Enzymatic Activity of Poliovirus RNA Polymerase Mutants with Single Amino Acid Changes in the Conserved YGDD Amino Acid Motif

SANDRA A. JABLONSKI, MING LUO, AND CASEY D. MORROW*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Received 20 November 1990/Accepted 10 May 1991

RNA-dependent RNA polymerases contain a highly conserved region of amino acids with a core segment composed of the amino acids YGDD which have been hypothesized to be at or near the catalytic active site of the molecule. Six mutations in this conserved YGDD region of the poliovirus RNA-dependent RNA polymerase were made by using oligonucleotide site-directed DNA mutagenesis of the poliovirus cDNA to substitute A, C, M, P, S, or V for the amino acid G. The mutant polymerase genes were expressed in *Escherichia coli*, and the purified RNA polymerases were tested for in vitro enzyme activity. Two of the mutant RNA polymerases (those in which the glycine residue was replaced with alanine or serine) exhibited in vitro enzymatic activity ranging from 5 to 20% of wild-type activity, while the remaining mutant RNA polymerases were inactive. Alterations in the in vitro reaction conditions by modification of temperature, metal ion concentration, or pH resulted in no significant differences in the activities of the mutant RNA polymerases relative to that of the wild-type enzyme. An antipeptide antibody directed against the wild-type core amino acid segment containing the YGDD region of the poliovirus polymerase reacted with the wild-type recombinant RNA polymerase and to a limited extent with the two enzymatically active mutant polymerases; the antipeptide antibody did not react with the mutant RNA polymerases which did not have in vitro enzyme activity. These results are discussed in the context of secondary-structure predictions for the core segment containing the conserved YGDD amino acids in the poliovirus RNA polymerase.

Poliovirus, a member of the *Picornaviridae*, has a single-stranded RNA genome of approximately 7,500 bases in length (13, 14). The viral enzyme responsible for the replication of the poliovirus genome is an RNA-dependent RNA polymerase designated 3D^{pol} (27). Previous studies have reported on the purification of 3D^{pol} from the cytoplasm and membranes of poliovirus-infected cells (2, 4, 8, 29). According to an analysis of purified enzyme preparations, 3D^{pol} consists of a single viral protein of 52,000 Da and is dependent on an RNA primer for enzymatic activity (4, 7, 29).

The poliovirus RNA polymerase shares features with RNA polymerases of animal viruses, plant viruses, and bacteriophages and with reverse transcriptases (6, 11, 12, 23). In particular, a 14-amino-acid sequence motif with a 4-residue core sequence, YGDD (amino acids 284 to 332 in poliovirus), has been identified (12). Structure predictions suggest that the aspartic acids in this core sequence are on an exposed "loop" in a β -hairpin structure, which may be involved in metal binding by the enzyme as well as catalytic activity (1). Although this region is well conserved, minimal experimental structure-function information is available on the role of the YGDD region in RNA polymerase enzyme activity. Previous studies on the well-characterized bacteriophage Q β RNA polymerase have demonstrated that single amino acid substitutions at the glycine position in the YGDD amino acids resulted in partial or complete loss of enzyme activity (9).

The entire RNA genome of poliovirus has been cloned, and the nucleic acid sequence has been determined (13, 24). The availability of cDNA clones for poliovirus has facilitated the use of recombinant DNA techniques to express specific

poliovirus genes (28). Studies from this laboratory, as well as others, have reported the successful expression of enzymatically active poliovirus RNA polymerase in *Escherichia coli* (21, 22, 25). The recombinant RNA polymerase exhibited enzymatic activity in the in vitro reactions similar to that of the native enzyme isolated from infected cells (9a, 26). Previous studies have described mutations in the RNA polymerase gene of poliovirus, although none were in the conserved YGDD amino acid motif (3). The great majority of these mutations, though, were actually amino acid differences in the two published sequences of Mahoney type 1 poliovirus and subsequently did not affect enzyme activity (3).

