An Aspartic Acid at Amino Acid 108 Is Required To Rescue Infectious Virus after Transfection of a Poliovirus cDNA Containing a CGDD but Not SGDD Amino Acid Motif in 3D^{pol}

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The poliovirus RNA-dependent RNA polymerase (3D^{pol}) contains a region of homology centered around the amino acid motif YGDD (amino acids 326 to 329), which has been postulated to be involved in the catalytic activity of the enzyme. Previous studies from this laboratory have used oligonucleotide site-directed mutagenesis to substitute the tyrosine amino acid at this motif with other amino acids (S. A. Jablonski and C. D. Morrow, *J. Virol.* 67:373-381, 1993). The viruses recovered with 3D^{pol} genes with a methionine mutation also contained a second mutation at amino acid 108 resulting in a glutamic acid-to-aspartic acid change (3D-E-108 to 3D-D-108) in the poliovirus RNA polymerase. On the basis of these results, we suggested that the amino acid at position 108 might interact with the YGDD region of the poliovirus polymerase. To further investigate this possibility, we have constructed a series of constructs in which the poliovirus RNA polymerases contained a mutation at amino acid 108 (3D-E-108 to 3D-D-108) as well as a mutation in which the tyrosine amino acid (3D-Y-326) was substituted with cysteine (3D-C-326) or serine (3D-S-326). The mutant 3D^{pol} polymerases were expressed in *Escherichia coli*, and in vitro enzyme activity was analyzed. Enzymes containing the 3D-D-108 mutation with the wild-type amino acid (3D-Y-326) demonstrated in vitro enzyme activity similar to that of the wild-type enzyme containing 3D-E-108. In contrast, enzymes with the 3D-C-326 or 3D-S-326 mutation had less in vitro activity than the wild type. The inclusion of the second mutation at amino acid 3D-D-108 did not significantly affect the in vitro activity of the polymerases containing 3D-C-326 or 3D-S-326 mutation. Transfections of poliovirus cDNAs containing the substitution at amino acid 326 with or without the second mutation at amino acid 108 were performed. Consistent with previous findings, we found that transfection of poliovirus cDNAs containing the 3D-C-326 or 3D-S-326 mutation in 3D^{pol} did not result in the production of virus. Surprisingly, transfection of the poliovirus cDNAs containing the 3D-D-108/C-326 double mutation, but not the 3D-D-108/S-326 mutation, resulted in the production of virus. The virus obtained from transfection of poliovirus cDNAs containing 3D-D-108/C-326 mutation replicated with kinetics similar to that of the wild-type virus. RNA sequence analysis of the region of the 3D^{pol} containing the 3D-C-326 mutation revealed that the codon for cysteine (UGC) reverted to the codon for tyrosine (UAC). The results of these studies establish that under the appropriate conditions, poliovirus has the capacity to revert mutations within the YGDD amino acid motif of the poliovirus 3D^{pol} gene and further strengthen the idea that interaction between amino acid 108 and the YGDD region of 3D^{pol} is required for viral replication.

Like all members of the picornavirus family, poliovirus is a plus-strand RNA virus with a genome of approximately 7,500 bp (20, 21). The viral genome is translated to generate a long polyprotein precursor which is subsequently processed by virus-encoded proteinases 2A and 3C^{pro} (34, 40). An RNA-dependent RNA polymerase, encoded by the virus, is used for replication of the genome. This protein, designated 3D^{pol}, has been purified from poliovirus-infected cells and corresponds to a protein with a molecular mass of 52 kDa (8, 13, 32, 37). The purified 3D^{pol} can copy a variety of RNA templates in vitro if a suitable RNA primer is provided (35, 36).

