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Mutation Analysis of the GDD Sequence Motif of a Calicivirus RNA-Dependent RNA Polymerase

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The RNA-dependent RNA polymerase from *rabbit hemorrhagic disease virus*, a calicivirus, is known to have a conserved GDD amino acid motif and several additional regions of sequence homology with all types of polymerases. To test whether both aspartic acid residues are in fact involved in the catalytic activity and metal ion coordination of the enzyme, several defined mutations have been made in order to replace them by glutamate, asparagine, or glycine. All six mutant enzymes were produced in *Escherichia coli*, and their in vitro poly(U) polymerase activity was characterized. The results demonstrated that the first aspartate residue was absolutely required for enzyme function and that some flexibility existed with respect to the second, which could be replaced by glutamate.

Rabbit hemorrhagic disease virus (RHDV) has been characterized as a member of the *Caliciviridae* (19, 21) and recently designated as the type species of the new genus *Lagovirus* (24). The viral genome is a positive polarity, single-stranded, polyadenylated RNA with approximately 7.4 kb, which has a virus-encoded VPg protein covalently attached to its 5′ end (15, 16, 28). Viral particles also encapsidate an abundant VPg-linked polyadenylated subgenomic RNA of about 2.2 kb (16). The data obtained from the in vitro translation (28), *Escherichia coli* expression studies (14), and detection of viral proteins after infection of cultured hepatocytes with RHDV (8) revealed that the viral RNA is translated into a polyprotein that is subsequently cleaved to give rise to mature structural and nonstructural proteins.

The extensive sequence similarities between the RNA-dependent RNA polymerase (RdRp) 3D of picornavirus and the RHDV polyprotein cleavage product p58 (8, 15, 28) suggested that this polypeptide could have a similar role in RHDV genome replication. Studies from our laboratory have reported the successful expression of enzymatically active RHDV RNA polymerase ($3D^{pol}$) in *E. coli* (13). The recombinant polypeptide exhibited rifampin- and actinomycin D-resistant poly(A)-dependent poly(U) polymerase as well as RNA polymerase activity by using synthetic RHDV subgenomic RNA as a template in the presence or absence of an oligo(U) primer (13).

The availability of a growing number of complete sequences from the genomes of positive, negative, and double-stranded RNA viruses of plant and animal origin has enabled many groups to carry out sequence alignments of the RdRps in an attempt to identify regions essential for polymerase function, which should appear as most conserved (1, 7, 23). Currently, eight conserved motifs have been described (23), four of which (A, B, C, and D) are now known to be located in the catalytic portion of the "palm" domain (4) of all classes of polymerases. Motif C, which formed a "β-strand, turn, β-strand" structure, contained a highly conserved GDD sequence found in all RdRps (7). This structure is very similar in all classes of polymerases and positions the two aspartate residues close to the conserved aspartate of motif A (4). The location of these residues in tight turns may be required for its proper orientation in the catalytic process.

The aim of this work was to use an oligonucleotide site-directed mutagenesis approach to study the structure-function relationship of RHDV RNA polymerase, focusing on the conserved GDD amino acid core segment.

The region of the $3D^{pol}$ gene targeted for mutagenesis (Fig. 1A) was located between nucleotides 4816 and 4827 of the AST/89 isolate of RHDV genome (EMBL accession no. Z49271). This region coded for the highly conserved $3D^{pol}$ sequence motif (YGDD) located at the catalytic site of the enzyme which has been proposed to be involved in metal binding (20).

In this work, we have made single mutations at the aspartic acid residues found at positions 1605 and 1606 of the deduced polyprotein amino acid sequence from the AST/89 RHDV isolate. Oligonucleotide site-directed mutagenesis using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) and the primers shown in Table 1 allowed the production of specific mutations in the previously described pGEX-3D plasmid (13). The resulting constructs were used to produce in *E. coli* six recombinant $3D^{pol}$ point mutants in which each aspartic acid residue of the YGDD motif was specifically mutated to glutamic acid (E), asparagine (N), or glycine (G).

The wild-type and mutant glutathione *S*-transferase (GST)-$3D^{pol}$ fusion proteins were purified from bacterial lysates by affinity chromatography using the bulk GST purification module (Pharmacia) as previously described (13). Wild-type and mutant $3D^{pol}$ were released from GST by thrombin cleavage, and the purified recombinant proteins were stored at −20°C after addition of 5% glycerol.