The aim of this study was to utilize a systematic approach to the structure-function analysis of the poliovirus RNA polymerase, focusing on the conserved YGDD amino acid core segment. In this study, we have utilized oligonucleotide site-directed mutagenesis to construct specific amino acid changes in the poliovirus 3D^{pol} in the conserved YGDD region. Each of the mutant RNA polymerase genes was expressed in *E. coli*, and the in vitro enzymatic activity was characterized. Our findings demonstrate that two of the mutant enzymes exhibit partial in vitro RNA polymerase activity ranging from 5 to 20% of that for the wild-type enzyme. To further characterize the structural features of this region of the polymerase encompassing the YGDD amino acid motif, we have generated antipeptide antibodies to this sequence. Interestingly, the antipeptide antibodies react only with mutant RNA polymerases which exhibit in vitro enzyme activity. Our findings, then, indicate that the structural integrity of the YGDD region of the poliovirus RNA polymerase is essential for enzyme activity, and the results are discussed in the context of structural predictions for the YGDD amino acid motif.

* Corresponding author.

MATERIALS AND METHODS

Materials. All chemicals, unless otherwise noted, were purchased from Sigma Chemical Co. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim. Modified T7 DNA polymerase (Sequenase) was purchased from U.S. Biochemicals. The peptide NH₂-MIAYGDDVIA-COOH was synthesized by the University of Alabama at Birmingham Cancer Center Peptide Synthesis Core Facility, and antipeptide antibodies were prepared by Southern Biotechnology (Birmingham, Ala.). All radioisotopes were obtained from Amersham, Inc.

Construction of mutant plasmids. The general techniques required for manipulation of recombinant DNA were carried out by using standard procedures (17).

The construction of the plasmid pProt-Pol-TRP has been previously described (21). A *Bam*HI-to-*Sal*I fragment of this plasmid, corresponding to nucleotides 5240 to 7400 of the poliovirus genome, was subcloned into the phagemid vector pUC119. The resulting plasmid, pUC119Prot-Pol, contains an intergenic region of the bacteriophage M13 and is of the appropriate size for packaging into M13 phage particles.

To obtain single-stranded DNA from pUC119Prot-Pol for mutagenesis, *E. coli* TG-1 (F') was transformed with this plasmid and infected with the helper phage M13K07 under standard conditions (30). Briefly, a culture of pUC119Prot-Pol was grown to early log phase in 2× YT broth supplemented with 10 mM magnesium chloride and 50 µg of ampicillin per ml. The culture was then infected with M13K07 at a multiplicity of infection of 10 and further incubated at 37°C for 120 min. At this time, kanamycin sulfate was added to a final concentration of 70 µg/ml, and the culture was grown overnight at 37°C. The cells were removed by centrifugation, and the phage was precipitated from the supernatant with 3% polyethylene glycol 6000–500 mM sodium acetate on ice for 60 min and collected by centrifugation at 20,000 × *g* for 30 min. The phage pellet was suspended in water, and the DNA was isolated by sequential phenol-chloroform (1:1 [vol/vol]) extractions followed by ethanol precipitation.

Oligonucleotide site-directed mutagenesis was performed with 0.4 pmol of single-stranded DNA template and 1 pmol of DNA oligonucleotide, previously phosphorylated by using 10 U of T4 polynucleotide kinase, in 40 mM Tris HCl (pH 7.5)–20 mM magnesium chloride–50 mM sodium chloride. The following synthetic DNA oligonucleotides were used to introduce the changes (the underlined nucleotides are those changed).

1. 5'–GCCTATGCTGATGATG-3' (YADD)
2. 5'–GCCTATTGCGATGATG-3' (YCDD)
3. 5'–GCCTATATGGATGATG-3' (YMDD)
4. 5'–GCCTATCCTGATGATG-3' (YPDD)
5. 5'–GCCTATAGTGATGATG-3' (YSDD)
6. 5'–GCCTATGTTGATGATG-3' (YVDD)

