Enzymatic Activity of Poliovirus RNA Polymerases with Mutations at the Tyrosine Residue of the Conserved YGDD Motif: Isolation and Characterization of Polioviruses Containing RNA Polymerases with FGDD and MGDD Sequences

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The poliovirus RNA-dependent RNA polymerase (3D^{pol}) shares a region of homology with all RNA polymerases, centered around the amino acid motif YGDD, which has been postulated to be involved in the catalytic activity of the enzyme. Using oligonucleotide site-directed mutagenesis, we substituted the tyrosine at this motif of the poliovirus RNA-dependent RNA polymerase with cysteine, histidine, isoleucine, methionine, phenylalanine, or serine. The enzymes were expressed in *Escherichia coli*, and in vitro enzyme activity was tested. The phenylalanine and methionine substitutions resulted in enzymes with activity equal to that of the wild-type enzyme. The cysteine substitution resulted in an enzyme with approximately 50% of the wild-type activity, while the serine substitution resulted in an enzyme with approximately 10% of the wild-type activity; the isoleucine and histidine substitutions resulted in background levels of enzyme activity. To assess the effects of the mutants in viral replication, the mutant polymerase genes were subcloned into the infectious cDNA clone of poliovirus. Transfection of poliovirus cDNA containing the phenylalanine mutation in 3D^{pol} gave rise to virus in all of the transfection trials, while cDNA containing the methionine mutation resulted in virus in only 3 of 40 transfections. Transfection of cDNAs containing the other substitutions at the tyrosine residue did not result in infectious virus. The recovered viruses demonstrated kinetics of replication similar to those of the wild-type virus, as measured by [³H]uridine incorporation at either 37 or 39°C. RNA sequence analysis of the 3D^{pol} gene of both viruses demonstrated that the tyrosine-to-phenylalanine or tyrosine-to-methionine mutation was still present. No other differences in the 3D^{pol} gene between the wild-type and phenylalanine-containing virus were found. The virus containing the methionine mutation also contained two other nucleotide changes from the wild-type 3D^{pol} sequence; one resulted in a glutamic acid-to-aspartic acid change at amino acid 108 of the polymerase, and the other resulted in a C-to-T base change at nucleotide 6724, which did not result in an amino acid change. To confirm that the second amino acid mutation found in the 3D^{pol} gene of the methionine-substituted virus allowed for replication ability, a mutation corresponding to the glutamic acid-to-aspartic acid change was made in the polymerase containing the methionine substitution, and this double-mutant polymerase was expressed in *E. coli*. The double-mutant enzyme was as active as the wild-type enzyme under in vitro assay conditions. Transfection of a cDNA containing the double-mutant 3D^{pol} into cells resulted in virus production in 100% of the transfection trials. These results demonstrate that poliovirus RNA polymerase retains in vitro enzymatic activity similar to that of the wild-type enzyme when specific amino acids are substituted for the tyrosine residue of the YGDD amino acid motif, and that infectious poliovirus can be recovered with the stable mutations in the YGDD amino acid motif of 3D^{pol}. These results are discussed with regard to the role that the tyrosine residue of the YGDD amino acid motif plays in both the enzymatic activity of the RNA polymerase and the capacity of 3D^{pol} to function in viral replication.

Poliovirus, a member of the family *Picornaviridae*, is a plus-stranded RNA virus with a genome of approximately 7,500 bases (19, 20). The viral genome is translated as a polyprotein precursor, with subsequent processing by virally encoded proteases. The capsid precursor protein, P1, is cleaved from the polyprotein precursor by the 2A protease (2A^{pro}) (37). The P1 polyprotein is further processed to the mature capsid proteins by 3CD, which is a precursor protein consisting of the 3C^{pro} proteinase and the 3D^{pol} RNA-dependent RNA polymerase (45). The further processing of 3CD releases 3C^{pro} and 3D^{pol} (19, 33). The form of the protease responsible for the proteolytic cleavages of the poliovirus polyprotein in the P2 and P3 regions is unknown, although it has been suggested to be 3C^{pro} (20). The poliovirus 3D^{pol} protein has been identified as an RNA-dependent

RNA polymerase and is required for the replication of the poliovirus genome (6, 9, 40). Poliovirus replication is thought to occur in a membrane-associated replication complex, with the involvement of other viral proteins, and potentially host cell proteins, still unclear (reviewed in reference 42). 3D^{pol} has been purified from poliovirus-infected cells and corresponds to a protein of molecular mass 52 kDa (6, 9, 40). Previous studies from this and other laboratories have described the expression of enzymatically active 3D^{pol} in *Escherichia coli* (28, 29, 31).