Previous studies from this as well as other laboratories, have demonstrated that 3D^{pol} expressed in *Escherichia coli* is enzymatically active (28, 29, 31). The availability of an expression system has prompted the use of DNA mutagenesis techniques to construct defined mutants in 3D^{pol}. In one study, the mu-

tagenesis of the 3D^{pol} corresponding to amino acid 424 resulted in a polymerase that was not defective for in vitro RNA synthesis but, when cloned into the infectious clone of poliovirus, resulted in a temperature-sensitive virus (4, 5). In another mutant, insertion of a leucine between amino acids 257 and 258 of 3D^{pol} resulted in an enzyme defective for the capacity to polymerize nucleoside triphosphates (NTPs) in vitro (6). Another study used "charged to alanine" mutagenesis to construct mutant 3D^{pol} genes (11). The mutant 3D^{pol} genes that were generated, when cloned into the poliovirus cDNA, gave rise to temperature-sensitive viruses. Previous studies from this laboratory have focused on a region that the poliovirus RNA-dependent RNA polymerase shares with other RNA polymerases from animal, plant, and bacterial viruses that consists of a hydrophobic region of amino acids centered around two aspartic acid residues (1, 9). In poliovirus, the conserved region in 3D^{pol} is between amino acids 326 and 329, corresponding to a YGDD amino acid motif. Substitutions of the glycine or one or both of the aspartic amino acids resulted

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FIG. 1. Poliovirus virus genome and locations of mutations in the 3D^{pol} region. (A) Schematic diagram of the poliovirus genome. The locations of the mutation in 3D^{pol} at amino acid 108 and the YGDD amino acid motif (amino acids 326 to 329) are depicted. The 5' end of the poliovirus virus genome, with a virus-encoded protein, Vpg (is noted by black circle), is depicted. The viral polyprotein composed of the capsid proteins (VP0, VP3, VP1) is located in P1 region. The expression of the poliovirus genome occurs via the translation of a long polyprotein that is proteolytically processed by the viral 2A proteinase (located in the P2 region). The viral proteinase 3C and the RNA-dependent RNA polymerase, 3D, are located in the P3 region of the poliovirus genome. The nucleotide sequence corresponds to that described by Kitamura et al. (20). (B) Nucleotide sequence of the YGDD amino acid motif. In this study, the codons for the tyrosine residue were mutated to codons for cysteine, serine, or histidine. The corresponding nucleotide changes required for the mutations are presented in parentheses.

in enzymes that were inactive or had greatly reduced in vitro RNA polymerase activity (16). Poliovirus cDNAs containing these mutant enzymes were noninfectious. In contrast, substitutions of a tyrosine amino acid (3D-Y-326) to phenylalanine (3D-F-326) or methionine (3D-M-326) resulted in the generation of enzymes that had in vitro enzymatic activity similar to that of the wild-type protein (17). Transfection of poliovirus cDNAs containing 3D^{pol} genes with the 3D-F-326 or 3D-M-326 mutation resulted in the production of infectious virus. Sequence analysis of the 3D^{pol} gene from the virus containing the 3D-M-326 mutation revealed that a second mutation at amino acid 108 in which a glutamic acid was changed to an aspartic acid (3D-D-108) had occurred. We demonstrated that the mutation to aspartic acid was essential for recovery of a virus containing the 3D-M-326 mutation (17).

In this study, we have further analyzed the relationship between amino acid 108 and the YGDD amino acid motif. We have reconstructed poliovirus 3D^{pol} genes containing double mutations in which the tyrosine of the YGDD amino acid motif has been changed to cysteine, serine, or histidine and the glutamic acid at amino acid 108 has been changed to aspartic acid (Fig. 1). The general procedures required for manipulation of recombinant DNA were carried out by using standard techniques (25). Single-stranded DNA was produced from plasmid pUC119 Prot-Pol, which was used as the template for mutagenesis (16). The DNA templates containing mutations in the codon for the YGDD motif in the poliovirus polymerase gene were used for these studies and have been described elsewhere (17). The pUC119 Prot-Pol single-stranded DNA containing a mutation of 3D-Y-326 to 3D-S-326, 3D-C-326, or 3D-H-326 in 3D^{pol} was used. Oligonucleotide site-directed mutagenesis was performed as described previously (17), using the

synthetic DNA oligonucleotide 5'-GAT GGT CTA GAT GCA CTT G-3' to change the codon at amino acid 108 from E to D. Mutants were identified by direct sequencing of double-stranded plasmid DNA by the Sanger dideoxy nucleotide technique as modified for use with Sequenase (33). To characterize the effects that the mutations in 3D^{pol} have on enzyme activity, each of the mutants as well as the wild-type enzyme was expressed in *E. coli*. For these studies, we amplified the 3D^{pol} gene by PCR followed by subcloning into a pET-11d vector. The DNA oligomers used for the amplification of the 3D^{pol} gene (nucleotides 5987 to 7369) were 5' CGACCATGGGTG AAATCCAGTGGATG 3' (5' oligomer) and 5' CTGGATCC TTAATGAGTCAAGCCAGGG 3' (3' oligomer).