The primer and template were annealed for 5 min at 65°C and then slowly cooled to room temperature for 60 min. The extension and ligation reactions were performed in the same buffer with added deoxynucleotide triphosphate (65 µM final concentration), dithiothreitol (6.5 µM final concentration), 13 U of Sequenase, and 9 U of T4 DNA ligase. The reaction mixtures were incubated at 37°C for 15 min, at 70°C for 5 min, and finally at 0°C for 5 min. The mixture was then used to transform competent *E. coli* (strain TG-1). Potential mutants were identified by colony hybridization using the [γ -³²P]ATP-phosphorylated oligonucleotide used for muta-

genesis as the probe. The filters were washed with 6× SSC (20× SSC is 3 M sodium chloride plus 300 mM sodium citrate, pH 7.0) at increasing temperature increments until they were 2°C below the theoretical dissociation temperature. The filters were then placed with X-ray film (Kodak X-OMAT) at –70°C to identify positive colonies. Confirmation of the desired mutation was obtained by isolation of the plasmid from *E. coli* and direct sequencing by the Sanger dideoxy chain termination method provided by U.S. Biochemicals. A synthetic DNA oligomer which is complementary to the poliovirus nucleotide sequence 6838 to 6852, approximately 120 bp 5' from the desired mutation, was used for the sequencing primer. Following sequence confirmation of the mutant, the region containing a partial 3C^{pro} gene and entire 3D^{pol} gene, from nucleotides 5601 to 7400 (a *Bgl*II-*Sal*I DNA fragment), was isolated and subcloned into the expression plasmid pPROT-Pol-TRP that had previously been digested with *Bgl*II and *Sal*I. For convenience, these plasmids will be referred to as *ptrp* 3D-wt (for wild type corresponding to the previously published pProt-Pol-Trp [21]) and *ptrp*-3D-X-327, where the X refers to the single amino acid change and its position in the polymerase protein. In all cases, the resulting plasmid was sequenced to reconfirm the mutation before analysis of protein expression.

Expression of poliovirus RNA polymerase in *E. coli* and preparation of lysates. The conditions required for the induction of the *trp* operon and RNA polymerase from *E. coli* were as previously described (21). Briefly, the cell pellets of induced *E. coli* were lysed with lysozyme–Triton X-100, and the crude supernatant containing RNA polymerase activity was purified by phosphocellulose chromatography. A single elution of the phosphocellulose column using 500 mM potassium chloride–TGNB (50 mM Tris [pH 8], 0.5% Triton X-100, 20% glycerol, 0.5 mM β-mercaptoethanol) was used to release the enzyme. The eluted proteins were concentrated against solid sucrose and followed by dialysis versus TGNB. The preparations were then frozen at –70°C and exhibited stability for at least 6 months under these conditions.

Electrophoretic blotting of polypeptides from induced *E. coli*. A Western immunoblot analysis was used to quantitate the relative levels of expressed 3D^{pol} (21). The proteins eluted from the phosphocellulose column were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose paper. After transfer, the blots were soaked in BLOTTO (50 mM Tris [pH 7.5], 10 mM NaCl, 5% nonfat dry milk [10]) for 30 min at room temperature. The blot was reacted with anti-3D^{pol} antibody which had been previously adsorbed with an extract from *E. coli* to reduce nonspecific background (21). In some cases, the blot was reacted with a rabbit antipeptide antibody specific for the amino acids NH₂-MIAYGDDVIA-COOH. The synthetic peptide which was used to produce the antipeptide antibody was coupled to keyhole limpet hemocyanin conjugate and used to immunize rabbits as previously described (20). The antipeptide serum was also treated with a bacterial lysate prior to use in Western blots to reduce nonspecific background. The blots were incubated with the antibodies in BLOTTO overnight at 4°C and then washed several times with BLOTTO to remove unbound antibody. The blot was reacted with ¹²⁵I-protein A (100,000 cpm/µg) for 1 h at room temperature, washed extensively with BLOTTO, dried, and autoradiographed at –70°C with a Dupont Cronex intensifier using Kodak XAR film. For quantitation, the autoradiographs were scanned with a Bio-

Rad model 620 video densitometer with a BioRad model 3392A integrator.

Assay for RNA polymerase and replication activities. 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 (21). The in vitro-synthesized product was precipitated with 10% trichloroacetic acid and then collected on Gelman filters (0.45-µm-pore-size), and the radioactivity was determined by scintillation counting. The concentration of [α - 32 P]UTP was adjusted such that 10,000 cpm correlated with 1 pmol of UTP incorporated into trichloroacetic acid-insoluble material.

