

# Tyrosine 66 of *Pepper vein banding virus* genome-linked protein is uridylylated by RNA-dependent RNA polymerase

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## Abstract

*Pepper vein banding virus* (PVBV), a member of the genus potyvirus, is a single-stranded positive-sense RNA virus and it primarily infects plants of the family *Solanaceae*. Genome organization and gene expression strategy of the potyviruses are similar to the picornaviruses, although they infect widely different hosts and have distinctly different morphologies. The genomic RNA of PVBV has a viral genome-linked protein (VPg) at the 5'-terminus and a poly(A) tail at the 3'-terminus. In order to establish the role of VPg in the initiation of replication of the virus, recombinant PVBV NIb and VPg were over-expressed in *Escherichia coli* and purified under non-denaturing conditions. PVBV NIb was found to be active as polymerase and it could uridylylate the VPg in a template independent manner. N- and C-terminal deletion analysis of VPg revealed that N-terminal 21 and C-terminal 92 residues of PVBV VPg are dispensable for in vitro uridylylation. The amino acid residue uridylylated by PVBV NIb was identified to be Tyr 66 by site-directed mutagenesis. It is possible that in potyviruses, replication begins with uridylylation of VPg which acts as primer for progeny RNA synthesis.

**Keywords:** Potyvirus; VPg; NIb; Uridylylation; Replication

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## Introduction

The genus *Potyvirus* is the largest plant virus group and is economically very important (Hull, 2002). *Pepper vein banding virus* (PVBV) belongs to the genus *Potyvirus* and is one of the most prevalent viruses infecting chilies in south India (Anindya et al., 2004; Joseph and Savithri, 1999; Ravi et al., 1997). Recently the complete genomic sequence of the virus was determined and a phylogenetic analysis clearly established that PVBV is a distinct member of the *Potyvirus* genus. The PVBV genome is 9771 nucleotides in length with VPg linked to the 5'-end of the genome and a poly-A tail at the 3'-end (Anindya et al., 2004). As in other potyviruses, PVBV genome contains a single large ORF, which can be translated to a polyprotein of size 340 kDa that undergoes proteolytic processing by three viral proteinases

viz. N-terminal proteinase (P1), helper component proteinase (HC-Pro) and NIa proteinase (Carrington et al., 1989; Riechmann et al., 1992). Although potyviruses are flexuous rods infecting plants and picornaviruses are icosahedral infecting animals, they are quite similar with respect to their genome organization and gene expression strategy (Domier et al., 1987). Many picornavirus proteins are structurally and functionally similar to potyvirus proteins despite little sequence similarities. For example, 2A protease (2A<sup>pro</sup>), 2C protein (2C<sup>ATPase</sup>), 3C proteinase (3C<sup>pro</sup>), and RNA-dependent RNA polymerase (3D<sup>pol</sup>) of poliovirus are functionally similar to potyviral P1 protein, cylindrical inclusion (CI) protein, nuclear inclusion protein-a (NIa), and nuclear inclusion protein-b (NIb), respectively (Domier et al., 1987; Koonin and Dolja, 1993). Due to this relatedness, they are often classified as 'picorna-like superfamily' of viruses and it has been postulated that picornaviruses and potyviruses might employ similar mechanism of replication (Hull, 2002; Strauss and Strauss, 1988; Urcuqui-Inchima et al., 2001). Both the virus groups have covalently

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linked VPg at the 5'-end of their genomes. However, the size of VPg is only ~2 kDa in poliovirus, whereas it is a 22-kDa protein in PVBV. It is therefore of interest to examine whether the role of VPg in the initiation of viral replication in PVBV would be similar to that observed in poliovirus.