The poliovirus RNA-dependent RNA polymerase shares highly conserved regions of sequence similarity with other RNA polymerases from animal, plant, and bacterial viruses, reverse transcriptases, and DNA polymerases (1, 7). One of the most highly conserved regions of homology is a hydrophobic region of amino acids centered around two aspartate residues (7). In poliovirus, this conserved region in 3D^{pol} is

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centered around a four-amino-acid sequence, YGDD, from amino acids 326 to 329. Structural predictions for this region suggest that it is a β -turn- β structure, containing the aspartates on the turn (1). Recently the crystal structure of the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase was resolved to 3.5 Å (0.35 nm). The reverse transcriptase, an RNA-dependent DNA polymerase, has a similarly conserved YMDD motif, which was found on a β -hairpin in the enzyme active site of the reverse transcriptase (21). This region of polymerases has been postulated to be involved in metal ion binding or otherwise involved at the catalytic site of the enzyme (1, 18, 21, 30, 35). In support of this idea is the fact that mutations of polymerases at this region result in significant loss of enzymatic activity (2, 3, 8, 10–12, 17, 24, 25, 27, 35, 41). In fact, because of the critical nature of this region in polymerase function, it has been difficult to obtain mutants with enzymatic activity. Previous studies in this laboratory have described the construction and characterization of 3D^{pol} in which the glycine of the YGDD conserved motif was changed. The mutant 3D^{pol} genes were expressed in *E. coli*, and in general, single amino acid substitutions at this site resulted in enzymes with greatly decreased or no enzymatic activity in vitro (12). In recent studies, we have also found that reconstruction of these mutants into the infectious clone of poliovirus results in noninfectious virus (13). The glycine residue is highly conserved in RNA-dependent RNA polymerases and, from the results of our studies, appears to be essential for function of the poliovirus 3D^{pol}.

Previous studies by Poch et al. have subgrouped the RNA-dependent RNA polymerases into polio-like and Sindbis-like groups (30). Although each group has a conserved GDD amino acid motif, the tyrosine residue is generally conserved among the polio-like group, while a great deal of diversity at this site exists in the Sindbis-like group. In particular, the tyrosine residue is substituted with an isoleucine, serine, or cysteine and, in rare cases, an asparagine, glycine, alanine, or methionine. Because of this finding, we speculated that substitutions of the tyrosine in the YGDD amino acid motif of the RNA polymerase might result in 3D^{pol} with significant enzyme activity differences, which, in turn, might lead to the isolation of viruses with different replicative characteristics. In this report, oligonucleotide site-directed mutagenesis was used to change the tyrosine residue of the YGDD conserved region of the poliovirus 3D^{pol}. The mutant polymerases were expressed in *E. coli*, and the in vitro enzymatic activity was characterized. To ascertain the effects of the mutant 3D^{pol} on virus replication, the mutant 3D^{pol} genes were subcloned into the infectious cDNA of poliovirus. The results of these studies demonstrate that substitution of the tyrosine at the YGDD amino acid motif has profound effects on the subsequent enzymatic activity of the RNA polymerase and the capacity of the 3D^{pol} to function in poliovirus replication.

MATERIALS AND METHODS

Materials. All chemicals, unless otherwise noted, were purchased from Sigma Chemical Co. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Modified T7 DNA polymerase (Sequenase) was purchased from U.S. Biochemical. The enhanced chemiluminescence kit for Western immunoblot analysis was purchased from Amersham, Inc. Avian myeloblastosis virus reverse transcriptase was purchased from LifeSciences, Inc. Radionucleotides were purchased from Amersham. Tissue

culture media and reagents were obtained from BRL-GIBCO. The synthetic DNA oligonucleotides used for mutagenesis and sequencing were prepared by the Cancer Center Oligonucleotide Synthesis facility at the University of Alabama at Birmingham.

Construction of mutant plasmids. The general techniques required for manipulation of recombinant DNA were carried out by using standard procedures (26).

Construction of the phagemid pUC119Prot-Pol has been previously described (12). Single-stranded DNA from pUC119Prot-Pol was prepared following infection of transformed *E. coli* TG-1 with M13 helper phage (K07), using previously described procedures (22, 23). Oligonucleotide site-directed mutagenesis was performed by using procedures which have been described previously (44, 46, 47). The synthetic DNA oligonucleotides used to introduce the changes were (changed nucleotides are underlined) as follows:

5'-GCC TGC GGT GAT GAT G-3' (CGDD)
 5'-GCC CAC GGT GAT GAT G-3' (HGDD)
 5'-GCC ATC GGT GAT GAT G-3' (IGDD)
 5'-GCC ATG GGT GAT GAT G-3' (MGDD)
 5'-GCC TTT GGT GAT GAT G-3' (FGDD)
 5'-GCC TCC GGT GAT GAT G-3' (SGDD)

Following mutagenesis, the plasmids were transformed into competent *E. coli* TG-1, individual colonies were isolated, and plasmid DNA was directly sequenced by the dideoxy-chain termination method (34), as modified for use with Sequenase (U.S. Biochemical), to confirm the desired mutation.

Expression of mutant polymerase proteins in vitro. The mutant polymerase proteins were expressed in *E. coli*, from the plasmid vector pProt-Pol-TRP, as previously described (12). Using unique *Bgl*II and *Sal*I restriction sites, the mutated region of plasmid pUC119Prot-Pol from poliovirus nucleotides 5601 to 7400, containing part of the 3C^{pro} gene and all of the 3D^{pol} gene, was subcloned into the expression plasmid pProt-Pol-TRP which had been previously digested with *Bgl*II and *Sal*I. The resulting plasmids were directly sequenced to confirm the presence of the introduced mutation before expression. The conditions for expression and partial purification of protein from this vector have been previously described (28). Cell pellets of induced *E. coli* were lysed with lysozyme-Triton X-100, and the lysate was passed over phosphocellulose. Following elution of the proteins bound to phosphocellulose with 0.5 M potassium chloride-TGNB (50 mM Tris [pH 8.0], 0.5% Triton X-100, 20% glycerol, 0.5 mM β -mercaptoethanol), the eluate was concentrated against solid sucrose and then dialyzed against TGNB, after which the preparations were frozen and stored at -70°C .