Construction of atomic models. Secondary-structure prediction followed that of Argos (1). The standard type I turn conformation was constructed for the YGDD sequence. The side chain conformation was selected from the Brookhaven protein structure data bank to be the most frequent conformation for that residue. The work was carried out on an IRIS computer (GTX) with a program package developed by Mike Carson at University of Alabama at Birmingham.

RESULTS

Generation of defined amino acid substitutions in the 3D^{pol} gene. The region of the poliovirus 3D^{pol} gene targeted for mutagenesis is located between nucleotides 6962 and 6974. Judging by an amino acid comparison of several putative RNA polymerases from animal, plant, and bacterial viruses and the RNA polymerase of *E. coli*, this region of 3D^{pol} corresponds to a conserved site consisting of four amino acids (tyrosine-glycine-aspartic acid-aspartic acid [YGDD]), which form a highly conserved motif found in many RNA polymerases and has been proposed to be involved in metal binding and the catalytic site of the enzyme. The glycine residue at position 2 of this sequence motif is not as strictly conserved between the RNA polymerases (11, 12, 23). Since our intention was to generate mutant 3D^{pol} with altered enzyme activity, we decided to focus our attention on the second position in the YGDD site because of the relative diversity of amino acids found at this position in RNA polymerases.

We subcloned the entire poliovirus gene segment used for expression, nucleotides 5240 to 7400 contained in a *Bam*HI-*Sal*I fragment, into the phagemid vector pUC119. Oligonucleotide site-directed mutagenesis was performed by using single-stranded DNA isolated from the phagemid. The nucleotides coding for the glycine residue were changed to give the predicted amino acid change from glycine to alanine, cysteine, methionine, proline, serine, or valine (Table 1). The nomenclature for the mutations is 3D-X-327, where X refers to the single-letter amino acid designation and 327 is the amino acid position in the polymerase. The mutant 3D^{pol} genes were then subcloned back into the expression plasmid pProt-Pol-TRP by using a unique *Bgl*II-*Sal*I restriction fragment (Fig. 1).

Expression of mutant 3D^{pol} genes in *E. coli*. To determine the effect of the amino acid substitutions on RNA polymerase activity, the mutant 3D^{pol} genes were expressed in *E. coli*. Previous studies from this laboratory have demonstrated that expression of the poliovirus genome from nucleotides 5240 to 7400 containing the intact 3C^{pro} and 3D^{pol} genes results in the synthesis of a polyprotein which is rapidly processed by 3C^{pro} to give enzymatically active 3D^{pol} (21). After induction of the *trp* operon, the *E. coli* cell pellets containing the expressed mutant RNA polymerases

TABLE 1. Mutations in 3D^{pol} at amino acid position 327^a

Designation	Amino acid sequence	Nucleic acid sequence
3D-G-327 (wt)	TATGGTGATGAT	YGDD
3D-A-327	GCT	YADD
3D-C-327	TGC	YCDD
3D-M-327	ATG	YMDD
3D-P-327	CCT	YPDD
3D-S-327	AGT	YSDD
3D-V-327	GTT	YVDD

^a Amino acid positions were calculated from the start of 3D^{pol} at nucleotide 5987 of the poliovirus genome. Glycine was mutated in each case by changing the nucleotide sequence to code for the new amino acid by using oligonucleotide site-directed mutagenesis.

were partially purified by phosphocellulose chromatography and analyzed by a Western blot analysis using an antibody specific for 3D^{pol} (Fig. 2). Immunoreactive material corresponding to a protein of 52 kDa, the molecular weight of mature 3D^{pol}, was detected in extracts from induced *E. coli* transformed with plasmids coding for wild-type and mutant enzymes, while no immunoreactive material was detected in extracts from induced *E. coli* transformed with the *trp* expression plasmid. Decreasing amounts of extract from *E. coli* transformed with the wild-type plasmid were used for quantitation of the Western blot (Fig. 2A). The autoradiograph was scanned, and a graph was generated from a plot of the intensity of immunoreactive material versus the amount of bacterial lysate containing 3D^{pol}. Extracts from *E. coli* transformed with the mutant and wild-type plasmids were diluted such that the levels of immunoreactive material were in the linear range of the Western blot. The level of immunoreactive material for each enzyme was then normalized as depicted in Fig. 2B.