Despite similarity in the genome organization and gene expression, not much is known about the mechanism of replication in potyviruses. This is largely due to difficulties in reconstituting the complete replication complex of potyviruses *in vitro* and lack of experimental evidence regarding the function of candidate viral proteins during replication. Potyvirus Nib protein was presumed to be an RNA-dependent RNA polymerase, based on sequence similarities with other polymerases (Domier et al., 1987). A direct evidence for the polymerase activity was obtained when Hong and Hunt (1996) showed that the recombinant Tobacco vein mottle virus (TVMV) Nib protein was able to utilize full-length TVMV RNA as a template for RNA synthesis.

The role of potyvirus VPg in the replication was shown indirectly. Tyr 60 of TVMV VPg (Tyr 1860 of the polyprotein) was shown to be covalently linked to the 5'-end of the RNA genome (Murphy et al., 1991) and a mutation of this residue to Ala completely abolished viral replication (Murphy et al., 1996). Similarly, substitution of the corresponding Tyr residue (Y62) with Ala in Tobacco etch virus also abolished genome amplification (Schaad et al., 1996). In a recent study it was shown that Potato virus A (PVA) Nib could uridylylate VPg. However, the amino acid residue involved in the uridylylation reaction was not identified (Puustinen and Makinen, 2004). In the present investigation we have addressed the role of VPg and Nib in the initiation of PVBV replication using *Escherichia coli* expressed proteins purified from the soluble fraction. The purified recombinant Nib was functionally active as polymerase and could uridylylate VPg *in vitro* in a template independent manner. The minimal region of PVBV VPg essential for uridylylation was mapped to be residues 22-100 of VPg. Tyr 66 of PVBV VPg present within this region was shown to be the amino acid residue that was uridylylated by Nib by site-directed mutagenesis. The uridylylated VPg might function as primer for the viral RNA synthesis.

## Results

### *Expression and purification of PVBV VPg, Nib, and their mutants*

PVBV VPg was cloned as a fusion protein with glutathione-S-transferase (GST) at the N-terminus and his-tag at the C-terminus using an anti-sense primer that coded for six histidines along with gene-specific sequence (Table 1). Such a GST-fused VPg was more stable compared to his-tag VPg. SDS-PAGE analysis of the

protein purified from the soluble fraction by Ni-NTA affinity chromatography showed a prominent 48 kDa band (Fig. 1A, lane 1) corresponding to the expected size of GST-VPg (26 kDa GST + 22 kDa VPg). Similarly the Y66T mutant of VPg was expressed and purified (Fig. 1A, lane 2). The identity of the proteins was further confirmed by the Western blot analysis using anti-GST polyclonal antibody (Fig. 1B). Majority of the lower molecular weight bands were due to degradation of fused GST protein. The yield of the recombinant protein was 1.0–1.5 mg/l of *E. coli* culture.

PVBV Nib was expressed in *E. coli* BL21(DE3) cells as N-terminal his-tag protein. SDS-PAGE analysis showed the induction of 62 kDa PVBV Nib protein and 50% of the protein was present in the soluble fraction (data not shown). The recombinant protein was purified by a two-step process. After extraction, the soluble protein fraction was loaded onto phenyl-sepharose column and washed successively with 50 mM Tris-HCl, pH 8.2 buffer containing 500 mM, 400 mM, 250 mM, and 100 mM NaCl. The bound proteins were eluted in 50 mM Tris-HCl, pH 8.2 buffer, and the purity was checked by SDS-PAGE analysis. As there were a few contaminant proteins in the extract, it was further purified by Ni-NTA affinity chromatography. The recombinant protein was eluted with 50 mM Tris-HCl, pH 8.2, containing 200 mM imidazole, dialyzed against final buffer (10 mM Tris-HCl, pH 8.2, 100 mM NaCl, containing 10% glycerol), and analyzed by SDS-PAGE (Fig. 1C, lane 1). In all the viral RNA-dependent RNA polymerases (RdRp) characterized till date, an active site motif Gly-Asp-Asp or (GDD) is conserved and in PVBV it corresponds to residues 351–353. By site-directed mutagenesis, GDD motif of PVBV Nib was mutated to GVD, i.e., Asp 352 was changed to Val. This D352V Nib was also expressed and purified by using a similar procedure (Fig. 1B, lane 2). The yield of the recombinant Nib and its mutant protein was in the range of 2.0–2.5 mg/l of *E. coli* culture.

