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Mutation of the Aspartic Acid Residues of the GDD Sequence Motif of Poliovirus RNA-Dependent RNA Polymerase Results in Enzymes with Altered Metal Ion Requirements for Activity

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The poliovirus RNA-dependent RNA polymerase, $3D^{pol}$, is known to share a region of sequence homology with all RNA polymerases centered at the GDD amino acid motif. The two aspartic acids have been postulated to be involved in the catalytic activity and metal ion coordination of the enzyme. To test this hypothesis, we have utilized oligonucleotide site-directed mutagenesis to generate defined mutations in the aspartic acids of the GDD motif of the $3D^{pol}$ gene. The codon for the first aspartate (3D-D-328 [D refers to the single amino acid change, and the number refers to its position in the polymerase]) was changed to that for glutamic acid, histidine, asparagine, or glutamine; the codons for both aspartic acids were simultaneously changed to those for glutamic acids; and the codon for the second aspartic acid (3D-D-329) was changed to that for glutamic acid or asparagine. The mutant enzymes were expressed in *Escherichia coli*, and the in vitro poly(U) polymerase activity was characterized. All of the mutant $3D^{pol}$ enzymes were enzymatically inactive in vitro when tested over a range of Mg^{2+} concentrations. However, when Mn^{2+} was substituted for Mg^{2+} in the in vitro assays, the mutant that substituted the second aspartic acid for asparagine (3D-N-329) was active. To further substantiate this finding, a series of different transition metal ions were substituted for Mg^{2+} in the poly(U) polymerase assay. The wild-type enzyme was active with all metals except Ca^{2+}, while the 3D-N-329 mutant was active only when $FeC_6H_7O_5$ was used in the reaction. To determine the effects of the mutations on poliovirus replication, the mutant $3D^{pol}$ genes were subcloned into an infectious cDNA of poliovirus. The cDNAs containing the mutant $3D^{pol}$ genes did not produce infectious virus when transfected into tissue culture cells under standard conditions. Because of the activity of the 3D-N-329 mutant in the presence of Fe^{2+} and Mn^{2+}, transfections were also performed in the presence of the different metal ions. Surprisingly, the transfection of the cDNA containing the 3D-N-329 mutation resulted in the production of virus at a low frequency in the presence of $FeSO_4$ or $CoCl_2$. The virus derived from transfection in the presence of $FeSO_4$ grew slowly, while the viruses recovered from transfection in $CoCl_2$ grew at a rate which was similar to that of the wild-type poliovirus. The nucleotide sequence of the virus obtained from transfection in the presence of Co^{2+} revealed that the 3D-N-329 mutation in the polymerase had reverted to a 3D-D-329. These results demonstrate that although the first aspartic acid residue is absolutely required for enzyme function, flexibility exists with respect to the requirement for the second aspartic acid residue. The activity of the 3D-N-329 mutant in the presence of different metal ions suggests the involvement of the aspartic acids in metal ion coordination during polymerization.

Poliovirus, a member of the family *Picornaviridae*, is a plus-stranded RNA virus with a length of approximately 7,500 bases (23, 24, 41). The virus utilizes a virally encoded RNA-dependent RNA polymerase, $3D^{pol}$, for replication of its genomic RNA (10, 15, 48). The $3D^{pol}$ first synthesizes an RNA of negative polarity, which serves as a template for the synthesis of large amounts of positive-stranded RNA. Several laboratories have reported on the expression of the viral $3D^{pol}$ gene in *Escherichia coli* (6, 7, 32, 34, 39, 40). The recombinant RNA polymerase is a single polypeptide which has a molecular mass of 52,000 Da and which exhibits an enzymatic activity similar to that of the enzyme isolated from infected cells (32, 34, 39, 40).

The poliovirus $3D^{pol}$ enzyme shares regions of sequence homology with other RNA polymerases from animal and plant viruses as well as bacteriophages (12, 13, 20, 22, 35). One of the most highly conserved sequence motifs is the YXDD motif, found in virtually all RNA polymerases, DNA polymerases, and reverse transcriptases (RTs) (12). In plus-stranded RNA viruses, the consensus sequence motif is GDD, whereas for negative-stranded RNA viruses the commonly found sequence is GDN, suggesting that the first aspartic acid of the motif is critical for enzyme function (12). Structural predictions have suggested that the secondary structure of this region appears to be in a β-turn-β conformation, with the aspartic acid residues exposed on the loop region (2). Functional studies utilizing site-directed mutagenesis at this region demonstrate its necessity for both DNA and RNA polymerase function (3, 4, 14, 16–18, 21, 26, 27, 30, 31, 38, 43, 45). On the basis of these results, the aspartic acids have been postulated to be involved in metal ion coordination at or near the catalytic active site of the enzyme (2, 12).

Previous studies from this laboratory have focused on a structure-function analysis of the conserved YGDD region of $3D^{pol}$. Site-directed mutagenesis was used to demonstrate the importance of the tyrosine and glycine residues in enzyme function (17, 18). In this study we have utilized site-directed mutagenesis to change the amino acid sequence at the site of the aspartic acid residues in RNA polymerase. Given the heterogeneity observed with regard to the GDD motif in RNA polymerases from negative-strand RNA viruses, we wanted to

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TABLE 1. DNA oligomers used to create mutations in $3D^{pol}$ gene

Amino acid sequence	Mutagenic oligonucleotide sequence[a]
YGDD	5′ GCCTATGGTGATGATGTAAATGC 3′
YGED	GCCTATGGTGAGGATG
YGHD	CCTATGGTCATGATG
YGND	GCCTATGGTAACGATG
YGQD	GCCTATGGTCAGGATG
YGEE	CCTATGGTGAGGAGGTAAATGC
YGDE	GCCTATGGTGATGAGG
YGDN	GCCTATGGTGATAACG

[a] The underlined nucleotides are those which were changed.

ascertain whether flexibility exists with respect to amino acid substitutions at this site of the poliovirus RNA polymerase. We utilized site-directed mutagenesis to construct several different combinations of mutations in the aspartic acids of the GDD motif. Each mutant was expressed in *E. coli*, and the enzymatic activities were characterized. The mutant $3D^{pol}$ genes were subcloned into the poliovirus infectious clone, followed by transfection to test for virus production. The results presented here demonstrate the absolute necessity for conservation of the first aspartic acid residue for the enzymatic function of poliovirus $3D^{pol}$. A single amino acid change in the second aspartic acid (to asparagine) resulted in a polymerase with a different metal ion preference for activity. The results are discussed in the context of previously described models of the GDD amino acid motif in polymerases.