The conditions for PCR amplification were as previously described (18). The resulting PCR-amplified DNA contained unique *Nco*I (5') and *Bam*HI (3') restriction sites. For subcloning into pET-11d, the PCR-amplified DNA was first digested with *Nco*I and *Bam*HI and then ligated directly into pET-11d DNA digested with the same enzymes. The correct plasmid containing the 3D^{pol} gene was determined by restriction digestion and by DNA sequence analysis. The plasmid was retransformed into *E. coli* BL21/DE3, which contains the T7 polymerase gene on the chromosome under control of the *lac* operon. To induce expression of the 3D^{pol}, the *E. coli* cells containing the plasmid were grown to an optical density at 600 nm of between 0.35 and 0.45, and then IPTG (isopropyl- β -thiogalactopyranoside) was added for an additional 2 h. The bacteria were harvested by centrifugation and resuspended in buffer A (50 mM Tris [pH 8.0], 1 mM EDTA, 5 mM β -mercaptoethanol, 10% glycerol). The bacteria were disrupted with a Heat System sonicator at a power setting of 7 and then centrifuged at 20,000 $\times g$ for 30 min at 4°C. The pellet, which contains the 3D^{pol} in the form of an insoluble inclusion protein, was solubilized by resuspension in buffer A with 4 M guanidine. The guanidine was removed by dialysis against buffer A containing 50 mM KCl. An insoluble precipitate forms after dialysis and was removed by centrifugation (1,500 $\times g$ for 30 min). The supernatant, which contains 3D^{pol}, was loaded onto a phosphocellulose column. The unbound proteins were washed from the column by buffer A with 50 mM KCl. The bound proteins were eluted by buffer A with 250 mM KCl. Fractions containing 3D^{pol} were identified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and silver staining (Bio-Rad kit). The 3D^{pol} is the predominant protein, present at greater than 90%. The enzymatic activity of each enzyme was determined by using the poly(A) $\cdot$ oligo(U) poly(U) polymerase assay (17). For these studies, we normalized the concentrations of the enzyme preparation to be approximately equal and in the linear range of the assay. Using equal amounts of enzymes, we analyzed the polymerase activities at 30 and 37°C (Fig. 2B). The wild-type enzyme and the 3D-E-108-to-3D-D-108 mutation had greater activities at either 30 or 37°C. Consistent with our previous studies, the 3D^{pol} enzymes containing the 3D-S-326 and 3D-C-326 amino acid changes had lower in vitro activities than the wild-type enzyme at either 30 or 37°C (17). The enzymes with the combination of 3D-D-108/S-326 and 3D-D-108/C-326 mutations also had less activity than the wild type. Interestingly, the 108D mutation resulted in slightly increased activity for the mutant enzyme with the 3D-S-326 mutation while slightly reducing the activity of the 3D^{pol} containing the 3D-C-326 mutation.

To ascertain the effects that the mutations in the poliovirus 3D^{pol} have on the capacity of the enzyme to function in replication, the mutant genes were subcloned into the poliovirus cDNA and transfected into COS-1 cells. The region of pUC119 Prot-Pol from nucleotides 5601 to 7400 of the polio-

FIG. 2. Expression of poliovirus 3D^{pol} mutants in *E. coli* and analysis of RNA polymerase activity. (A) The 3D^{pol} gene of polioviruses containing different mutations were subcloned into the pET vector. Following expression of the viral RNA polymerase, the proteins were purified as detailed in Materials and Methods. Equal amounts of the proteins (approximately 10 μ g) were analyzed by polyacrylamide gel electrophoresis followed by silver staining. Lanes: 1, wild-type 3D^{pol}; 2, 3D-D-108; 3, 3D-C-326; 4, 3D-D-108/C-326; 5, 3D-S-326; 6, 3D-D-108/S-326. The molecular mass markers and 3D^{pol} are noted. (B) RNA-dependent RNA polymerase activity of the 3D^{pol} expressed in *E. coli*. The enzymatic activities of the wild-type and mutant RNA polymerases were determined using the poly(A) $\cdot$ oligo(U) poly(U) polymerase assay at reaction temperatures of 30 and 37°C. By using 20 μ l of a normalized enzyme extract (corresponding to approximately the same amount of protein run on the SDS-polyacrylamide gel in panel A), the enzyme activities of the wild-type (WT) and mutants were determined.