Identification of 3D^{pol} mutants from induced *E. coli*. A Western immunoblot analysis was used to identify and quantitate relative levels of expressed 3D^{pol} (28). The eluate from the phosphocellulose columns was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to separate the proteins, which were then electrophoretically transferred to nitrocellulose paper. After transfer was complete, the blots were soaked in BLOTTO (50 mM Tris [pH 7.5], 10 mM sodium chloride, 5% nonfat dry milk [14]) for 30 min. The blots were incubated overnight at 4°C with a 3D^{pol} antibody (31) which had been previously adsorbed with an extract from *E. coli* to reduce nonspecific background (28). The blots were washed with BLOTTO to

remove unbound antibody and incubated at room temperature for 1 h with a donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Amersham). The blots were washed again with BLOTTO to remove unbound second antibody and were then developed with the detection reagents supplied in the Amersham enhanced chemiluminescence kit. The blot was then subjected to Kodak XAR film to visualize the specific protein bands. The Western blots were used to approximate quantities of 3D^{pol} proteins expressed from *E. coli* for further analysis of the enzymes (12).

Assay for RNA polymerase activity. The poly(A) · oligo(U) polymerase assay for RNA polymerase activity was performed as previously described except that rifampin (20 µg/ml) was included in the reaction mixture for assays with enzymes derived from *E. coli* (28). The product was precipitated with 10% trichloroacetic acid and collected on 0.45-µm-pore-size Gelman filters, and the radioactivity was determined by scintillation counting.

Transfection of poliovirus infectious clone mutants. The *Bgl*II-to-*Sall* restriction fragment of pUC119Prot-Pol (nucleotides 5601 to 7400) was subcloned into the poliovirus infectious clone pIC, which contains a complete infectious clone of the poliovirus genome along with the simian virus 40 origin/early promoter and two G bases immediately 5' to the poliovirus cDNA (5). Plasmid DNA (2 µg per well in a 24-well plate) was transfected into COS-1 cells by the DEAE-dextran method (39) as described by Choi et al. (5). The cells were incubated at 37°C for 5 days and then were subjected to three freeze-thaw cycles, and the supernatant was passed onto fresh BSC40 cells to check for virus production as evidenced by cytopathic effect. Two further passages onto fresh BSC40 cells were done after 5 days of incubation to allow for identification of a potentially low-titer virus.

Isolation of viral RNA from cells and RNA sequencing. Viral RNA was isolated from infected cells essentially as described by Rico-Hesse et al. (32). Briefly, cells were infected with virus and allowed to proceed to complete lysis. Cells were subjected to three freeze-thaw cycles, and then cell debris was removed by centrifugation (15,000 × g). Virions were precipitated from the supernatant with 7% polyethylene glycol 8000 and 2.3% sodium chloride at 4°C. The polyethylene glycol precipitate was collected by centrifugation (15,000 × g) and resuspended in 50 mM Tris-HCl (pH 7.8)–50 mM sodium chloride. Insoluble material was removed by centrifugation (15,000 × g), and the supernatant was loaded onto a cushion of 30% sucrose, 1 M sodium chloride, 20 mM Tris-acetate (pH 7.5), and 0.1% bovine serum albumin to pellet the virions. The samples were centrifuged in an SW 55Ti rotor at 45,000 rpm at 4°C for 90 min. The virion pellet was resuspended in TSE buffer (10 mM Tris-HCl [pH 8.0], 50 mM sodium chloride, 1 mM EDTA), the suspension was adjusted to 1% each SDS and β-mercaptoethanol, and the virions were heat dissociated at 60°C for 90 s. The virions were extracted three times with acid-phenol-chloroform, the aqueous phase was adjusted to contain 0.2 M lithium chloride, and viral RNA was precipitated with ethanol. The RNA pellet was collected by centrifugation, washed twice with 80% ethanol, and dried. RNA sequencing was performed by the dideoxy method (34), using reverse transcriptase (LifeSciences). Primers used for sequencing the 3D^{pol} gene were as follows: nt 6117, 5'-GTG AGGACTGCTGG-3'; nt 6239, 5'-GCTGGCCAGCATAG-3'; nt 6282, 5'-GGCATCCTCC-3'; nt 6410, 5'-GCATTTCT TAGTGTC-3'; nt 6507, 5'-CTGGATTTCCTCC-3'; nt 6623, 5'-CGCACCCCACTGC-3'; nt 6729, 5'-GCCTCGAACCAA

nucleotide sequence:	GCC	TAT	GGT	GAT	GAT	GTA
amino acid sequence:	A	Y	G	D	D	V
changes made:	Y to	C H I M F S	(TGC) (CAC) (ATC) (ATG) (TTT) (TCC)			

FIG. 1. Conserved YGDD region of poliovirus 3D^{pol} and mutations made at tyrosine residue. The nucleotide sequence of poliovirus RNA-dependent RNA polymerase (3D^{pol}) from nucleotides 6959 to 6976 is depicted. The amino acid changes (and the corresponding codon changes) are also shown.