The purified recombinant proteins (wild type and mutants) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (9), showing in all cases the expected molecular mass. Since some differences in protein yield were observed for the mutant proteins, the protein concentration of the purified preparations was adjusted in order to use equivalent amounts of protein for testing enzyme activity (Fig. 1B).

To measure wild-type and mutant $3D^{pol}$ enzymatic activity, the preparations were tested by using the poly(A)-dependent

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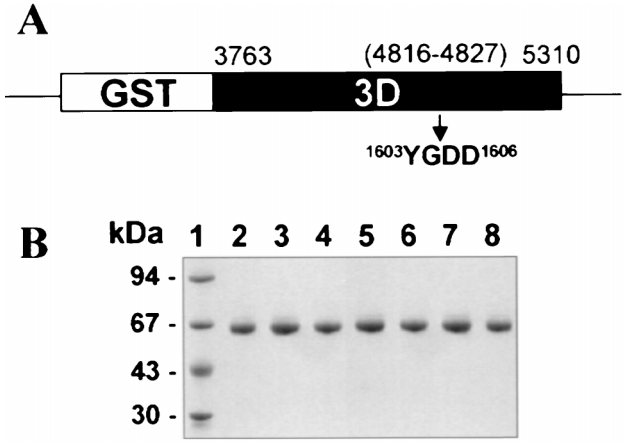

FIG. 1. Diagram of the expression system used for the production of $3D^{pol}$ in *E. coli*. (A) The region of the RHDV genome from nucleotides 3763 to 5310 ($3D^{pol}$ coding region) was cloned into expression plasmid pGEX-2T fused to the GST gene (13). The location of the sequence coding for the conserved YGDD motif is indicated in parentheses. (B) SDS-PAGE analysis of wild-type and mutant recombinant $3D^{pol}$ purified preparations, after correction for protein concentration. Lane 1, molecular mass markers; lane 2, wild-type $3D^{pol}$; lanes 3 to 8, $3D^{pol}$ mutants $D^{1605}E$, $D^{1605}G$, $D^{1605}N$, $D^{1606}E$, $D^{1606}G$, and $D^{1606}N$, respectively.

oligo(U)-primed poly(U) polymerase assay. Oligo(U) primer was prepared by alkali hydrolysis of poly(U) as described elsewhere (22). The size of the resulting oligo(U) was determined by end labeling with [γ-^{32}P]ATP (ICN) and electrophoresis on a 6% polyacrylamide gel.

The poly(A)-dependent oligo(U)-primed poly(U) polymerase assay was performed as previously described (13, 26) except for some assays in which magnesium acetate (MgAcO), which was used at a concentration of 3 mM in the standard assays, was replaced by $MnCl_2$ or $FeSO_4$. [α-^{32}P]UTP (400 Ci/mmol) (Amersham) was used as a tracer to measure the amount of UMP incorporated to the oligo(U) primer to form the reaction product. The reaction was carried out at 30°C for approximately 120 min in 50-μl samples. The in vitro-synthesized product was precipitated with 10% trichloroacetic acid, collected onto 0.45-μm-pore-size Whatman GF/C filters, and vacuum dried. The radioactivity on the filters was measured by scintillation counting.

Considering that previous studies from our laboratory had demonstrated that the amount of product synthesized by using this type of assay was a linear function of enzyme concentration from 0.7 to 3.5 μM (13), the reactions described in this work were performed with 1.4 μM concentrations of the wild-type or mutant $3D^{pol}$ enzymes.

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

Point mutation	Amino acid sequence	Mutagenic oligonucleotide sequence[a]
Wild type	YGDD	5′ CTACACGTATGGTGATGACGGCGTGTATGCC 3′
$D^{1605}E$	YGED	5′ CACGTATGGTGAAGACGGCGTG 3′
$D^{1605}G$	YGGD	5′ CTACACGTATGGTGGTGACGGCGTGTATGCC 3′
$D^{1605}N$	YGND	5′ CACGTATGGTAATGACGGCGTG 3′
$D^{1606}E$	YGDE	5′ CGTATGGTGATGAAGGCGTGTATG 3′
$D^{1606}G$	YGDG	5′ CTACACGTATGGTGATGGCGGCGTGTATGCC 3′
$D^{1606}N$	YGDN	5′ CGTATGGTGATAACGGCGTGTATG 3′

[a] Mutated nucleotide residues are underlined.