Enzymatic activity of mutant 3D^{pol} proteins. To determine if the mutant 3D^{pol} proteins have enzymatic activity, the preparations were tested using the poly(A) · oligo(U) polymerase assay. The concentrations of the wild-type and mutant 3D^{pol} were estimated by comparing autoradiograms of the immunoreactive 52-kDa material, and the enzyme preparations were adjusted so that all of the extracts had similar amounts of immunoreactive material. The enzymatic activity of each of the 3D^{pol} preparations was tested over a range of concentrations by using the standard polymerase assay (Fig. 3). The differences observed between the extracts of wild-type 3D^{pol} (3D-wt) and of vector alone were on the order of 100-fold (approximately 5×10^5 cpm versus 5×10^3 cpm, respectively). The single change of glycine to alanine or serine (3D-A-327 and 3D-S-327, respectively) resulted in RNA polymerases with levels of enzyme activity

FIG. 1. Expression plasmid pProt-Pol-TRP (hereafter called *ptrp* 3D-wt) used for the expression of poliovirus 3D^{pol} in *E. coli*. A DNA fragment corresponding to nucleotides 5240 to 7400 was subcloned into the phagemid vector pUC119 for mutagenesis. The mutated 3D^{pol} genes at the YGDD amino acids (nucleotides 6962 to 6973) were resubcloned into the expression plasmid by exchange of the *Bgl*II (5601)-*Sal*I restriction fragment.

FIG. 2. Immunoblot analysis of wild-type and mutant 3D^{pol} expressed in *E. coli*. (A) Standardization of 3D^{pol} Western blot. Decreasing amounts of phosphocellulose-purified extracts from *E. coli* transformed with *ptrp*-3D-wt were analyzed by Western blot with a polyclonal anti-3D^{pol} antibody which has been previously demonstrated to react with the whole molecule (25). The autoradiogram was scanned by a densitometer, and by using a plot of band intensity versus extract concentration, the wild-type and mutant RNA polymerase samples were diluted to be in the linear range. Lanes contained 10 μ l of control extract (lane 1) or 10 μ l (lane 2), 5 μ l (lane 3), 2.5 μ l (lane 4), 1.25 μ l (lane 5) or 0.625 μ l (lane 6) of *ptrp*-3D-wt extract. (B) Analysis of wild-type and mutant 3D^{pol} proteins. Phosphocellulose-purified protein preparations from induced *E. coli* transformed with plasmids containing the vector, wild-type, or mutant 3D^{pol} genes were separated on a resolving 10% sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred to nitrocellulose. Immunoblots were performed by using the same anti-3D^{pol} antibody as in panel A. Lanes: 1, *E. coli* transformed with vector plasmid; 2, 3D-wt; 3, 3D-A-327; 4, 3D-C-327; 5, 3D-M-327; 6, 3D-P-327; 7, 3D-S-327; 8, 3D-V-327. The molecular weight markers were electrophoresed in parallel lanes, and the 3D^{pol} position is marked.

between 5 and 20% of wild-type levels. The extracts from RNA mutant polymerases with 3D-C-327, 3D-M-327, 3D-P-327, and 3D-V-327 mutations resulted in a complete loss of enzyme activity over a wide range of concentrations. At least three independent enzyme preparations were generated and tested; results comparable to those shown in Fig. 3 were obtained.

The YGDD amino acid motif has been proposed to be involved in metal ion binding and to act catalytically in the polymerization process (1, 5, 23). Thus, we predict that the mutant polymerases might exhibit different phenotypic properties if the experimental conditions for the RNA polymerase assay are varied. In the initial experiment, the mutant enzymes were compared with the wild type under conditions in which the metal ion requirements were varied over a range of 0 to 10 mM magnesium acetate (Fig. 4). The pattern of the enzyme activity was consistent in that both the 3D-S-327 and 3D-A-327 RNA polymerases exhibited 20 and 5%, respectively, of the wild-type activity, regardless of the magnesium acetate concentration. We compared the enzymatic activities of the mutant and wild type polymerases at 30, 37, and 42°C (Fig. 5A) and over an assay pH range of 7.0 to 9.0 (Fig. 5B). No significant differences in the patterns of wild-type and mutant enzyme reactivities at different temperatures or with different assay pHs were detected.