MATERIALS AND METHODS

Materials. All chemicals, unless otherwise noted, were purchased from Sigma Chemical Company. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Modified T7 DNA polymerase (Sequenase) was purchased from U.S. Biochemicals. The enhanced chemiluminescence kit for Western blot (immunoblot) analysis and all radioisotopes were obtained from Amersham, Inc. Tissue culture media and RT-PCR reagents were obtained from BRL-Gibco. The RNA isolation reagents were obtained from Molecular Research Center, Inc. The kit used for subcloning the PCR-amplified DNA was obtained from Invitrogen. 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 or purchased from Oligos Etc.

Construction of mutants. The general procedures required for the manipulation of recombinant DNA were carried out by standard techniques (29). The construction of the plasmid pUC119Prot-Pol has been previously described (17). Single-stranded DNA was produced from this plasmid as previously indicated (17), and this was used as the template for mutagenesis. Oligonucleotide site-directed mutagenesis was performed as previously described (17), utilizing the synthetic DNA oligonucleotides shown in Table 1 to introduce the changes at the aspartic acids of the YGDD motif. Mutants were identified by direct sequencing of double-stranded plasmid DNA by the Sanger dideoxy nucleotide technique (42) as modified for use with Sequenase. The region of pUC119Prot-Pol from nucleotides 5601 to 7400 of the poliovirus genome (a *Bgl*II-*Sal*I DNA fragment) was isolated and subcloned into the expression plasmid pProt-Pol-TRP which had previously been digested with *Bgl*II and *Sal*I. The plasmids will be referred to as p*trp*-3D-wt (for the wild-type, corresponding to the previously published pProt-Pol-TRP [32]) and p*trp*-3D-X-328, where X refers to the single amino acid change, and the number refers to its position in the polymerase.

Expression and detection of poliovirus $3D^{pol}$ mutants in *E. coli*. The conditions required for the induction of the *trp* operon to produce $3D^{pol}$ from *E. coli* were as previously described (32). A Western immunoblot analysis was used to visualize and quantitate the relative levels of expressed $3D^{pol}$ (17). Lysate eluted from the phosphocellulose column was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to nitrocellulose. The proteins were detected with an anti-$3D^{pol}$ antibody (39) which had been previously adsorbed with an extract from *E. coli* to reduce nonspecific background, and then an anti-rabbit horseradish peroxidase-conjugated antibody was reacted with the blots to detect the protein bands with enhanced chemiluminescence. A series of twofold dilutions of bacterial extracts expressing the wild-type polymerase was first assayed, to assure that the detection of $3D^{pol}$ was in the linear range. The levels of immunoreactive material were then normalized for subsequent analysis.

Enzymatic activity of mutant $3D^{pol}$ proteins. The bacterial lysates were tested in a poly(A) · oligo(U) polymerase assay to determine RNA polymerase activity (32). The in vitro product was precipitated with 10% trichloroacetic acid and then collected on 0.45-μm-pore-size Gelman filters. Radioactivity was determined by scintillation counting.

Analysis of mutant polymerase function in vivo. The *Bgl*II-*Sal*I fragment containing the mutations in the pUC119Prot-Pol DNA was also subcloned into a plasmid, pT7IC, which contains the infectious clone of poliovirus. This plasmid contains the entire poliovirus genome, preceded by the simian virus 40 origin and early promoter and two guanines (8). Transfection of 2 μg of the mutant plasmid DNA per well in a 24-well plate of COS-1 cells was accomplished by the DEAE-dextran method (8, 47). The cells were incubated at 37°C for 5 days and subjected to three freeze-thaw cycles to release the virus, and the supernatant was passaged onto fresh BSC40 cells to check for virus production by cytopathic effect. Two further passages of the cellular lysate on BSC40 cells were performed to allow for amplification of the virus. Transfections done in the presence of metal ion were performed in the same manner, except that metal ion at a final concentration of 100 μM was present at all times.

Metabolic labeling of mutant viruses and subsequent immunoprecipitation. Virus obtained in the presence of $FeSO_4$ was used to infect 35-mm-diameter plates of near-confluent BSC-1 cells. At 1 day postinfection, cells were labeled with 100 μCi of ^{35}S-Translabel per well for 24 h, and cell lysates were harvested in radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM sodium chloride, 25 mM Tris-HCl [pH 7.34]). Immunoprecipitations were performed as previously described with antisera to whole poliovirus (1). Briefly, samples were incubated overnight with antisera, and immune complexes were collected with protein A-Sepharose beads (10%, wt/vol), washed three times with radioimmunoprecipitation assay buffer, and separated on SDS–10% PAGE and fluorographed.

For the wild-type virus, 35-mm-diameter plates were infected with 1 PFU of virus per cell. The cells were labeled for 60 min at 5 h postinfection with 50 μCi of ^{35}S-Translabel per well. After labeling, the cells were disrupted in radioimmunoprecipitation assay buffer. Immunoprecipitations and SDS-PAGE were performed as previously described (1).