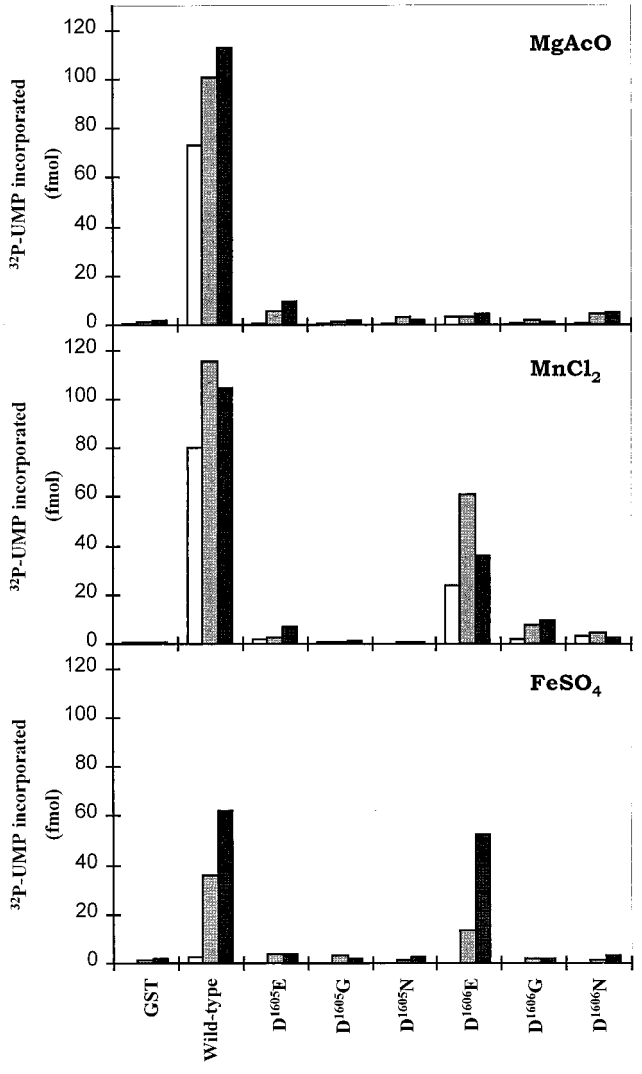

FIG. 2. Poly(U) polymerase activity of GST (control) and wild-type and mutant RHDV $3D^{pol}$ in the presence of increasing concentrations (white, gray, and black bars for lower to higher ion concentrations, respectively) of MgAcO (0.5, 1, and 2 mM), $MnCl_2$ (0.25, 0.5, and 1 mM), or $FeSO_4$ (0.08, 0.2, and 0.5 mM).

The results indicated that none of the mutant polymerases had enzymatic activity above background levels in the presence of MgAcO under standard assay conditions (Fig. 2). Since the GDD amino acid sequence has been postulated to be involved in enzyme-metal interaction, it could be predicted that mutations at the aspartic acid residues would produce changes in the levels of observed enzyme activity or modify the enzyme cation preferences. To test these hypotheses, the enzyme assay was performed with various amounts of Mg^{2+} (0.5 to 2 mM). 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 $3D^{pol}$ in the range tested (Fig. 2).

The wild-type RHDV RNA polymerase can also use Mn^{2+} in the in vitro reaction, although the optimum concentration (0.5 mM) was found to be lower than that measured for Mg^{2+} (2 to 3 mM). The in vitro enzyme activity of the wild-type enzyme was similar with optimum concentrations of Mg^{2+} or Mn^{2+} (Fig. 2). Wild-type and the six mutant $3D^{pol}$ proteins

were tested for enzyme activity over a range of $MnCl_2$ concentrations (Fig. 2). Surprisingly, the $D^{1606}E$ mutant showed significant enzymatic activity in the presence of $MnCl_2$ at an optimal concentration of 0.5 mM. Under these assay conditions, the $3D^{pol}$ mutant was 53% as active as the wild-type polymerase.

On the basis of these results, assays were performed with other divalent metals (Ca, Co, and Zn; data not shown). The wild-type and mutant enzymes were found to be inactive in the presence of all assayed metals except for Fe^{2+} (Fig. 2). At the optimal Fe^{2+} concentration (0.5 mM), the $D^{1606}E$ mutant showed 84% of the wild-type enzyme activity in contrast to the remarkable differences found between these two enzymes assayed with Mg^{2+} and Mn^{2+}. It should be mentioned that in the presence of Fe^{2+}, the wild-type $3D^{pol}$ exhibited only 55% of the activity found with Mg^{2+} or Mn^{2+}.