virus genome (contained on a *Bgl*II-to-*Sal*I DNA fragment) was isolated and subcloned into the plasmid containing the infectious clone of poliovirus, pT7-IC (7). For these studies, we routinely do 20 independent transfections and ascertain the frequency of recovery of infectious virus (16, 17). No difference in the results are obtained if RNA obtained from in vitro transcription is transfected instead of DNA (18a). The cells were incubated at 37°C for 5 days and subjected to three freeze-thaw cycles to release virus, and the supernatant was passaged onto subconfluent BSC1 cells. After 5 days, the cultures were frozen-thawed three times and passaged on BSC1 cells. Virus production was monitored by visual inspection for cytopathic effect. Under these experimental conditions, transfection of the poliovirus infectious clone (pT7-IC) resulted in the recovery of virus in all (20 of 20) of the transfected cells (percent virus recovery was calculated as $100 \times [\text{number of wells with viral cytopathic effect}/\text{total number of wells transfected}]$). Consistent with our previous studies, the transfection of the poliovirus cDNAs containing the 3D-S-326, 3D-C-326, or 3D-H-326 mutation did not result in the production of infectious virus. In contrast, transfection of poliovirus cDNAs which contained the double mutation 3D-D-108/C-326 re-

FIG. 3. Replication kinetics of viruses isolated from transfection of poliovirus cDNAs containing mutant 3D^{pol} genes. Viruses were obtained from the transfection of the wild-type poliovirus infectious cDNA, the cDNA containing the 3D-E-108-to-3D-D-108 mutation, and cDNAs containing the 3D-D-108/C-326 double mutation. The viruses were plaque purified, and the replication kinetics were determined by using [³H]uridine incorporation. For these studies, cells were infected as described in Materials and Methods; at designated times postinfection, aliquots of the infected cultures were removed and the radioactivity was determined.

sulted in the production of virus after passage onto BSC1 cells in 100% (20 of 20) of the transfection trials. Transfection of poliovirus cDNAs containing the 3D-D-108/S-326 or 3D-D-108/H-326 mutation, though, did not result in the production of infectious virus in any of the transfection trials (data not shown).

The virus obtained after transfection of the infectious clone containing the 3D-D-108/C-326 mutation was plaque purified to obtain a working stock. In a first set of experiments, the replication kinetics of the virus obtained after transfection of the poliovirus cDNA containing 3D-D-108/C-326 was compared with the kinetics of the wild-type virus as well as a second virus containing a 3D-D-108 change in the 3D^{pol} gene. BSC1 cells were infected with designated viruses at 10 PFU per cell. After a 30-min adsorption, the inoculum was removed and replaced with complete medium. After an additional 60 min at 37°C, actinomycin D (10 μ g/ml) was added for 15 min, and then 50 μ Ci of [³H]uridine per ml was added. Samples were harvested at the designated times and processed as previously described (17), and the radioactivity was determined by scintillation counting. The replication of the virus isolated from transfection of the plasmid containing the 3D-D-108/C-326 mutation was comparable to that of the wild-type virus and the virus containing the 3D-E-108-to-3D-D-108 change (Fig. 3).