RT-PCR isolation of polymerase genes. Total cellular RNA was isolated from infected cell lysates with TRI-REAGENT-LS (Molecular Research Center, Inc.), followed by treatment with DNase I to remove any plasmid DNA. RT-PCR isolation was performed with the SuperScript Preamplification System for first-strand cDNA synthesis. The primer used for reverse transcription of viral RNA and subsequent 3′-end amplification was 5′ GGTACAATGTTGAGTACTCT GGGAGCAATA 3′. This corresponds to the minus-sense poliovirus sequence from nucleotides 7349 to 7320, the end of the $3D^{pol}$ coding region. The primer for the 5′-end amplification was 5′ GCAAAGAAGTGGAGATCTTGGATGC CAAAG 3′. This corresponds to the plus-sense poliovirus sequence from nucleotides 5589 to 5618, in the $3C^{pro}$ gene. PCRs were run for 30 cycles, with 94°C dissociation for 1 min, 37°C annealing for 1 min, and 72°C extension for 3 min, followed by a final 7-min extension at 72°C. The PCR products were gel purified and directly cloned into the TA cloning vector (Invitrogen). DNA sequencing of the $3D^{pol}$ gene from the recombinants containing the PCR-amplified product was carried out with a Prism ready-reaction dideoxy termination cycle sequencing kit and an Applied Biosystems automated sequencer. Any changes that were found were confirmed by manual DNA sequencing (42). The oligonucleotides used for sequencing were as previously described (18), with the following additions: (i) 5′ CCA ACC AGA GCA GGA CAG TGT GG 3′ (complementary to nucleotides 5858 to 5880 of the poliovirus genome); (ii) 5′ GGA TCG CAC CCC ACT GCT GAA CC 3′ (complementary to nucleotides 6627 to 6605 of the poliovirus genome); and (iii) 5′ GTC TTT TCC TGA TTG GGC TAG G 3′ (complementary to nucleotides 7033 to 7012 of the poliovirus genome). Note that oligonucleotide i will read 5′ to 3′ in the plus sense while oligonucleotides ii and iii read 5′ to 3′ in the minus sense. In addition to these DNA oligonucleotides, the M13 forward and reverse primers were used for sequencing, since the TA vectors have the sequences complementary to these DNA oligomers flanking the cloning sites.

RESULTS

Expression of poliovirus $3D^{pol}$ in *E. coli*. The region of $3D^{pol}$ which has been targeted for mutagenesis is the highly conserved YGDD amino acid motif located between nucleotides 6962 and 6973 (Fig. 1A). In these studies, we have focused on the aspartic acids (DD) for mutagenesis. The region containing the entire $3D^{pol}$ gene (the *Bgl*II-*Sal*I fragment) was subcloned into the phagemid pUC119. Oligonucleotide site-directed mutagenesis was used to construct single or double mutations as described in Table 1. The $3D^{pol}$ mutants were cloned into the

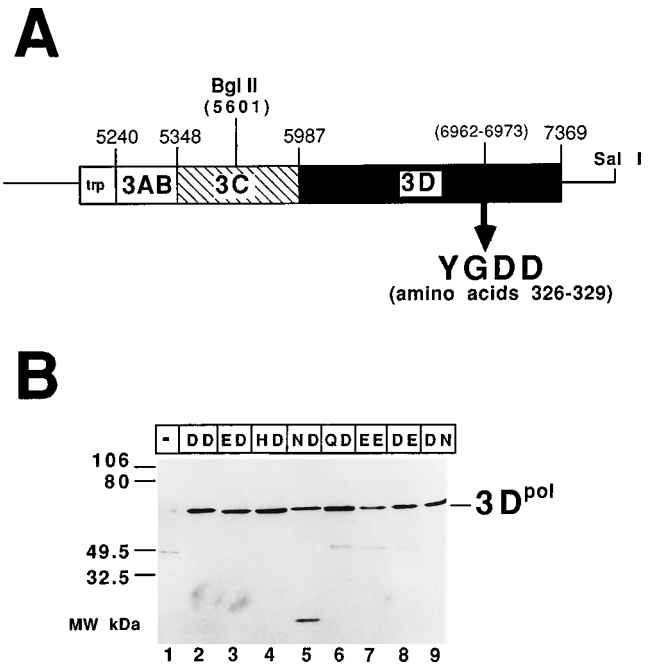

FIG. 1. Expression of $3D^{pol}$ in *E. coli*. (A) The region of the poliovirus genome from nucleotide 5240 to 7369 was subcloned into expression plasmid p*trp* (32). Induction of the *trp* operon results in the expression of the 3ABCD fusion protein, which is subsequently processed by the 3C protein to yield $3D^{pol}$. The location of the conserved YGDD amino acid motif (nucleotide 6962 to 6973) is noted. For site-directed mutagenesis, the region from 5240 to the *Sal*I restriction site was subcloned into pUC119. The mutant genes were subcloned by using a *Bgl*II-*Sal*I restriction fragment into p*trp* or T7IC (poliovirus infectious cDNA). (B) Immunoblot analysis of wild-type and mutant $3D^{pol}$ expressed in *E. coli*. Extracts from induced *E. coli* transformed with plasmids containing the vector, wild-type, or mutant $3D^{pol}$ genes were partially purified by phosphocellulose chromatography. After concentration, the eluates from the phosphocellulose column 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 (39). Lanes: 1, *E. coli* transformed with vector plasmid; 2, p*trp*-3D-328 (wild-type); 3, p*trp*-3D-E-328; 4, p*trp*-3D-H-328; 5, p*trp*-3D-N-328; 6, p*trp*-3D-Q-328; 7, p*trp*-3D-EE-328-9; 8, p*trp*-3D-E-329; 9, p*trp*-3D-N-329. The positions of molecular mass markers electrophoresed in parallel are indicated.