All positive-strand RNA viruses encode an RdRp which acts as the catalytic subunit, in concert with host and sometimes viral proteins, in the replication of the viral genome (10). Most of these polymerase proteins have been identified solely on the basis of sequence conservation. Catalytic activity has been experimentally demonstrated in only a handful of these viral proteins, including poliovirus $3D^{pol}$ (17, 18, 22, 25), encephalomyocarditis virus $3D^{pol}$ (26), hepatitis C virus NS5B (2, 3, 11), tobacco vein mottling virus NIb protein (5), and RHDV $3D^{pol}$ (13).

Structural studies of RdRps have lagged behind those of DNA-dependent RNA polymerases (DdRps), DNA-dependent DNA polymerases (DdDps), and reverse transcriptases. More recently, the crystal structure of the poliovirus $3D^{pol}$ was reported (4), allowing comparisons with other polymerases. The overall structure of the poliovirus polymerase appeared to resemble a right hand in which the palm subdomain, which contained the amino acid sequence motifs A, B, C, and D (23), was remarkably similar to those observed in the other three classes of polymerases. It is likely that the high degree of conservation of the aforesaid regions reflects their crucial importance for polymerase activity.

Motif C contained a β-strand, turn, β-strand structure, in which the two aspartic acid residues of the highly conserved GDD motif are located in the turn region. The first aspartate of the GDD motif is thought to be involved in coordination of a second divalent cation (20) and appeared to be strictly required, as any changes made at this position, including a replacement for a negatively charged glutamate, were almost never tolerated for in vivo viral replication and/or in vitro RNA synthesis (6, 11, 12).

In agreement to this, we have found that replacement of the RHDV $3D^{pol}$ 1605 aspartate residue by glutamate, asparagine, or glycine residues resulted in a complete loss of enzymatic activity by using a poly(A)-dependent oligo(U)-primed poly(U) polymerase assay.

The second aspartate of the GDD motif was not absolutely conserved in all classes of polymerases, suggesting some flexibility at this position. The Klenow fragment of *E. coli* DNA polymerase I (a DdDp) has a GDE motif (20), whereas a GDN sequence, which appeared as a classical feature in all polymerase sequences from unsegmented minus-strand RNA viruses (23), was observed in the putative reverse transcriptase from *Chlamydomonas reinhardtii*. However, mutation analysis indicated a fairly strict requirement for an aspartate at this position in the RdRps. Subtle changes, such as $D^{319}N$ or $D^{319}E$, in hepatitis C virus NS5B RdRp were tolerated only at low levels in vitro (11). Similar changes made in poliovirus $3D^{pol}$, $D^{329}N$ and $D^{329}E$ (6), or $D^{334}E$ in encephalomyocarditis virus $3D^{pol}$ (27) were also not tolerated. The requirement for the second aspartate was relaxed only in the presence of alternate cations (manganese and iron), as has been found in the poliovirus mutant $D^{329}N$ (6). In contrast to these data, our results indicate a complete loss of activity in the mutant $D^{1606}N$, whereas mutant $D^{1606}E$ was active when Mg^{2+} was replaced by Mn^{2+} or Fe^{2+}. This is a significant functional difference between the picornavirus and a calicivirus $3D^{pol}$ enzyme. However, it should be stressed that we have measured RdRp activity by using a purified recombinant $3D^{pol}$ in the absence of other viral or host proteins, as a consequence of the lack of a permissive cell culture system for RHDV. It should be then taken into account that enzymatic activity could be significantly modified in vivo by cellular or viral cofactors which could compensate for the lower activity exerted by a less favorable residue.

