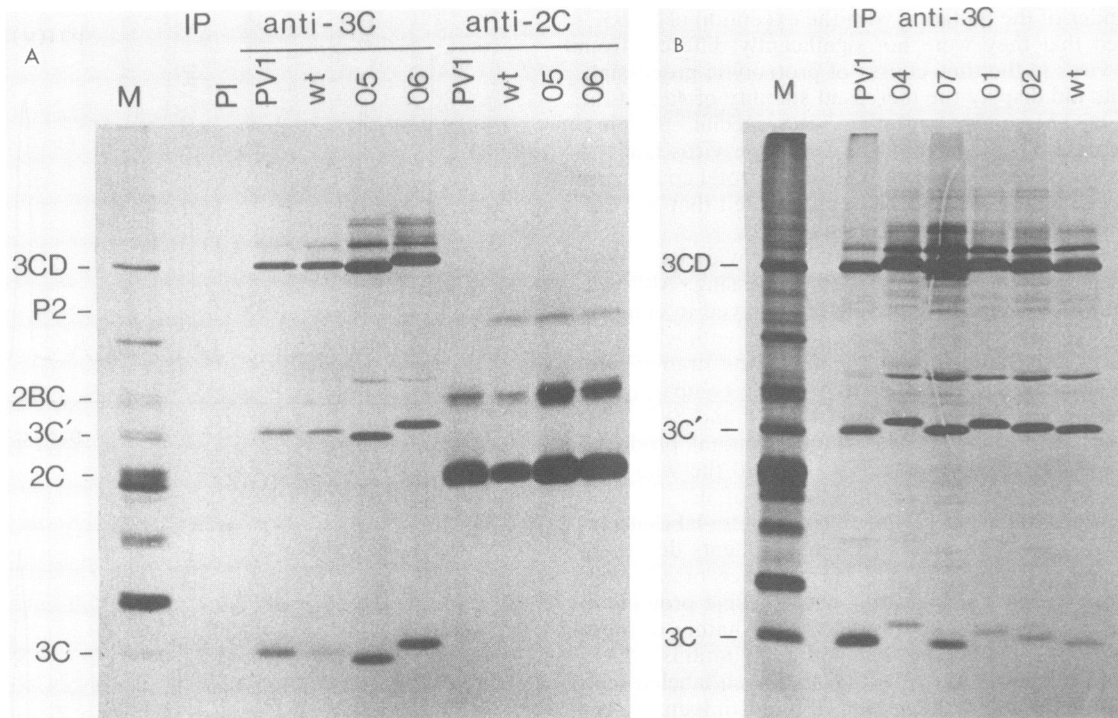


FIG. 7. Immunoprecipitation of viral polypeptides from extracts of [^{35}S]methionine-labeled, infected cells. Extracts of infected cells were prepared and immunoprecipitated with virus-specific antisera as described in Materials and Methods. (A) Extracts of HeLa cells infected with the following viruses: M, Marker lane of poliovirus proteins prepared as described in Fig. 5; PI, labeled extract of wild-type poliovirus-infected cells immunoprecipitated with preimmune serum; PV1, third-passage stock of poliovirus type 1 (Mahoney); wt, transfection-derived wild-type poliovirus; 05, Se1-3c-05; 06, Se1-3C-06. (B) Extracts of HeLa cells infected with the following viruses: 04, Se1-3C-04; 07, Se1-3C-07; 01, Se1-3C-01; 02, Se1-3C-02. The autoradiogram in this panel was overexposed to demonstrate the altered electrophoretic mobilities of the mutant forms of 3C.

among the picornaviruses to an Asp residue yielded a viable mutant virus, whereas more conservative changes to Val or Ala were lethal within our limits of detection. The Lys → Arg, Ile, or Thr changes that we introduced at residue 52 resulted in the production of viable mutants in all three cases. Amino acid residue 52 is not a highly conserved site in the picornavirus alignments of 3C sequences made by Argos et al. (1), and thus it is not surprising that such substitutions would lead to an active 3C proteinase.