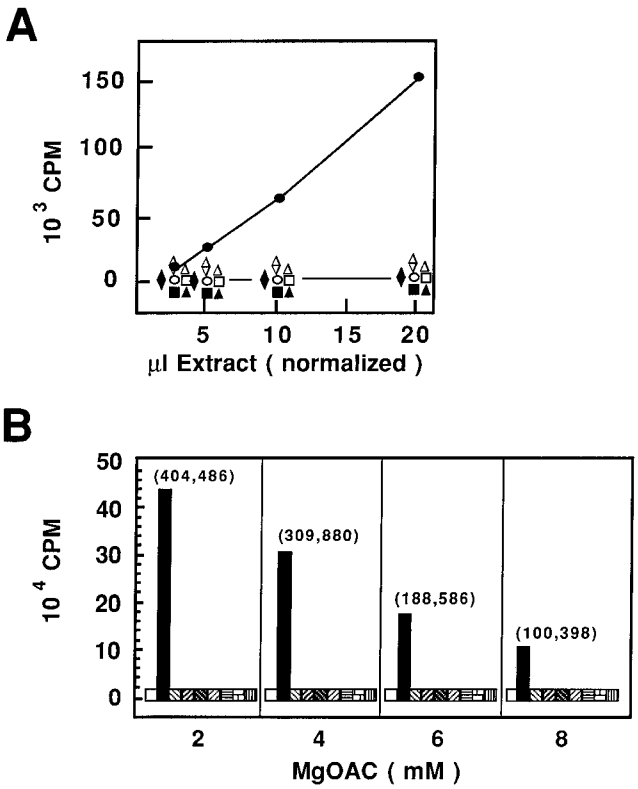

FIG. 2. Enzymatic activity of wild-type and mutant RNA polymerases. (A) Extracts from induced *E. coli* transformed with plasmids containing the various polymerase genes were purified by phosphocellulose chromatography. The amounts of $3D^{pol}$ in each preparation were estimated by immunoblot analysis, and equal amounts of immunoreactive $3D^{pol}$ (normalized extracts) were used in the poly(A) · oligo(U) polymerase assay under standard conditions. The total protein concentrations in each preparation were estimated to be within 50% of one another. The preparations tested are indicated as follows: ○, vector; ●, p*trp*-3D-D-328 (wild type); ▲, p*trp*-3D-E-328; □, p*trp*-3D-H-328; ◊, p*trp*-3D-N-328; ■, p*trp*-3D-Q-328; △, p*trp*-3D-EE-328-9; ◆, p*trp*-3D-N-329. The enzyme activity of the preparation containing the 3D-E-329 mutant was also not above that for extracts prepared from *E. coli* transformed with the vector alone. (B) Effect of variation of Mg^{2+} ion concentration on enzymatic activity of wild-type and mutant $3D^{pol}$ polymerases. Phosphocellulose-purified wild-type or mutant enzymes were tested by the poly(A) · oligo(U) assay, with different concentrations of Mg^{2+} (magnesium acetate [MgOAC]). Equivalent amounts of immunoreactive $3D^{pol}$ (by Western blot analysis) were tested for each enzyme. The samples are indicated (in order) as follows: □, extract from *E. coli* with vector alone; ■, 3D-D-328 (wild type); ▨, 3D-E-328; ▨, 3D-H-328; ▨, 3D-N-328; ▨, 3D-Q-328; ▤, 3D-EE-328-9; ▤, 3D-E-329; ▥, 3D-N-329. The results of a representative experiment are shown. This assay was repeated three times with similar results. The counts per minute incorporated in the poly(U) assay for the wild-type enzyme are noted. All other enzymes had values comparable to that obtained from extracts of *E. coli* with vector alone (less than 5,000 cpm incorporated).

pProt-Pol-TRP expression vector; this step was followed by induction of the *trp* operon (32). The RNA polymerase was partially purified by chromatography on phosphocellulose. The eluates from phosphocellulose were subjected to Western blot analysis with anti-$3D^{pol}$ antibody, as described in Materials and Methods. Immunoreactive material, corresponding to a protein with a molecular mass of 52 kDa, was detected in extracts of *E. coli* expressing wild-type or mutant poliovirus polymerase and used to normalize the amount of polymerase in each sample as previously described (17, 18) (Fig. 1B).

Enzymatic activity of mutant $3D^{pol}$ proteins. The enzymatic activity of the polymerase preparations was tested over a wide range of in vitro enzyme concentrations (Fig. 2A). The differences observed between the extracts of wild-type $3D^{pol}$ and of the vector alone were approximately 100-fold. None of the mutant polymerases had enzymatic activity above background levels under standard in vitro conditions. Since the GDD amino acid motif has been postulated to be involved in the interaction of metal with the enzyme, it was possible that mutations in the aspartic acids would affect the metal ion required for in vitro activity. To test this hypothesis, the in vitro assay was done with varying amounts of Mg^{2+} (Fig. 2B). Although the wild-type enzyme was active over a wide range of Mg^{2+} concentrations, no enzyme activity was detected for any of the mutant enzymes in the range tested. The wild-type poliovirus RNA polymerase will also use Mn^{2+} in the in vitro reaction, although the optimum concentration (0.6 mM) is considerably lower than that for Mg^{2+}. The in vitro enzyme activity of the wild-type enzyme at the optimum concentration of Mg^{2+} or Mn^{2+} was similar (18a). To determine enzymatic activity in the presence of Mn^{2+}, wild-type and mutant polymerases were tested over a range of $MnSO_4$ concentrations (Fig. 3A). Surprisingly, the 3D-N-329 polymerase demonstrated enzymatic activity in the presence of $MnSO_4$ at an optimal concentration of 0.06 to 0.08 mM. On the basis of these results, assays were performed with a similar range of concentrations in which