GCAGGG-3'; nt 6858, 5'-GAGCAGCCAGATGGC-3'; nt 6935, 5'-CTATGCCCTTG-3'; nt 7097, 5'-CATTCTCCCAT GTGAC-3'; nt 7215, 5'-GTGTTCCCTAGG-3'; nt 7349, 5'-GGTACAATGTTGAGTACTCTGGGAGCAATA-3'; and nt 7444, 5'-TTTTCTCCGAATTAAAG-3'.

Analysis of viral RNA replication. BSC40 cells in 35-mm-diameter plates were infected with 20 PFU of the appropriate virus per cell. Virus was allowed to adsorb to cells in 0.5 ml of Dulbecco modified Eagle medium for 30 min at 37 or 39°C. After 30 min, 1.5 ml of complete medium (Dulbecco modified Eagle medium with 5% fetal bovine serum and 5% newborn calf serum) was added, and the cells were allowed to incubate at the indicated temperature for 1 h. Actinomycin D (BRL-GIBCO) was added to a final concentration of 5 µg/ml, and cells were incubated for 1 h. The medium was removed, and 1 ml of fresh medium containing actinomycin D (5 µg/ml) and [³H]uridine (10 µCi/ml; Amersham) was added to each plate. Cells were incubated at the indicated temperature for 90 min and then harvested starting at 4 h postinfection. A plate was harvested every hour to 9 h postinfection. Medium was removed, and the cells were scraped into 1 ml of 20% trichloroacetic acid. The samples were filtered onto 0.45-µm-pore-size Gelman filters, and radioactivity was determined by scintillation counting.

RESULTS

Construction and expression of 3D^{pol} containing mutations in the YGDD amino acid motif. The region of the 3D^{pol} gene targeted for mutagenesis is the highly conserved YGDD region, located between nucleotides 6962 and 6973. This region has been found to be a highly conserved motif in many RNA polymerases and putative polymerases from animal, plant, and bacterial viruses (1, 18, 30, 43). The region of the poliovirus genome containing the entire 3D^{pol} gene and a portion of the 3C^{pro} gene was cloned into the phagemid pUC119 for production of single-stranded DNA. Oligonucleotide site-directed mutagenesis was performed, and the nucleotides were changed to give the predicted amino acid change from tyrosine to cysteine, histidine, isoleucine, methionine, phenylalanine, or serine (Fig. 1). The nomenclature for the mutations is 3D-X-326, where X refers to the single-letter amino acid designation and 326 is the amino acid position in the 3D^{pol}. The mutant 3D^{pol} genes were then subcloned back into the pProt-Pol-TRP expression plasmid and expressed in *E. coli*. The *E. coli* cell lysates were passed over a phosphocellulose column, and the eluates were analyzed by Western blotting with an antibody specific for 3D^{pol} (31). Initially, a series of twofold dilutions of extracts from bacteria expressing the wild-type polymerase was assayed to ensure that detection of 3D^{pol} was in the linear

FIG. 2. Immunoblot analysis of wild-type and mutant 3D^{pol} expressed in *E. coli*. Phosphocellulose-purified extracts from induced *E. coli* transformed with plasmids containing the vector, wild-type, or mutant 3D^{pol} genes were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. Immunoblots were performed with a polyclonal anti-3D^{pol} antibody which had been previously demonstrated to react with the entire molecule (31). Lanes: 1, *E. coli* transformed with vector plasmid; 2, *ptrp*-3D-wt; 3, *ptrp*-3D-C-326; 4, *ptrp*-3D-H-326; 5, *ptrp*-3D-I-326; 6, *ptrp*-3D-M-326; 7, *ptrp*-3D-F-326; 8, *ptrp*-3D-S-326. Positions of molecular weight standards which had been electrophoresed in parallel are indicated, as is the position of 3D^{pol}.

range (data not shown). Immunoreactive material corresponding to a protein of molecular mass 52 kDa, the molecular mass of correctly processed 3D^{pol}, was found in extracts of *E. coli* expressing the mutant or wild-type 3D^{pol} genes, while no immunoreactive material was found in extracts of cells which had been transformed with the *trp* expression plasmid alone (Fig. 2). The levels of immunoreactive material were normalized by the Western blot analysis to give approximately equal levels of polymerase for testing of enzymatic activity (12).

To determine the *in vitro* enzymatic activity of the mutant 3D^{pol} proteins, the phosphocellulose-purified extracts were tested by the poly(A) · oligo(U) polymerase assay. The enzyme preparations were adjusted so that the extracts contained similar amounts of immunoreactive material as determined by Western blotting; the enzymatic activities of the mutant polymerases were tested over a range of concentrations in the standard polymerase assay (Fig. 3). The

FIG. 3. RNA polymerase activities of wild-type and mutant 3D^{pol} enzymes expressed in *E. coli*. Extracts from induced *E. coli* transformed with plasmids containing the various polymerase genes were purified by phosphocellulose chromatography. The amounts of 3D^{pol} in each preparation were estimated by immunoblot analysis, and equal amounts of immunoreactive 3D^{pol} were used in the poly(A) · oligo(U) polymerase assay under standard conditions. Symbols: ○, vector; ●, *ptrp*-3D-wt; ▲, *ptrp*-3D-C-326; □, *ptrp*-3D-H-326; ◇, *ptrp*-3D-I-326; ■, *ptrp*-3D-M-326; △, *ptrp*-3D-F-326; ◆, *ptrp*-3D-S-326.