Since the YGDD region is postulated to be involved in the

FIG. 3. RNA polymerase activities of wild-type and mutant 3D^{pol} enzymes. 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 identical amounts of immunoreactive 3D^{pol} were used in the poly(A) · oligo(U) polymerase assay under standard conditions. The different preparations tested were as follows: ○, vector; ●, 3D-wt; □, 3D-A-327; ■, 3D-C-327; △, 3D-M-327; ▲, 3D-P-327; ◇, 3D-S-327; ◆, 3D-V-327.

enzyme active site, it was possible that the 3D-S-327 and 3D-A-327 enzymes required extended time to synthesize RNA in vitro. If this were the case, the kinetics of synthesis would reveal that mutant enzymes will synthesize levels of poly(U) similar to that synthesized by the wild type after extended time. To address this question, extended reaction time kinetics were performed on each enzyme preparation from 30 min (standard) to 2 h (Fig. 6). The amounts of the trichloroacetic acid-precipitable products [poly(U)] from the reactions containing the wild-type and mutant 3D-A-327 and

FIG. 4. Effect of magnesium concentration on RNA polymerase activities of wild-type and mutant 3D^{pol}. For this study, the RNA polymerase activity of 5 μ l of the normalized enzyme was determined by using various concentrations of magnesium acetate in the poly(A) · oligo(U) polymerase assay. The preparations tested were as follows: ○, vector; ●, 3D-wt; □, 3D-A-327; ■, 3D-C-327; △, 3D-M-327; ▲, 3D-P-327; ◇, 3D-S-327; ◆, 3D-V-327.

FIG. 5. (A) Effect of different assay temperatures on RNA polymerase activity. For these studies, the activity of 5 μ l of the RNA polymerase in the indicated normalized enzyme preparations was determined in the standard poly(U) polymerase assay at three different temperatures: □, 30°C; ■, 37°C; and ▨, 42°C. (B) Effect of different assay pHs on RNA polymerase activity. Activity at each pH value was measured at 30°C. The different preparations tested were as follows: ○, extract from *E. coli* transformed with vector; ●, 3D-wt; □, 3D-A-327; ■, 3D-C-327; △, 3D-M-327; ▲, 3D-P-327; ◇, 3D-S-327; ◆, 3D-V-327.

3D-S-327 RNA polymerases increased in a linear fashion for up to 30 min. Further incubation resulted in no significant increase of poly(U) synthesized from reactions with either the wild-type or mutant RNA polymerase. At extended reaction times, the levels of poly(U) detected actually decreased, most probably because of contaminating nucleases in the preparations. Additionally, no RNA polymerase activity was detected for the other mutants over the reaction times examined.

Reactivity of wild-type and mutant RNA polymerases with antipeptide antibody. Secondary-structure predictions for the region containing the YGDD motif suggest a β hairpin with the aspartic acids on an exposed loop (1). To obtain insights into the nature of this structure, a peptide, NH₂-MIAYGDDVIA-COOH, which corresponds exactly to that

FIG. 6. Reaction time kinetics for RNA polymerases. The phosphocellulose-purified preparations were analyzed for RNA polymerase activity over the time course indicated using standard conditions for the poly(A) · oligo(U) polymerase assay. The preparations tested were as follows: ○, extracts prepared from *E. coli* transformed with vector; ●, 3D-wt; □, 3D-A-327; ■, 3D-C-327; △, 3D-M-327; ▲, 3D-P-327; ◇, 3D-S-327; and ◆, 3D-V-327.

found in the poliovirus RNA polymerase, was synthesized and conjugated to a larger carrier protein for use as an immunogen to produce antipeptide antibodies. This antipeptide antibody was used in a Western blot analysis with bacterial extracts containing identical amounts of immunoreactive polymerase as determined by reactivity to the entire polymerase molecule (Fig. 7). The antipeptide antibody reacted with the wild-type enzyme, detecting predominantly a 52-kDa protein, and with the 3D-A-327 and 3D-S-327 mutants, which demonstrated reduced enzymatic activity in vitro. The level of reactivity was significantly lower than that observed for the wild-type enzyme even though equal amounts of total immunoreactive material were analyzed. The antipeptide antibody did not react with the other mutants to any significant extent in this Western blot assay. We conclude from these results that the reactivity of the anti-