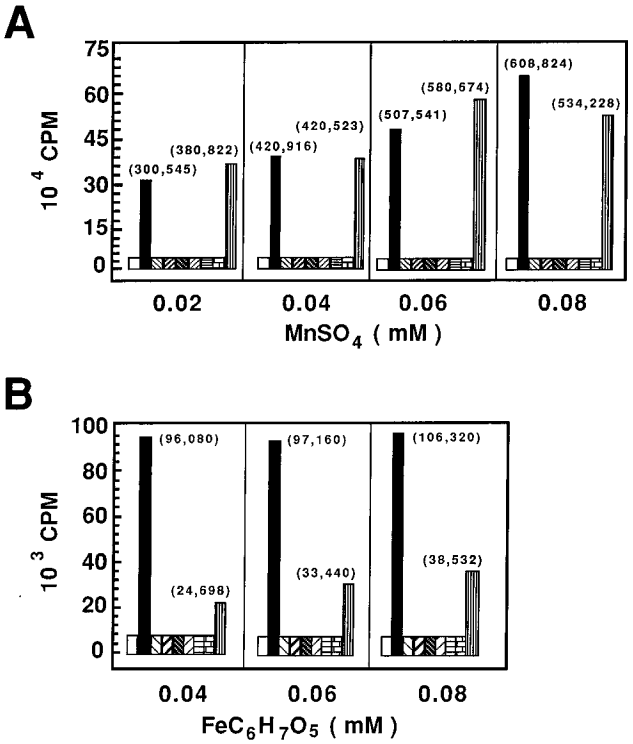

FIG. 3. Effects of Mn^{2+} and Fe^{2+} on enzymatic activity of wild-type and mutant enzymes. Phosphocellulose-purified extracts of *E. coli* containing the wild-type or mutant enzymes were tested by the poly(A) · oligo(U) assay, with different concentrations of Mn^{2+} ($MnSO_4$) (A) or Fe^{2+} ($FeC_6H_7O_5$) (B). Equivalent amounts of enzymes as determined by levels of immunoreactive $3D^{pol}$ (by immunoblot analysis) were tested. The samples are indicated (in order) as follows: □, extract from *E. coli* with vector alone; ■, 3D-D-328 (wild type); ▨, 3D-E-328; ▨, 3D-H-328; ▨, 3D-N-328; ▨, 3D-Q-328; ▤, 3D-EE-328-9; ▤, 3D-E-329; ▤, 3D-N-329. The results of a representative experiment are shown. The assay was repeated three times with similar results. The counts per minute incorporated for the wild-type enzyme and 3D-N-329 are noted. The counts per minute incorporated for all other enzymes as well as for the extract from *E. coli* with vector alone were below 5,000 (A) and 8,000 cpm (B).

$CaCl_2$, $CoCl_2$, $CuCl_2$, $FeC_6H_7O_5$, $NiCl_2$, and $ZnCl_2$ were substituted for $MnSO_4$. Although the wild type polymerase was active in the presence of all metals except $CaCl_2$, the 3D-N-329 mutant was active only in the presence of $FeC_6H_7O_5$ (Fig. 3B). The other mutant enzymes were inactive in the presence of the metal ions. The mutant enzymes were also inactive when the reaction pH (7 to 9) and temperature were varied (data not shown).

Infectious potential of poliovirus cDNAs containing polymerase mutations. To test the capacity of the mutant polymerases to support replication in vivo, the mutant $3D^{pol}$ genes were subcloned into an infectious clone of poliovirus and the DNA was transfected into tissue culture cells. For these studies, we routinely set up 20 independent transfection trials with the same preparation of plasmid DNA containing the poliovirus cDNA. Under these conditions, transfection of the cDNA containing the wild-type polymerase resulted in the appearance of viruses in 20 of 20 transfections. In contrast, no virus was recovered from the transfection of cDNAs containing the mutant enzymes, including the 3D-N-329 mutant (data not shown). Since the 3D-N-329 polymerase was active in vitro in the presence of Mn^{2+} and Fe^{2+}, a second set of transfections was done in the presence of several different metal ions (at a final concentration of 100 μM). Viruses were isolated from transfection of the cDNA containing the 3D-N-329 mutation

TABLE 2. Virus recovery after transfection in the presence of metal ions of poliovirus containing 3D-N-329 mutation

Metal ion		% Virus recovery[a]
Source	Concn (μM)	
$CaCl_2$	100	0
$CoCl_2$	100	5
$CuCl_2$	100	0
$CuCL_2$	50[b]	0
$FeSO_4$	100	15
$FeC_6H_7O_5$	100	0
$MnSO_4$	100	0
$NiCl_2$	100	0
$ZnCl_2$	100	0

[a] Percent virus recovery was calculated from 20 independent transfection trials with the following equation: 100 × (number of wells positive for viral cytopathic effect/20 wells).

[b] $CuCl_2$ (100 μM) was toxic for cells, so transfections were also performed with 50 μM $CuCl_2$.

only in the presence of $FeSO_4$ and $CoCl_2$ (Table 2). The virus recovered in the presence of $FeSO_4$ was quite different from the wild-type virus, in that it was a very slow-growing virus, with little cytopathic effect. The virus did not form plaques, and we were unable to derive high-titer stocks. To characterize the virus isolated from the transfection of the cDNA containing the 3D-N-329 mutation in the presence of $FeSO_4$, cells were infected with an extract from a third serial passage. At 3 days postinfection, the cells were metabolically labeled overnight, followed by immunoprecipitation with antipoliovirus antibody (Fig. 4A). Capsid proteins were detected, indicating the presence of poliovirus, although the levels were considerably lower than that for the wild-type virus. In one of the virus samples, only the capsid protein VP3 was detected at a very low level (Fig. 4A, lane 3). All the capsid proteins were immunoprecipitated from cells infected with a second isolate of the virus grown in the presence of $FeSO_4$ (Fig. 4A, lane 4). Repeated attempts to amplify by PCR and subclone the $3D^{pol}$ gene from the virus derived from transfection in the presence of $FeSO_4$ were unsuccessful.