TABLE 1. Virus recovery after transfection and passage

cDNA transfected	% Virus recovery ^a
pIC-wt	100 ^b
pIC-3D-C-326	0 ^b
pIC-3D-H-326	0 ^b
pIC-3D-I-326	0 ^b
pIC-3D-M-326	7.5 ^c
pIC-3D-F-326	100 ^c
pIC-3D-S-326	0 ^b
pIC-3D-D-108/M-326	100 ^c

^a Calculated by following equation: $100 \times (\text{number of wells positive for virus by cytopathic effect} / \text{total number of wells transfected})$.

^b Calculated from 20 independent transfection trials with the same preparation of DNA.

^c Calculated from 40 independent transfection trials with the same preparation of DNA.

wild-type 3D^{pol} extract was appreciably active over background levels of vector alone (approximately 400-fold). The mutant with substitution of phenylalanine for the tyrosine residue, 3D-F-326, and the methionine substitution mutant, 3D-M-326, both had activity levels equal to or greater than that of the wild-type 3D^{pol} enzyme extract. Mutant 3D-C-326, which had a cysteine residue in place of the tyrosine, was approximately 50% as active as the wild-type polymerase, while 3D-S-326, with the serine substitution, was 10% as active as the wild type. The enzymatic activities of the other two mutants, 3D-H-326 and 3D-I-326, were slightly above background levels.

The YGDD amino acid motif has been proposed to be at or near the catalytic active site of the enzyme (1, 21, 35). To determine whether the mutant polymerases would exhibit different enzymatic properties, the Mg²⁺ ion concentration, reaction pH, and temperature were varied in the *in vitro* assays. The Mg²⁺ ion concentration was varied from 2 to 10 mM (magnesium acetate), the pH was varied from 7.0 to 9.0, and assay temperatures of 30, 37, and 42°C were tested. Under the different assay conditions tested, none of the mutants exhibited any difference in activity from the standard reaction conditions (data not shown).

Infectious potential of viruses containing mutants in the YGDD motif of 3D^{pol}. To ascertain the effects that the mutations in the YGDD amino acid motif of 3D^{pol} have on the capacity of the enzyme to function in replication of the poliovirus genome, the mutant polymerase genes were subcloned into an infectious clone of poliovirus and transfected into COS-1 cells. Cells were allowed to incubate for 5 days, and then the cell lysate was passaged onto BSC40 cells to identify the cytopathic effect of virus infection. The cell lysate was then passaged two more times on BSC40 cells to allow for identification of low-titer virus. Under the experimental conditions used, transfections of the poliovirus infectious clone, pIC, resulted in the production of virus in all of the trials. Similarly, transfection of pIC-3D-F-326 resulted in production of virus in all transfection trials. Surprisingly, transfection of pIC-3D-M-326 resulted in production of virus in only 3 of 40 trials (Table 1). An isolate of each of these viruses was then used to generate plaque-purified virus stocks for further analysis of each of the mutants. Transfection of the cDNAs containing the other 3D^{pol} mutants at the tyrosine residue did not result in the appearance of virus in any of the 20 independent transfection trials (Table 1).

The phenotypes of the mutant viruses were characterized. By convention, the viruses will be designated vIC-3D-F-326

FIG. 4. RNA replication analysis of wild-type and mutant viruses. [3 H]uridine incorporation in infected cells in the presence of actinomycin D was measured over a 9-h period of infection at 37°C (A) or 39°C (B). Background levels of incorporation in mock-infected cells have been subtracted. Symbols: ●, wild-type poliovirus; ▲, v1C-3D-F-326; □, v1C-3D-M-326.

and vIC-3D-M-326. Both mutant viruses displayed a plaque phenotype at 37°C similar to that of the wild type (data not shown). Furthermore, the expression of viral proteins and proteolytic processing of the recovered viruses was not demonstrably different from that of the wild-type virus (data not shown). Finally, replication of vIC-3D-F-326 and vIC-3D-M-326 was compared with that of the wild type by measuring [³H]uridine incorporation at either 37 or 39°C (Fig. 4). No difference was found between the replication of the wild-type and mutant viruses at either temperature as measured by [³H]uridine incorporation.