### *Polymerase activity of PVBV Nib*

The purified recombinant PVBV Nib was initially checked for its polymerase activity using the assay procedure described for poliovirus 3D<sup>pol</sup> (Neufeld et al., 1991). Incorporation of label from [ $\alpha$ -<sup>32</sup>P]UTP into high molecular weight poly-nucleotides was analyzed either by agarose gel electrophoresis or by DE81 filter binding assay. As shown in Fig. 2A (lane 3), in the presence of poly(A) template and oligo(dT) primer, PVBV Nib catalyzed the incorporation of radio-labeled UMP into poly(U). The efficiency of UMP incorporation was rather less when MgCl<sub>2</sub> was used as cofactor as compared to MnCl<sub>2</sub> (Fig. 2A, lane 2) and no incorporation was observed when either of poly(A), oligo(dT), or Nib was omitted from the reaction mixture (Fig. 2A, lanes 4, 1, and 5, respectively). Further when mutant D352V Nib was used instead of wild-type Nib, no poly(U) formation was observed (Fig. 2A, lane 6).

Table 1  
Description of the oligonucleotides used in this study

Primer	Sequence	Remarks
VPg-sen	5'GCACAAAAGAAGAACAAGCGACAG 3'	Sense primer for wtVPg
VPg-anti	5'ATGATGATGATGATGATGGGATCGAAACCGGTCATG 3'	Anti-sense primer with codons for hexa-histidine tag followed by VPg-specific sequence
Y66T VPg-sen	5'GGATCGAAACCGGTCATGTTTCACG 3'	Sense primer for VPg Y66T mutation
Y66T VPg-anti	5'CGTGAACATGACCGGTTTCGATCC 3'	Anti-sense primer for VPg Y66T mutation
ΔN21VPg-sen	5'GTTGGAAGAATCATTGTTTCATGACG 3'	Sense primer for N-terminal 21 residue deletion of VPg
ΔN38VPg-sen	5'GGTAGTGCTTACACGAAGAAAGGC 3'	Sense primer for N-terminal 38 residue deletion of VPg
ΔC64VPg-anti	5'ATGATGATGATGATGATGTGTACTAGCATTACATCTGC 3'	Antisense primer for C-terminal 64 residue deletion of VPg
ΔC92VPg-anti	5'ATGATGATGATGATGATGTTGAATTCCAGGATTGCTG 3'	Antisense primer for C-terminal 92 residue deletion of VPg
Nlb-sen	5'GCAAGTGATAAATGGCGTTATG 3'	Sense primer for amplification of Nlb
Nlb-anti	5'CATACAGCCATTTATCACTTGCCAC 3'	Antisense primer for amplification of Nlb
D352V Nlb-sen	5'GACAATTAAGCTGACACCATTGGC 3'	Sense primer for Nlb D352V mutation
D352V Nlb-anti	5'GCCAATGGTGTGCGACTTAATTGTC 3'	Antisense primer for Nlb D352V mutation

These results clearly establish that purified PVBV Nlb can act as a polymerase in vitro. The activity was quantitated by DE81 filter-binding assay and the amount of UMP incorporated was measured as a function of time. The reaction was found to be linear up to 45 min (Fig. 2B, open circles). Only marginal incorporation of labeled UTP was observed when MgCl<sub>2</sub> was used instead of MnCl<sub>2</sub> (Fig. 2B, closed squares). Almost no incorporation was observed when mutant D352V Nlb was used instead of wild-type recombinant Nlb (Fig. 2B, open triangles), suggesting that purified recombinant PVBV Nlb has an intrinsic polymerase activity. Similarly, no activity was observed in the absence of oligo-dT primer (Fig. 2B, closed triangles, merged with the former profile, Fig. 2B). This functionally active Nlb was used to characterize the uridylylation reaction.