Virus was also evident in 1 of the 20 transfections of the cDNA with the 3D-N-329 mutation in the presence of $CoCl_2$. The virus resembled the wild-type virus with respect to cytopathic effect in tissue culture cells. Using [^{3}H]uridine labeling of infected cells, we determined that the kinetics of replication were similar to those of the wild-type virus. Furthermore, there were no differences with regard to production of viral proteins between wild-type poliovirus grown in the presence of $CoCl_2$ and the 3D-N-329-derived virus (data not shown). Since in vitro growth characteristics of the virus derived from transfection of the cDNA containing the 3D-N-329 mutation in the presence of $CoCl_2$ were similar to those of the wild-type virus, we wanted to determine if a reversion in the $3D^{pol}$ gene had occurred. For these studies, we utilized RT-PCR to amplify the polymerase gene from viral RNA and subcloned the amplified DNA into the TA cloning vector. The $3D^{pol}$ genes from five independent clones of the 3D-N-329 mutant were sequenced. The virus derived from transfection of the 3D-N-329 mutant in the presence of $CoCl_2$ was found to have a reversion from 3D-N-329 to 3D-D-329 (wild-type) (Fig. 4B). In parallel, the $3D^{pol}$ gene for the wild-type virus grown in the presence of $CoCl_2$ was amplified and subcloned. The sequences of the 3D-D-329 region for twelve independent clones of the wild-

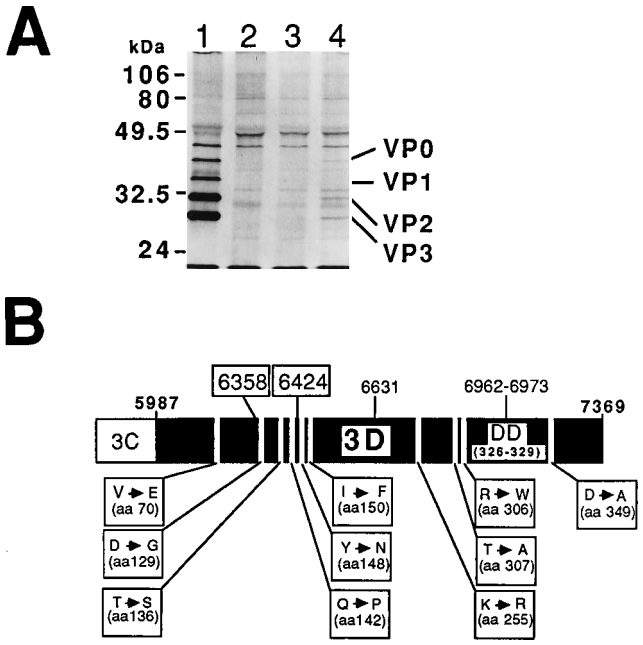

FIG. 4. Characterizations of virus recovered from transfection in the presence of Fe^{2+} or Co^{2+} of poliovirus cDNA containing the 3D-N-329 mutation in $3D^{pol}$. (A) Detection of poliovirus after transfection in the presence of $FeSO_4$. Virus was recovered after transfection of the poliovirus cDNA containing the 3D-N-329 mutation in the presence of $FeSO_4$. After three serial passages, the supernatant was used to infect BSC-1 cells. Since very little cytopathic effect was observed, infections were allowed to proceed for 3 days prior to metabolic labeling for 24 h with ^{35}S-Translabel. The cells infected with the wild-type virus were labeled after 6 h of infection. The viral proteins were immunoprecipitated with antipoliovirus antibodies, followed by SDS-PAGE and fluorography. Lanes: 1, wild-type poliovirus-infected cells; 2, mock-infected cells labeled for 24 h; 3, cells infected with a passage 3 stock of virus isolated in the presence of $FeSO_4$ and labeled for 24 h (note that only very low levels of the capsid protein VP3 were detected); 4, cells infected with a second independent passage 3 stock of virus isolated in the presence of $FeSO_4$. The positions of the molecular mass standards are noted. The positions of the poliovirus capsid proteins, VP0, VP1, VP2, and VP3 are also noted. (B) Summary of changes in the $3D^{pol}$ gene obtained from virus isolated after transfection of poliovirus cDNA containing the 3D-N-329 mutation in the presence of Co^{2+}. RT-PCR was used to amplify the $3D^{pol}$ gene of the virus isolated after transfection of the poliovirus cDNA containing the 3D-N-329 mutation in the presence of Co^{2+}. The PCR-amplified DNA was subcloned into the TA cloning vector. As a control, the $3D^{pol}$ gene from the wild-type virus, passaged in the presence of 100 μM $CoCl_2$, was also amplified and subcloned. The $3D^{pol}$ genes from five independent clones of each sample were sequenced by *Taq* DNA polymerase cell cycle sequencing in conjunction with an automated DNA sequencer. Thirteen changes were found in the $3D^{pol}$ gene from the virus isolated from transfection of the cDNA containing the 3D-N-329 mutation compared with the wild-type sequence. Two nucleotide changes that did not result in amino acid changes (6358 and 6426 [boxed]) were found in more than one clone. The additional nucleotide change (6631) and the amino acid changes noted were found in one of the five clones; all of the five clones contained at least two changes from the wild-type.