Sequences of 3D^{pol} genes from recovered viruses. Since the phenotype of the viruses was similar to that of the wild type, it was important to determine whether vIC-3D-F-326 and vIC-3D-M-326 had retained the original mutation in the 3D^{pol} gene. To do so, the viral RNA was isolated from each of the mutants, and the initial sequencing was focused on the region containing the YGDD amino acid motif (Fig. 5). The RNA sequence of vIC-3D-F-326 revealed the presence of the original mutation, a tyrosine-to-phenylalanine change. This was expected because transfection of the cDNA containing the 3D-F-326 mutation resulted in virus production in all of the trials. A surprising result was obtained from the analysis of vIC-3D-M-326 RNA in that the original mutation, tyrosine to methionine, was still present. On the basis of the infrequent recovery of virus from transfection of pIC-3D-M-326, we suspected that the recovered virus would have reverted to wild type. Since the original mutation was still present, it

FIG. 5. Strategy for sequencing the polymerase gene and mutations found. (A) Locations along the genome where primers were generated to sequence the polymerase gene (sequencing primers are listed in the text). The YGDD region of the genome is noted. (B) Sequence changes found in the vIC-3D-F-326 and vIC-3D-M-326 mutant polymerase genes. Nucleotide and amino acid changes are indicated.

was possible that additional mutations in the 3D^{pol} gene were present in this virus. To address this possibility, the sequence for the entire 3D^{pol} gene for each virus was determined and compared with the wild-type sequence. No difference other than the tyrosine-to-phenylalanine change was found between the vIC-3D-F-326 virus and wild-type sequences. However, two additional differences from the wild-type sequence were observed in the vIC-3D-M-326 sequence: a change from C to T at nucleotide 6724, which would not change the amino acid (phenylalanine at amino acid 245), and a change from A to T at nucleotide 6310, resulting in an amino acid change at position 108 of the polymerase from glutamic acid to aspartic acid (Fig. 5).

Significance of the glutamic acid-to-aspartic acid change in the 3D^{Pol} of vIC-3D-M-326. Since the methionine-containing cDNA resulted in only a low frequency of virus production and the virus which arose was similar to the wild type in its replication capability, it was important to determine which of the second-site mutations were responsible for this effect. The effect of the glutamic acid-to-aspartic acid change on the enzymatic activity of the RNA polymerase containing the 3D-M-326 mutation and on the capacity to generate infectious virus in pIC-3D-M-326 was next investigated. To do so, a mutation to change the glutamic acid to an aspartic acid at amino acid 108 was made in pUC119Prot-Pol containing the methionine substitution. The mutation was made by using the mutagenic primer 5'-GAT GGT CTA GAT GCA CTT G-3' (the changed nucleotide is underlined). This double mutant was then subcloned into pProt-Pol-TRP, and 3D^{Pol} was expressed in *E. coli*. 3D^{Pol} was partially purified by phosphocellulose chromatography, and the resultant enzyme was identified by Western blot analysis (data not shown). Normalized amounts of enzyme were tested for

FIG. 6. Poly(A) · oligo(U) polymerase assay of wild-type, mutant 3D-M-326, and double-mutant enzymes containing the second-site mutation at amino acid position 108 (glutamic acid-to-aspartic acid change). Equivalent amounts of extracts, as determined by levels of immunoreactive 3D^{pol} (by Western blot analysis), were tested. Symbols: ○, vector extract from *E. coli*; ●, *ptrp*-3D-wt; ▲, *ptrp*-3D-M-326; □, *ptrp*-3D-D-108/M-326.

activity at 30°C (Fig. 6). As expected, the glutamic acid-to-aspartic acid change did not significantly affect the in vitro enzymatic activity in comparison with that of 3D-M-326 or the wild-type enzyme. The double-mutant 3D^{pol} gene was then cloned back into the poliovirus cDNA. Forty independent transfection trials were performed in COS-1 cells, and the frequency of recovered virus was determined (Table 1). The cDNA containing the polymerase double mutant resulted in virus recovery in 100% of the transfection trials.

DISCUSSION

In this report, we have examined the contribution of the tyrosine at the conserved YGDD amino acid motif to the RNA polymerase activity of 3D^{pol} of poliovirus. Site-directed mutagenesis was used to change the nucleotides to give the corresponding amino acid changes from tyrosine to cysteine, histidine, isoleucine, methionine, phenylalanine, or serine. The enzymes were expressed in *E. coli* and tested for in vitro RNA polymerase activity. The in vitro RNA polymerase activities of the resultant enzymes ranged from activity similar to or greater than wild-type levels, intermediate levels of activity, and little or no activity. To test for the effects of the mutations on virus replication, the mutant genes were subcloned into the infectious cDNA of poliovirus. Transfection of poliovirus cDNA with the 3D-F-326 mutation resulted in production of infectious virus in all of the transfection trials. However, transfection of cDNA with the 3D-M-326 mutation resulted in production of infectious virus in only 3 of 40 trials. Both recovered viruses displayed kinetics of replication similar to those of the wild-type virus. The nucleic acid sequence of the 3D^{pol} gene from each of the two mutant viruses was determined and compared with the wild-type sequence. Both mutant viruses contained the original mutations, but vIC-3D-M-326 had two other nucleotide changes, one at nucleotide 6310, an A-to-T change resulting in a glutamic acid-to-aspartic acid change at amino acid 108 of 3D^{pol}, and another at nucleotide 6724, a C-to-T change that was silent at the amino acid level. To confirm that the second-site amino acid change resulted in an enzyme with complete polymerase activity, the corresponding change was made in the methionine-substituted mutant, resulting in a double mutant containing both amino acid

changes found in the polymerase of the virus isolated from transfection of pIC-3D-M-326 cDNA. This double-mutant polymerase was subcloned into the bacterial expression vector, and the polymerase isolated from induced *E. coli* was found to have activity similar to that of the wild-type enzyme produced in *E. coli*. Furthermore, the double mutant, when recloned back into the poliovirus cDNA and transfected into COS-1 cells, resulted in the production of virus in all of the transfection trials.