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REFERENCES

1. **Argos, P.** 1988. A sequence motif in many polymerases. Nucleic Acids Res. **16:**9909–9916.
2. **Behrens, S.-E., L. Tomei, and R. De Francesco.** 1996. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J. **15:**12–22.
3. **De Francesco, R., S.-E. Behrens, L. Tomei, S. Altamura, and J. Jiricny.** 1996. RNA-dependent RNA polymerase of hepatitis C virus. Methods Enzymol. **275:**58–67.
4. **Hansen, J. L., A. M. Long, and S. C. Schultz.** 1997. Structure of the RNA-dependent RNA polymerase of poliovirus. Structure **5:**1109–1122.
5. **Hong, Y., and A. G. Hunt.** 1996. RNA polymerase activity catalyzed by a potyvirus-encoded RNA-dependent RNA polymerase. Virology **226:**146–151.
6. **Jablonski, S. A., and C. D. Morrow.** 1995. 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. J. Virol. **69:**1532–1539.
7. **Kamer, G., and P. Argos.** 1984. Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. Nucleic Acids Res. **12:**7269–7282.
8. **König, M., H.-J. Thiel, and G. Meyers.** 1998. Detection of viral proteins after infection of cultured hepatocytes with rabbit hemorrhagic disease virus. J. Virol. **72:**4492–4497.
9. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
10. **Lai, M. M. C.** 1998. Cellular factors in the transcription and replication of viral RNA genomes: a parallel to DNA-dependent RNA transcription. Virology **244:**1–12.
11. **Lohmann, V., F. K. Körner, U. Herian, and R. Bartenschlager.** 1997. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J. Virol. **71:**8416–8428.
12. **Longstaff, M., G. Brigneti, F. Boccard, S. Chapman, and D. Baulcombe.** 1993. Extreme resistance to potato virus X infection in plants expressing a modified component of the putative viral replicase. EMBO J. **12:**379–386.
13. **López Vázquez, A., J. M. Martín Alonso, R. Casais, J. A. Boga, and F. Parra.** 1998. Expression of enzymatically active rabbit hemorrhagic disease virus RNA-dependent RNA polymerase in *Escherichia coli*. J. Virol. **72:**2999–3004.
14. **Martín Alonso, J. M., R. Casais, J. A. Boga, and F. Parra.** 1996. Processing of rabbit hemorrhagic disease virus polyprotein. J. Virol. **70:**1261–1265.
15. **Meyers, G., C. Wirblich, and H.-J. Thiel.** 1991. Rabbit hemorrhagic disease virus. Molecular cloning and nucleotide sequencing of a calicivirus genome. Virology **184:**664–676.
16. **Meyers, G., C. Wirblich, and H.-J. Thiel.** 1991. Genomic and subgenomic RNAs of rabbit hemorrhagic disease virus are both protein linked and packaged into particles. Virology **184:**677–686.
17. **Morrow, C. D., B. Warren, and M. R. Lentz.** 1987. Expression of enzymatically active poliovirus RNA-dependent RNA polymerase in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84:**6050–6054.
18. **Neufeld, K. L., O. C. Richards, and E. Ehrenfeld.** 1991. Purification, characterization, and comparison of poliovirus RNA polymerase from native and recombinant sources. J. Biol. Chem. **266:**24212–24219.
19. **Ohlinger, V. F., B. Haas, G. Meyers, F. Weiland, and H.-J. Thiel.** 1990. Identification and characterization of the virus causing rabbit hemorrhagic disease. J. Virol. **64:**3331–3336.
20. **O'Reilly, E. K., and C. C. Kao.** 1998. Analysis of RNA-dependent RNA polymerase structure and function as guided by known polymerase structures and computer predictions of secondary structure. Virology **252:**287–303.

21. **Parra, F., and M. Prieto.** 1990. Purification and characterization of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. J. Virol. **64:**4013–4015.
22. **Plotch, S. J., O. Palant, and Y. Gluzman.** 1989. Purification and properties of poliovirus RNA polymerase expressed in *Escherichia coli*. J. Virol. **63:** 216–225.
23. **Poch, O., I. Sauvaget, M. Delarue, and N. Tordo.** 1989. Identification of four conserved motifs among the RNA-dependent RNA polymerase encoding elements. EMBO J. **8:**3867–3874.
24. **Pringle, C. R.** 1998. Virus taxonomy—San Diego 1998. Arch. Virol. **143:** 1449–1459.
25. **Rothstein, M. A., O. C. Richards, C. Amin, and E. Ehrenfeld.** 1988. Enzymatic activity of poliovirus RNA polymerase synthesized in *Escherichia coli* from viral cDNA. Virology **164:**301–308.
26. **Sankar, S., and A. G. Porter.** 1991. Expression, purification, and properties of recombinant encephalomyocarditis virus RNA-dependent RNA polymerase. J. Virol. **65:**2993–3000.
27. **Sankar, S., and A. G. Porter.** 1992. Point mutations which drastically affect the polymerization activity of encephalomyocarditis virus RNA-dependent RNA polymerase correspond to the active site of *Escherichia coli* DNA polymerase I. J. Biol. Chem. **267:**10168–10176.
28. **Wirblich, C., H.-J. Thiel, and G. Meyers.** 1996. Genetic map of the calicivirus rabbit hemorrhagic disease virus as deduced from in vitro translation studies. J. Virol. **70:**7974–7983.