type polymerase gene which were amplified from infection in the presence of $CoCl_2$ were determined, and no sequence changes were found, suggesting that viral growth in the presence of $CoCl_2$ did not result in spontaneous sequence changes in this region. An additional 13 mutations in the $3D^{pol}$ gene were also found in the revertant virus. Three of these mutations were silent, in that no amino acid substitutions occurred as a result of the mutation. In contrast, 10 of the mutations resulted in amino acid changes. Of the 10 amino acid changes, 9 changes occurred 5′ of the codon for the 3D-D-329 mutation (nucleotides 6962 to 6973). The only change 3′ of the 3D-N-329 mutation was at nucleotide 7011. Of the 10 amino acid substitutions, 5 substitutions were located between amino acids 129 and 150 of $3D^{pol}$. These results suggest that the wild-type growth characteristics of the revertant virus were probably due to the 3D-N-329-to-3D-D-329 mutation and that the additional mutations probably arose during the process of reversion.

DISCUSSION

Seven enzymes with a mutation at the conserved GDD amino acid motif of the poliovirus RNA-dependent RNA polymerase were constructed to ascertain their role in the polymerase function. The first aspartic acid was replaced with a glutamic acid, a histidine, an asparagine, or a glutamine; the second aspartic acid was replaced with a glutamic acid or an asparagine; or both aspartic acids were replaced with glutamic acids. Polymerase genes containing the various mutations were expressed in *E. coli* and tested for in vitro enzymatic activity. In all cases, a mutation in one or both of the aspartic acids resulted in a complete loss of enzymatic activity under standard in vitro conditions. However, the polymerase containing the 3D-N-329 mutation was enzymatically active if Mg^{2+} was replaced with Mn^{2+} or Fe^{2+}. The mutant RNA polymerase genes, when subcloned into the infectious cDNA clone of poliovirus, did not produce virus when transfected into tissue culture cells under standard conditions. Transfection of cDNA containing the 3D-N-329 mutation in the presence of $CoCl_2$ or $FeSO_4$ resulted in the production of virus. The virus derived from transfection in the presence of $FeSO_4$ grew slowly. In contrast, the virus obtained from transfection in the presence of $CoCl_2$ was similar to the wild-type virus with respect to replication kinetics. Sequence analysis of the $3D^{pol}$ gene revealed that the original 3D-N-329 mutant had reverted back to aspartic acid. Sequence analysis of five independent clones revealed 13 additional nucleotide differences from the wild-type $3D^{pol}$.

The goal of this experimental approach was to evaluate the role of the conserved aspartic acids in poliovirus $3D^{pol}$ enzyme function. In order to do this, we targeted the experiments to a highly conserved pair of aspartic acids to determine whether substituting the aspartic acids with amino acids found in other RNA polymerases, such as GDN in negative-strand viruses, or to DE similar to that found for DNA-dependent DNA polymerase (i.e., Klenow), would result in functional enzymes. Previous studies of RNA-dependent RNA polymerases from other viruses, RTs, or DNA polymerases have addressed the role of aspartic acid by using a limited number of mutations. In a study of the RNA-dependent RNA polymerase of encephalomyocarditis virus, each of the aspartic acid residues in the YGDD sequence was replaced with a glutamic acid and the resultant enzymes had little or no activity in vitro (43). The GDD region of the L-A double-stranded RNA virus of *Saccharomyces cerevisiae* was replaced with AEE, and this enzyme was also rendered nonfunctional in vivo (38). T7 RNA polymerase, a DNA-directed RNA polymerase, was also found to have diminished activity in vitro when substitutions were made at the corresponding IHDS sequence (5). Larder et al. substituted the first aspartic acid at this region in the human immunodeficiency virus type 1 reverse transcriptase with histidine and the second aspartic acid with asparagine and found that neither of the mutant reverse transcriptases were functional in vivo or in vitro (26, 27). In a related study, each of the aspartic acid residues of the RT of human immunodeficiency virus type 1 was changed to asparagine, either singly or as a double mutant, and the mutant enzymes produced did not have appreciable reverse transcriptase activity in vitro (28). Changes at the aspartic acid residues in the YGDTDS sequence motif of

the adenovirus DNA polymerase resulted in enzymes with little or no activity in vitro (21). Substitutions of the first aspartic acid of herpesvirus 1 DNA polymerase with asparagine or of the second aspartic acid with alanine resulted in nonfunctional mutants in vitro (14, 16). Mutations made in the active site of the Klenow fragment of DNA polymerase I also support the idea that these residues are involved in catalytic activity. Mutations at the aspartic acid or glutamic acid residues of the VHDE sequence in the Klenow fragment disrupt the catalytic activity (36, 37). The results of this study have demonstrated that a modest change of the GDD amino acid motif to GDN resulted in an RNA polymerase with different in vitro activity. In an analogous study, Sleat and Banerjee substituted the GDN sequence of vesicular stomatis virus RNA polymerase with GDD and found that the mutant polymerase was active in transcription but at a reduced level (45). Taken together, the previous study and the present one clearly show the requirement for the first aspartic acid residue and a very limited flexibility in substitution of the second aspartic acid for enzyme function.