The YXDD amino acid motif is a hallmark for RNA polymerases and reverse transcriptases, while the YGDTDS motif is a distinguishing feature of DNA polymerases (1, 7, 18, 30, 43). Because of the highly conserved nature of this amino acid motif, several studies have focused on mutagenesis at this conserved region in an effort to gain knowledge about its functional significance in polymerase activity (2, 3, 8, 10–12, 17, 24, 25, 27, 35, 41). The overriding theme of all of these studies is that this region is important in catalytic function of the polymerase, and most mutations in these conserved amino acids result in nonfunctional polymerases. Previous studies on viral DNA polymerases from herpes simplex virus type 1 (8, 10, 27), adenovirus (17), or ϕ 29 (2, 3) have highlighted the functional significance of this region by demonstrating that mutations at or near YGDTDS resulted in a loss of enzyme activity. The change of the tyrosine to phenylalanine or serine in ϕ 29 DNA polymerase resulted in a polymerase which did not replicate primed DNA (3). The change of the tyrosine residue of adenovirus DNA polymerase to phenylalanine also resulted in a polymerase with reduced in vitro polymerase activity (17). Because these are large DNA viruses, it has been difficult to assess the effects of these mutations on virus viability. In the case of the HIV-1 reverse transcriptase, previous studies have indicated that mutations of the tyrosine resulted in a loss of in vitro activity as well as a noninfectious provirus (24, 25). In the present study, some of the mutations engineered at the tyrosine residue were chosen because of the motifs found in other polymerases; a cysteine is found in black beetle virus and tobacco mosaic virus (1, 30), isoleucine is found in Sindbis virus and Middleburg virus, methionine is found in southern bean mosaic virus (1), and serine is found in yellow fever virus and brome and alfalfa mosaic viruses (1, 30). Finally, the tyrosine-to-histidine change represents a substantially different amino acid substitution and would be expected to result in an inactive enzyme. A phenylalanine at this position is found in some retroelements (RNA-dependent DNA polymerases [43]) and is also a very conservative change from tyrosine. The results of our studies on the poliovirus RNA-dependent RNA polymerase lend further support to the significance of the tyrosine residue of the YGDD in enzyme function. In a previous study, a 14-amino-acid sequence, containing the YGDD motif in the center, was predicted to have a β -turn- β structure (1). In support of the structural prediction of the polymerase at this region, the RNA-dependent DNA polymerase (reverse transcriptase) of HIV-1 was recently reported as a crystal structure complexed with an inhibitor of the enzyme (21). The highly conserved YMDD region of reverse transcriptase was found on a β -hairpin loop of the protein, located in the putative active site of the enzyme. The predicted β -turn- β structure was previously used to model mutations at the glycine of the YGDD motif of the poliovirus RNA polymerase (12). This modelling has been extended to the mutants at the tyrosine residue of the poliovirus polymerase YGDD region. A six-amino acid sequence, AYGGDV, was modelled in a type I turn conformation after that predicted by Argos (1), using

the computer program FRODO (15). The substitutions at the tyrosine residue were modelled with the program ATOM (16) by searching the protein data base for the most likely conformation for that side chain based upon known protein structures. They were then depicted in van der Waals space-filling models with the program Ribbons (4). As would be expected, the phenylalanine substitution was in a conformation similar to that predicted for the tyrosine; however, the conformation predicted for the methionine substitution was vastly different from the tyrosine conformation (12, 13). The differences in orientation of the substituted side chains could be responsible for reduced enzymatic activity and could support the idea that compensating changes elsewhere in the polymerase are necessary for complete replication ability of the enzyme.

The development of an infectious cDNA clone for poliovirus has provided the opportunity to assess the effect of mutant 3D^{pol} genes on viral replication. In particular, we were interested in determining whether the 3D^{pol} mutants would give rise to viruses with different replication characteristics. Viruses were recovered from transfection of poliovirus cDNAs containing the 3D-F-326 and 3D-M-326 mutations in the 3D^{pol} gene. Importantly, these were the two 3D^{pol} mutants which exhibited the same relative amounts of in vitro enzyme activity as did the wild-type enzyme. The 3D-F-326 mutation appears to be readily tolerated in the infectious clone, in that virus was obtained following every transfection trial. Sequence analysis of the 3D^{pol} gene from this virus revealed that the phenylalanine mutation was still present, and no additional differences from the wild-type 3D^{pol} gene sequence were found. The surprising result was the fact that the 3D-M-326 mutation, when subcloned into the infectious clone, resulted in the infrequent isolation of virus after transfection of the cDNA. Given the fact that 3D^{pol} with 3D-F-326 and 3D-M-326 mutations demonstrated similar in vitro enzyme activities, this result was somewhat perplexing. A sequence analysis of one of the isolates of vIC-3D-M-326 revealed the presence of the original MGDD mutation as well as a second amino acid change (glutamic acid to aspartic acid at amino acid 108) and a single base change at nucleotide 6724. This represents a significant observation, because we have, for the first time, identified a region of the poliovirus 3D^{pol} which possibly interacts with the YGDD region in a functional capacity. The amino acid at position 108 is not strictly conserved among picornaviral RNA polymerases. A glutamic acid is found in wild-type poliovirus and coxsackievirus 3D^{pol} genes, which is not surprising, since previous studies have noted the similarity between the coxsackievirus 3B and poliovirus replicase genes (36). Interestingly, the amino acid sequence of hepatitis A virus and encephalomyocarditis virus at the position corresponding to amino acid 108 of the polymerase is an aspartate (30). The interaction between the aspartic acid at position 108 and the MGDD motif does not seem to be important for the RNA-dependent RNA polymerase activity as measured by the in vitro assays. That is, no difference was observed in the in vitro assays between the RNA polymerases containing 3D-M-326 and 3D-D-108/M-326. However, it is important to note that this assay measures only the capacity of the polymerase to recognize and elongate from an artificial template/primer [poly(A) · oligo(U)] and probably does not represent the initiation reaction for poliovirus RNA replication.