The wild-type-like replication kinetics of the virus obtained from transfection of the poliovirus cDNA containing 3D-D-108/C-326 was inconsistent with the in vitro RNA polymerase activity of 3D^{pol} with the 3D-D-108/C-326 double mutation. To resolve this discrepancy, we isolated the viral genomic RNA and directly sequenced the region of the viral 3D^{pol} gene containing the 3D^{pol} mutation (17) (Table 1). The virus recovered contained a mutation from the starting cDNA in which the UGC codon, corresponding to cysteine, had reverted to the codon for tyrosine, UAC. Therefore, the original 3D^{pol} mutation had reverted from cysteine to tyrosine, thus regenerating a consensus YGDD amino acid motif. The reversion was a result of a single nucleotide change (G to A). Since the codon for tyrosine (UAC) was different from that used by the wild-type virus (UAU), the virus isolated was a true revertant rather than a contaminant wild-type virus. Sequence analysis of the

TABLE 1. Reversion of 3D-D-108/C-326 to 3D-D-108/Y-326

Poliovirus cDNA nucleotide sequence	GCC	<u>TAT</u> ^a	GGT	GAT	GTA
Amino acid sequence	A	Y	G	D	D
3D-D-108/C-326 mutant sequence	GCC	<u>TGC</u>	GGT	GAT	GTA
Amino acid sequence	A	C	G	D	D
3D-D-108/C-326 revertant sequence	GCC	<u>TAC</u>	GGT	GAT	GTA
Amino acid sequence	A	Y	G	D	D

^a Poliovirus cDNA sequence at the YGDD amino acid motif. The sequence of the poliovirus cDNA corresponding to the tyrosine or cysteine is underlined, and the corresponding amino acid is in boldface.

3D^{pol} gene at amino acid 108 revealed that the codons for the E-to-D change were still present (data not shown).

The YXDD amino acid motif is one of the hallmarks of RNA polymerases and reverse transcriptases (1, 9). Numerous studies have focused on mutagenesis of the conserved region in an effort to gain insights into the significance of this region with regard to polymerase activity (2, 3, 12, 14–16, 19, 22, 23, 26, 33, 38). Previous studies from this laboratory have described mutations in which the tyrosine amino acid was substituted with methionine, phenylalanine, serine, or cysteine. As evident from our previous study and the results presented in this study, substitutions of the tyrosine residue have profound effects on the enzymatic activity of the poliovirus polymerase. In this study, the mutations in which the tyrosine was changed to cysteine or serine resulted in a reduction of RNA polymerase activity as measured in our *in vitro* assays. The choice of the cysteine and serine amino acid substitutions was based on the fact that polymerases from other viruses contain the CGDD and SGDD amino acid motifs. A cysteine is found in black beetle virus and tobacco mosaic virus, and serine is found in yellow fever virus and in alfalfa mosaic virus (1, 30). Although the poliovirus enzymes containing 3D-S-326 and 3D-C-326 mutations had detectable levels of RNA polymerase activity *in vitro*, the complete poliovirus genomes containing these mutant 3D^{pol} genes were not infectious when transfected into cells. In a previous study, we identified a second-site mutation at amino acid 108 of the poliovirus 3D^{pol} gene in viruses recovered from transfection of poliovirus cDNAs containing a 3D-M-326 mutation (17). We demonstrated that transfection of poliovirus cDNA containing a 3D-D-108/M-326 mutation resulted in the production of virus in all transfections. To further explore this result, we constructed 3D^{pol} genes which contained a 3D-D-108 mutation and a 3D-C-326 or 3D-S-326 mutation. The *in vitro* enzymatic activities of 3D^{pol} containing the single or double mutation differed slightly depending on the mutation. In some cases, the activity of 3D-C-326, the enzyme with the double mutation, was lower than that of the enzyme with the single mutation; the activity of the enzyme with the 3D-S-326 mutation was slightly higher with the double mutation. The actual significance of the differences in enzyme activity is not clear since virus containing the 3D-D-108 mutation had replication kinetics similar to that of the wild-type virus (Fig. 3). The important new result of this study was that the inclusion of the 3D-D-108 mutation in the 3D^{pol} gene with the 3D-C-326 but not 3D-S-326 mutation resulted in an infectious poliovirus cDNA. This result was surprising because the *in vitro* enzyme activity of the 3D^{pol} containing the 3D-D-108/C-326 mutation was actually lower than that of the enzyme with either the 3D-C-326 or 3D-S-326 mutation alone or the 3D-D-108/S-326 double mutation. A low level of 3D^{pol} enzyme activity therefore is probably sufficient for viral genome replication. This conclusion is consistent with the earlier suggestion by Lundquist and Maizel that only a subpopulation of poly-