One of the surprising results of our study was the fact that the change of the second aspartic acid to asparagine resulted in a polymerase that utilizes a different metal ion for enzymatic activity. Metal ion coordination by $3D^{pol}$ is undoubtedly important in the polymerization reaction. The negatively charged phosphate backbone of the template RNA could be complexed with metal ions, and these could interact structurally with the polymerase in positioning the template. It is possible that the catalytic reaction itself is driven by the presence of metal ion, by stabilizing an intermediate in the enzyme reaction which makes the phosphodiester bond. Structural predictions suggest that the GDD region is in a β-turn-β structure, with the aspartic acids exposed on the loop portion (2). In a comparison of secondary structural predictions of polymerases with the known three-dimensional structure of the Klenow fragment of *E. coli* DNA polymerase I, the aspartic acid residues of the conserved GDD motif have been proposed to correspond to β-strands 12 and 13 of the Klenow structure, with the aspartic acid residues in the connecting loop of the β-hairpin (12). If this is true, it would allow the inclusion of at least one of the aspartates in the catalytic active site of the molecule. A recent determination of the crystal structure of the RNA-dependent DNA polymerase (RT) of human immunodeficiency virus type 1 shows the conserved YMDD region at the loop connecting β-strands 9 and 10, and this aligns with β-strands 12 and 13 of the Klenow structure (25). The sequence at this region of Klenow includes D882 and E883, which bind divalent metal ions with the additional interaction of D705. If the two catalytic domains are compared, D185 and D186 of human immunodeficiency virus type 1 reverse transcriptase appear superimposed upon Klenow D882 and E883. Further support for a common structure of the catalytic active site of polymerases is evidenced by recent reports of the crystal structures of *E. coli* RNA polymerase holoenzyme (9), bacteriophage T7 RNA polymerase (46), and DNA polymerase β (11, 33, 44). Mutations at critical acidic residues of enzyme active sites have resulted in the change of metal ion preference for enzymatic activity. For example, the enzyme xylose (glucose) isomerase from *Actinoplanes missouriensis* contains two metal binding sites and requires either Mg^{2+}, Co^{2+}, or Mn^{2+} for activity (19). Since the crystal structure of the enzyme has been solved, the amino acid residues involved in metal ion binding have been identified. Mutagenesis of the amino acids involved in metal ion binding resulted in isomerases that demonstrated a different requirement for Mg^{2+}, Co^{2+}, or Mn^{2+} for enzyme activity than did the wild type.

In order to corroborate the results of our in vitro enzyme studies, we tested the capacity of the poliovirus cDNAs containing the mutant polymerase gene to give rise to infectious virus following transfection. Overall, the results from our transfections were in agreement with the in vitro enzyme experiments. Under our standard transfection conditions, no viruses were recovered from transfection of the cDNAs containing the mutant polymerase genes. If the culture conditions were modified to include divalent metal ions, viruses were recovered in cultures transfected with cDNAs containing the polymerase gene with the 3D-N-329 mutation. Consistent with our in vitro experiments, we detected viruses derived from transfections of the cDNA containing the 3D-N-329 mutation in the presence of $FeSO_4$. Due to the low level of replication, though, we could not amplify the virus stock for a detailed characterization. We also recovered a virus from transfection of the cDNA containing the 3D-N-329 mutation in the presence of $CoCl_2$. This was surprising because the polymerase with the 3D-N-329 mutation was enzymatically inactive in the presence of Co^{2+}. A virus was not recovered if the transfections of the cDNA containing the 3D-N-329 mutation were done in the presence of Mn^{2+}, even though the 3D-N-329 mutant enzyme was active in vitro in the presence of $MnCl_2$. The reason for this discrepancy is not clear. It is important to note that all of the transfections were done in the presence of excess Mg^{2+} that is present in the tissue culture media. In preliminary experiments, we found that the presence of Mg^{2+} inhibits the Mn^{2+}-stimulated in vitro enzyme activity of the polymerase (data not shown). Thus, even in the presence of $MnCl_2$ in the transfections, the enzyme might be inhibited because of the intracellular levels of Mg^{2+}. The reason why transfection in the presence of $CoCl_2$ gave rise to virus, albeit infrequently, is unknown. It is possible that Co^{2+} and Mg^{2+} in the cell might synergize to allow low levels of replication that are enough to generate a reversion (3D-N-329 to 3D-D-329). Although the polymerase containing the 3D-N-329 mutation was inactive in the presence of Co^{2+} and Mg^{2+} in the in vitro assay, it is possible that a polymerase precursor (i.e., 3CD) alone or in combination with cellular factors might provide enough low-level replication to result in the reversion to 3D-D-329.

Numerous silent and single amino acid changes were found in addition to the 3D-D-329 mutation in the $3D^{pol}$ gene of the virus recovered from transfection in the presence of $CoCl_2$, suggesting that an initial mutation (one or several) might have occurred prior to the 3D-N-329 reversion to aspartic acid. In support of this idea is our finding in previous studies that a single amino acid change (3D-E-108 to 3D-D-108) was required for the replication of a virus containing a 3D-M-326 mutation in $3D^{pol}$ (18). The transfection of viral cDNA containing the 3D-M-326 mutation alone resulted in the infrequent appearance of virus. Although several mutations were found in the polymerase genes isolated from these viruses, only the substitution at amino acid 108 was necessary for the production of infectious virus; transfections of cDNA clones containing a double-mutant polymerase (3D-D-108 and 3D-M-326) were found to produce infectious virus in all transfection trials. Interestingly, 50% of the amino acid changes found in the virus obtained from transfection in the presence of $CoCl_2$ were between amino acids 129 and 150. The nucleotide changes that did not result in amino acid changes (nucleotides 6358 and 6474), which were found in multiple clones, were also located in this region. This finding, as well as the interaction found previously between amino acids 108 and 326 of the poliovirus polymerase (18), suggests that the region between amino acids 108 and 150 of $3D^{pol}$ might interact with the

catalytic active site of $3D^{pol}$ located at or near the conserved GDD motif (amino acids 328 and 329). To test this possibility, studies are ongoing to construct $3D^{pol}$ genes containing one or all of the additional changes with the 3D-N-329 mutant to ascertain whether this increases the frequency of virus recovery upon transfection in the presence of $CoCl_2$.

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