To further explore this problem, the double 3D^{pol} mutation, 3D-D-108/M-326, was reconstructed into the infectious clone, and the frequency of virus isolated from independent

transfections was determined. Transfection of pIC-3D-M-326 cDNA containing the second-site mutation, which replaced the glutamic acid at position 108 with an aspartic acid, resulted in production of infectious virus with a frequency similar to that of the wild-type cDNA. Thus, the second-site mutation at position 108 in the poliovirus 3D^{pol} restored the ability of the polymerase to function in viral replication.

Determination of the crystal structure of the HIV-1 reverse transcriptase allows for speculation as to the role of the second-site mutation found in the virus which resulted from transfection of the cDNA containing the methionine substitution at the tyrosine residue of the poliovirus YGDD sequence. The structure of the reverse transcriptase was compared with the only other known structure of a polymerase, that of the Klenow fragment of the *E. coli* DNA-dependent DNA polymerase, polymerase I (21). The two proteins had regions of striking similarity, most importantly at the catalytic active site of the polymerases. In the comparison, D185 and D186 of the reverse transcriptase aligned with D882 and E883 of the Klenow fragment. An additional aspartic acid residue of the reverse transcriptase, D110, aligned with D702 of the Klenow fragment in the comparison of the three-dimensional structure of the protein. In the Klenow fragments, these three carboxylate residues have been shown to interact with a divalent metal ion, and this interaction presumably could be important in enzyme function. The two aspartic acid residues which are proximally distant in the sequences of the proteins align in another conserved region of polymerases, motif A, which aligns around a conserved aspartic acid residue at a loop between a β -sheet and an α -helix also in the polymerase active site (7, 30). In poliovirus, the region which is aligned with conserved motif A is proposed to be between amino acids 228 and 251 (7). It could possibly be that E108 of poliovirus is the actual residue that interacts with the aspartic acids at 328 to 329, and substitution of the tyrosine residue at position 326 with a methionine disrupts the structure enough that the glutamic acid residue at position 108 needs to change to an aspartic acid in order for the polymerase to function in viral replication. Additionally, D185 and D186 are thought to be located near the primer terminus of the template/primer complex in the HIV-1 reverse transcriptase (21). If the aspartic acid residues at position 328 and 329 of the poliovirus polymerase are likewise involved in template/primer interactions of the protein, this could explain why the original methionine substitution resulted in a polymerase with complete elongation capacity but no viral replication capability. The disruption of the structure locally at the YGDD region by the methionine substitution could result in a polymerase unable to correctly interact with the other components of the replication complex, ultimately disrupting the initiation of viral RNA synthesis. Indeed, previous studies have suggested a role for 3D^{pol} in the uridylylation of VPg, which is required as a primer for RNA synthesis (38). Alternatively, the second-site mutation in 3D^{pol} at position 108 could be required for one of the other functions of 3D^{pol}, possibly in the 3CD precursor protein, which is the protease that processes the viral capsid proteins (45). Finally, the other base change located in the polymerase, which did not result in a change in the amino acid sequence, could also be important for replication of the viral RNA in terms of RNA sequence and polymerase interactions. Sequence analysis of the virus isolated from the double-mutant polymerase-containing cDNA transfections is currently under way to determine whether the C-to-T change is present in this virus at nucleotide 6724.

In summary, we have investigated the role of the tyrosine residue of the conserved YGDD motif in the enzyme function of the poliovirus RNA polymerase. Amino acids have been identified that, when substituted for the tyrosine, result in inactive enzymes or enzymes with intermediate or wild-type activity in vitro. Interestingly, we have identified and characterized mutant 3D^{pol} genes which have similar activities in vitro but differ in the capacity to produce infectious virus. Finally, a second region in the RNA polymerase which is affected by mutations in the YGDD amino acid motif has been identified. This region is not essential for in vitro RNA polymerase activity but is important for replication of the viral RNA. Further studies of the characterization of the interaction of this region with the YGDD amino acid motif will be needed to understand the role of this interaction in 3D^{pol} enzyme function and replication of viral RNA.

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