The third site that we targeted for mutagenesis was amino acid residue 54 in the poliovirus 3C polypeptide. This site has been previously shown to be conserved among picornaviruses (encephalomyocarditis virus and foot-and-mouth disease virus) and a related plant virus (cowpea mosaic virus) because it contains an uncharged amino acid (1). Our mutagenesis protocol produced Val → Ala and Val → Glu substitutions at position 54 that both lead to viable virus. Thus, lack of a charged residue at position 54 is not the sole determinant for a correctly folded, active 3C polypeptide. In addition, we have shown that residue 54 is required for virus infectivity, since deletion of the wild-type Val codon led to loss of infectivity (Table 1).

On initial characterization, all mutant viruses displayed a small-plaque phenotype but were not temperature sensitive for growth at 37°C. We examined the protein processing characteristic of the various mutants in more detail by conducting in vivo pulse-chase labeling of infected cells. Whereas in most cases the overall kinetics of protein processing in the mutants were not significantly affected, we were able to detect virus-specific proteins not normally seen in wild-type infections. The elevated levels of 4a in the mutant infections can be explained either by increased stability or greater frequency of cleavage of the Gln-Gly pair that gives rise to the amino terminus of the protein. The latter possibility appears more likely because the amino acid pair is located at residues 122 and 123 within the 3C proteinase sequence and is only cleaved infrequently during the course of wild-type poliovirus infection. This site is located approximately 70 amino acids downstream from the mutagenized region, and it is possible that the amino acid substitutions have altered the configuration of the precursor in such a way that the Gln-Gly pair has been rendered more accessible to proteolytic cleavage by the 3C proteinase activity. Conversely, the mutant proteinases may be more efficient at recognizing this potential cleavage site as a result of an altered active site configuration of 3C.

The amino acid substitutions quite unexpectedly resulted in P3 region proteins exhibiting altered electrophoretic mobility in SDS-PAGE gels. Aberrant mobility of 3C and capsid proteins has been observed in clinical isolates of all three serotypes of poliovirus (15, 20), but these differences have not been correlated to specific amino acid changes. The altered electrophoretic mobility of 3C-related polypeptides that we have observed in the infectious proteinase mutants could be a result of altered cleavage specificity of the proteinase 3C. However, we discount this explanation on the following grounds. Most importantly, the electrophoretic mobility of the cleavage counterparts (3AB, 3D′, and 3D) to 3C, 3C′, and 3CD remained unchanged from that of wild-type virus, suggesting that the proteolytic cleavages gener-