FIG. 7. Immunoblot analysis of RNA polymerase preparations with antipeptide antibody specific for the core YGDD amino acid segment of poliovirus RNA polymerase. Preparations of RNA polymerase purified by phosphocellulose column chromatography were electrophoresed through a 10% sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred to nitrocellulose. Equal amounts of immunoreactive 3D^{pol} protein as judged by immunoblot analysis using anti-3D^{pol} (Fig. 2) were used for each sample. Following transfer, the blot was reacted with an antipeptide antibody specific for the core YGDD amino acid segment. Lanes: 1, extracts from *E. coli* transformed with vector; 2, 3D-wt; 3, 3D-A-327; 4, 3D-C-327; 5, 3D-M-327; 6, 3D-P-327; 7, 3D-S-327; 8, 3D-V-327. The molecular weight markers were electrophoresed in parallel, and the position corresponding to 3D^{pol} is marked.

peptide antibody with wild-type and different mutant polymerases in this Western blot analysis correlates with the levels of observed *in vitro* RNA polymerase enzyme activity and is consistent with a conservation of structure in these active mutants (see Discussion).

DISCUSSION

In this study, we describe a structure-function analysis of the poliovirus RNA-dependent RNA polymerase focused on the highly conserved YGDD amino acid core segment. We have utilized oligonucleotide site-directed mutagenesis to construct six mutant RNA polymerases which were expressed in *E. coli*. Two of the mutants, 3D-S-327 and 3D-A-327, exhibited reduced but clearly detectable RNA polymerase activities *in vitro* under a variety of experimental assay conditions, while the remaining mutations in the RNA polymerase resulted in inactive enzymes. We have also utilized an anti-peptide antibody specific for the YGDD core segment to obtain insights into the structure of this amino acid segment. The anti-peptide antibody in a Western blot analysis reacts only with those RNA polymerases exhibiting enzymatic activity. These studies indicate that structural motifs detected by the anti-peptide antibody correlate, in part, with those required for the enzymatic activity of the RNA polymerase.

Previous studies have identified several amino acid changes throughout the polymerase gene (3). These are not in the YGDD core segment and do not affect enzyme activity. Most of these are single amino acid changes identified as a result of minor differences between similar strains of poliovirus (3). As might be expected, amino acid insertions in the RNA polymerase protein, which undoubtedly have drastic effects on the protein structure, generally result in the complete loss of RNA polymerase enzyme activity (3). The results of our study are unique because this is the first systematic analysis of the YGDD core segment in the function of an animal virus RNA-dependent RNA polymerase. The targeting of glycine, the second amino acid in the core YGDD segment, for mutagenesis was based on previous studies demonstrating the effects of amino acid changes on replicase activity of the bacteriophage Q β (9). Changes in the conserved YGDD core segment of the Q β replicase, in contrast to change in the poliovirus RNA polymerase, resulted in a complete loss of *in vivo* replicase activity as measured by the capacity to complement replicase-deficient phages. This appears to be a sensitive assay, because Mills et al., reporting on mutant Q β replicases generated by oligonucleotide linker insertion, found that even low levels of *in vitro* RNA synthesis are sufficient to support phage infection and produce a positive phenotype in the *in vivo* assays (19). We have subcloned the mutant poliovirus RNA polymerase genes back into an infectious clone of poliovirus. Preliminary results suggest that transfection of these genomes results in the production of infectious virus, and we are in the process of determining the nucleic acid sequence to ascertain whether viruses have maintained the mutation or reverted (9a).

The results of these studies provide evidence for the importance of this core YGDD sequence in RNA polymerase enzymatic activity, since single amino acid changes in this region drastically affected the enzymatic activity of the RNA polymerase. The single amino acid changes result in an intrinsic change in the RNA polymerase, as evidenced by the fact that the activity of the mutants is not altered by changes in reaction temperature, pH, metal ion, or length of incuba-