merase molecules in a given cell participate in RNA replication (24) and recent studies by Dewalt and Semler, who have demonstrated that virus containing a mutation in 3C^{pro}, which reduces the level of 3D^{pol} in infected cells, replicates to levels similar to those of the wild-type virus (10). The complexity of poliovirus replication, though, is highlighted by the result that poliovirus cDNAs containing the 3D-D-108/S-326 mutation were not infectious even though the *in vitro* analysis demonstrated that enzymes containing these mutations were more active than enzymes containing the 3D-D-108/C-326 mutation. Although we believe the viruses containing 3D-D-108/S-326 were able to replicate the viral genome, it is possible that under our tissue culture conditions the viruses containing 3D^{pol} with this low enzyme activity do not replicate to levels sufficient to produce enough virus for continued infection. In support of this idea is the fact that even though the virus containing the 3D-D-108/C-326 mutation was replication competent, we detected this virus only because the original mutation was not stable and 3D-C-326 reverted to 3D-Y-326 by a single G-to-A change (Table 1). In order for the virus containing the 3D-D-108/S-326 mutation to revert, the codon for serine, UCC, would need to be changed to UAC, the codon for tyrosine, by a single C-to-A change. Alternatively, UCC could have mutated to a UUC, the codon for phenylalanine, by a single C-to-U change, since previous studies from this laboratory demonstrated that viruses containing the 3D-F-326 mutation were replication competent (17). It is possible, then, that the poliovirus RNA polymerase favors G-to-A changes rather than the change of C to U or A needed by the virus containing the 3D-D-108/S-326 mutation for reversion. Previous studies have determined that the error frequency for the poliovirus 3D^{pol} is between 7×10^{-4} and 5.4×10^{-3} (39). Since these studies were done with *in vitro* homopolymer templates, it is difficult to access if a preference would be observed for a change of G to A rather than of C to U or A. Interestingly, studies have reported a G-to-A hypermutation in retroviral genomes (27). These mutations are a result of the substitution of dCTP by dTTP as a result of a localized depletion of intracellular dCTP. Recent studies have provided further support for this idea by demonstrating the hypermutagenesis of RNA by reverse transcription in *in vitro* reactions by modifying the dNTP concentrations (27). Further studies will be required to determine if poliovirus infection results in a depletion of cellular NTP pools that leads to the G-to-A changes observed with the 3D^{pol} mutants.

Finally, the results of these experiments, when combined with our previous studies, suggest a possible order for the sequence of mutations required for the reversion of the mutations in the YGDD region of 3D^{pol}. We believe that the 3D^{pol} genes containing mutations at 3D-Y-326 must maintain enough 3D^{pol} activity for multiple cycles of genome replication so that a mutation at the codon for amino acid 108 from GAA to GAU occurs, resulting in a glutamic acid-to-aspartic acid change. To date, we have found only one mutation, 3D-Y-326 to 3D-M-326, that retained sufficient enzyme activity for this mutation to occur. Even in this case, the transfection of poliovirus cDNAs containing the 3D-M-326 mutation in 3D^{pol} resulted in the production of virus in only 5% of the transfection trials (17). Once a 3D-E-108-to-3D-D-108 mutation occurs, the resulting viral genome is infectious, as evidenced by the isolation of a virus containing a stable 3D-D-108-to-M-326 change in 3D^{pol} (17). If we engineer a 3D^{pol} gene with a 3D-E-108 to 3D-D-108 change, RNA genomes with mutations in the YGDD amino acid motif (i.e., 3D-C-326 and 3D-S-326) are replication competent. However, a strong selective pressure must also exist for the production of poliovirus with 3D^{pol}

enzyme activity similar to that of the wild-type enzyme because to date, we have been unable to isolate poliovirus with mutations in the 3D^{pol} that grow more slowly than the wild-type virus. It is possible, though, that viruses containing the 3D-D-108 change with a second mutation in the YGDD amino acid motif, alterations that result in enzymes with activities greater than those of enzymes with a 3D-C-326 or 3D-S-326 mutation, might be stable. Studies are ongoing with additional mutants to identify these mutant 3D^{pol} genes.

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