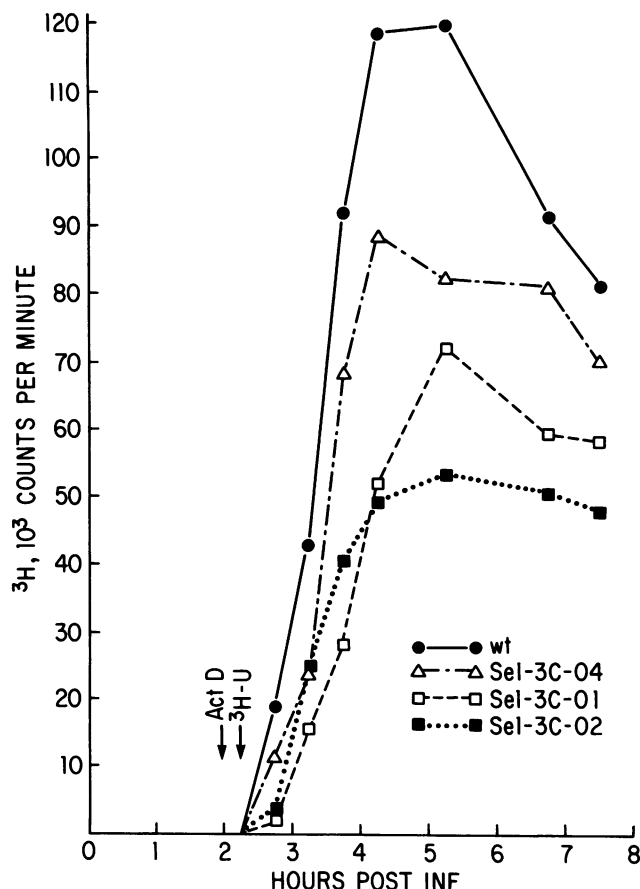


FIG. 8. Kinetics of RNA synthesis by wild-type poliovirus and proteinase mutants. HeLa cells were infected at a multiplicity of 25 with either wild-type or mutant poliovirus and labeled with [^{3}H]uridine as described in Materials and Methods. RNA synthesis was measured by liquid scintillation counting of trichloroacetic acid-precipitable material. Radioactivity incorporated by uninfected cells has been subtracted from the values shown in this figure. Act D, Actinomycin D.

ating these proteins have occurred normally. In addition, for any given mutant the mobility of 3C, 3C', and 3CD appears to be shifted to the same degree rather than as a reflection of the presence or absence of specific sequences. We have been able to use the characteristic mobilities of these proteins as phenotypic markers for the mutants and in periodic checks for reversion in later passages of the virus stocks. These markers should be very useful in experiments involving recombination between different strains at the RNA level.

The mutant virus that exhibited the most interesting phenotype was Sel-3C-02, which contained a conservative Val → Ala substitution at residue 54 in 3C. This virus produced extremely low levels of the 3CD cleavage products 3C and 3D, yet it appeared to generate wild-type levels of the alternative cleavage products 3C' and 3D'. RNA labeling experiments demonstrated that this defect manifested itself in decreased levels of viral RNA synthesis. Taking this into account, it is surprising that the mutant virus grew to nearly wild-type titers (10^8 to 10^9 PFU/ml) as determined by plaque assay. The single-step growth curve obtained for Sel-3C-02 is indistinguishable from that of wild-type transfection-derived poliovirus (data not shown). This observation, together with the apparently normal proteolytic processing of the mutant capsid proteins, argues against any major defect in virion assembly resulting from the low levels of mature proteinase 3C in the mutant. Upon subsequent passages of the original mutant virus stock, we observed heterogeneity in plaque size morphology (Fig. 4). However, pulse-chase analysis of the polypeptides produced by one such stock showed that the mutant virus had maintained the protein-processing phenotype of the original stock of Sel-3C-02 and produced elevated levels of protein 4a. This later passage of Sel-3C-02 also continued to exhibit the altered electrophoretic mobility of 3C-related polypeptides.

It is possible that the very low amounts of 3D that the mutant Sel-3C-02 does produce are sufficient for replication of the genome. A previous report of a poliovirus mutant containing an amino acid insertion in protein 2A demonstrated that a virus deficient in the synthesis of viral proteins still can produce wild-type levels of viral RNA (3). In addition, Lundquist and Maizel (18) have suggested that only a small fraction of the RNA polymerase polypeptides produced during poliovirus infection actually participate in RNA replication. This simple explanation raises the question as to why wild-type poliovirus, which is so economical with its genetic information, would be wasteful with respect to protein and RNA synthesis. An intriguing hypothesis is that other P3 region proteins, to which no functions have as yet been ascribed, may compensate for the depressed levels of 3D and 3C produced by the mutant. That 3C' and 3D' may serve as alternate proteinase and replicase, respectively, is a possibility that has been alluded to but not substantiated by direct evidence (27). The precursor 3CD may also play a role as a replicase with characteristics different from those of 3D. Since we have generated viable 3C mutants that affect the processing of P3 region polypeptides, it should be feasible to design experiments that will test these possibilities.

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