FIG. 8. Atomic models of YGDD core amino acid segment. Secondary-structure predictions for the core 14-amino-acid segment containing the YGDD and mutants are presented. (A) Ball-and-stick model of the middle 8 amino acids of the core segment with the YGDD sequence centered in the β -turn- β configuration, generated as indicated in Materials and Methods. (B through F) van der Waals space-filling models of sequences of wild type and four mutants: 3D-wt (B), 3D-A-327 (C), 3D-S-327 (D), 3D-C-327 (E), and 3D-M-327 (F). Green, carbon; red, oxygen; blue, nitrogen; yellow, sulfur groups.

tion time. Recent studies with the DNA polymerase from herpes simplex virus type 1 (18) and human immunodeficiency virus type 1 reverse transcriptase (15) have also highlighted the functional importance of the YXDD amino acid motif. Indeed, the mutation of glycine to alanine in the YGDTD core amino acid motif of the herpes simplex virus type 1 DNA polymerase resulted in a lethal mutation in the *pol* gene. We have also found that mutations in the second amino acid (YMDD) of this motif of the human immunodeficiency virus type 1 reverse transcriptase results in drastic effects on *in vitro* enzyme activity as well as on virus infectivity (9b). Taken together, these studies support the notion that the YXDD motif is at or near the enzyme active center. Computer modeling and sequence comparison by Delarue et al. have utilized the known tertiary structure of the Klenow fragment of *E. coli* polymerase I to predict the spatial arrangement for this conserved amino acid core segment in other polymerases, and they also support the notion that the YGDD core amino acid segment is at or near the catalytic site (5). In this model, it is the first aspartate which is presumed to be important in the catalytic site of the

polymerase. Consistent with this prediction is the fact that mutation of the first aspartate to histidine in the poliovirus RNA polymerase results in a complete loss of enzyme activity (9a).

The fact that the serine and alanine are partially tolerated as substitutes for glycine in the enzymatically active RNA polymerase is interesting. Since antipeptide antibodies generally react with the linear amino acid structure (reviewed in reference 16), this result suggests a similarity at this level between the wild-type, 3D-S-327, and 3D-A-327 RNA polymerases. To explore these potential structural similarities, we have utilized secondary-structure predictions for this core segment in order to understand possible interactions between contiguous amino acids in this region (Fig. 8). A region of 14 amino acids, with the conserved YGDD sequence at the center, was predicted by Argos to have a β -turn- β structure (1). The ball and stick represent a structural model with YGDD in the type I turn conformation. The side chains of each residue were placed according to the most commonly occurring conformation in the protein structure data bank. The ball-and-stick model of this segment, in a β -turn- β structure, is shown in Fig. 8A. The space-filling model of the wild-type sequence, showing the van der Waals volume occupied by the amino acids, is shown in Fig. 8B. Only minor structural perturbations were observed in this region by the substitution of alanine or serine for glycine (cf. Fig. 8C and D with B). However, significant changes of the volume at the top of the loop were predicted when the glycine was replaced by cysteine or methionine (Fig. 8E and F), and perturbations were starting to affect the neighboring tyrosine and aspartic acid residues. The increasing volume occupied by the larger side chains and the structural effects of the larger side chains, additions of hydrophobic amino acids (e.g., V), or disruptions in the primary structure by amino acids (e.g., P) correlate well with the decrease of enzymatic activity and loss of reactivity with the antipeptide antibody. While this analysis represents a limited number of mutants, the model structures do help visualize potential interactions between neighboring amino acids and offer a possible explanation for the observed experimental results. We are extending this analysis to the other conserved amino acids (tyrosine and aspartic acids) to determine if the enzyme activity correlates with reactivity with the antipeptide antibody.

At present, we do not have any information on the interactions between the conserved YGDD segment and other regions of the RNA polymerase. On the basis of structural comparisons with the Klenow fragment, however, we expect that this region would be internal in the protein structure and might cooperate with other regions of the RNA polymerase to form the catalytically active site (5). The fact that we have not been able to inhibit the enzyme activity of the RNA polymerase by using the antipeptide antibodies is consistent with this prediction (9a). To further understand the nature of the interaction between the YGDD core amino acid sequence and other regions of the polymerase, we are undertaking a sequence analysis of the polymerase genes of the polioviruses obtained from the transfection of cDNA clones containing the mutant RNA polymerase genes. Ultimately, the further details of the interactions between amino acids await the three-dimensional structure of the RNA polymerase obtained from X-ray crystallography.

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