### Uridylylation of VPg by PVBV Nlb

Initiation of RNA synthesis by RdRp during replication of picornaviruses requires priming by the viral protein VPg (Paul et al., 1998; Rothberg et al., 1978). This reaction involves formation of phosphodiester bond between UMP and hydroxyl group of a Tyr residue of the VPg and this results in uridylylated VPg (Paul et al., 1998, 2000). Potyviruses have a genome organization similar to that of picornaviruses. It was therefore of interest to examine whether PVBV VPg can be uridylylated by PVBV Nlb in vitro and analyze the product of such a uridylylation reaction. PVBV VPg (6 μg) was incubated with Nlb (1 μg) in the presence of 0.5 μg of poly(A), 5 μCi (3000 Ci/mmol) of [<sup>32</sup>P]UTP, and 15 μM UTP in reaction buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM MnCl<sub>2</sub>) at 30 °C for 45 min. The reaction was terminated by addition of SDS-PAGE sample loading buffer and the reaction products were analyzed by 12% SDS-PAGE and subjected to autoradiography. As evident from Fig. 3A, lane 1, the VPg was radioactively labeled when all the components of the reaction mixture were present. It was also observed that no radioactive products were detected in the absence of PVBV Nlb (Fig. 3A, lane 4) or in the presence of inactive D352V Nlb mutant (Fig. 3A, lane 5), establishing that VPg uridylylation was catalyzed by viral Nlb and not by any contaminant *E. coli* proteins present in the PVBV VPg or Nlb preparations. The uridylylation was also not observed when recombinant GST with C-terminal his-tag was used as substrate instead of GST-VPg (Fig. 3A, lane 7) further supporting that the reaction is specific for the PVBV VPg.

Taken together, these results suggest that the purified recombinant PVBV Nlb can specifically uridylylate PVBV VPg in vitro. Interestingly, the incorporation of labeled uridylyl residues into recombinant PVBV VPg was also observed in the absence of template poly(A) RNA (Fig. 3A, lane 3). This result is in contrast to the observations made with polio and rhinovirus (Gerber et al., 2001; Paul et al., 1998), where uridylylation of VPg occurred only in the presence of poly(A) RNA template. Similar observation

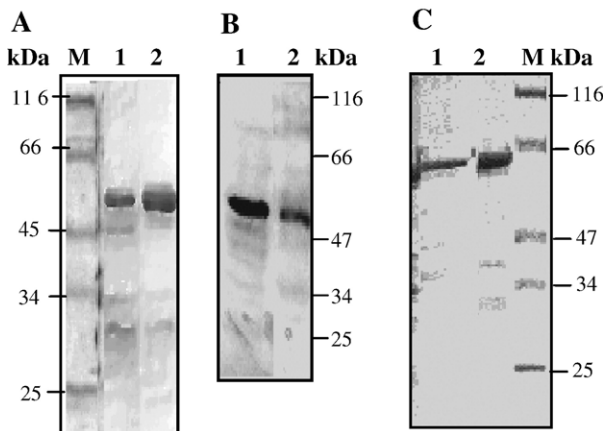


Fig. 1. SDS-PAGE analysis of recombinant proteins. (A) Purification of PVBV VPg and Y66T VPg. Lanes 1 and 2 represent Ni-NTA agarose affinity purified wild-type PVBV VPg and Y66T VPg, respectively. M denotes protein molecular wt markers. (B) Western blot analysis of Ni-NTA agarose affinity purified PVBV VPg (lane 2) and Y66T VPg (lane 1) using anti-GST polyclonal antibody. The position of the molecular wt. markers are as shown. (C) Expression and purification of PVBV Nlb and D352V Nlb mutant. Lanes 1 and 2 represent Ni-NTA agarose affinity purified wild-type PVBV Nlb and D352V Nlb, respectively. M denotes protein molecular wt markers.

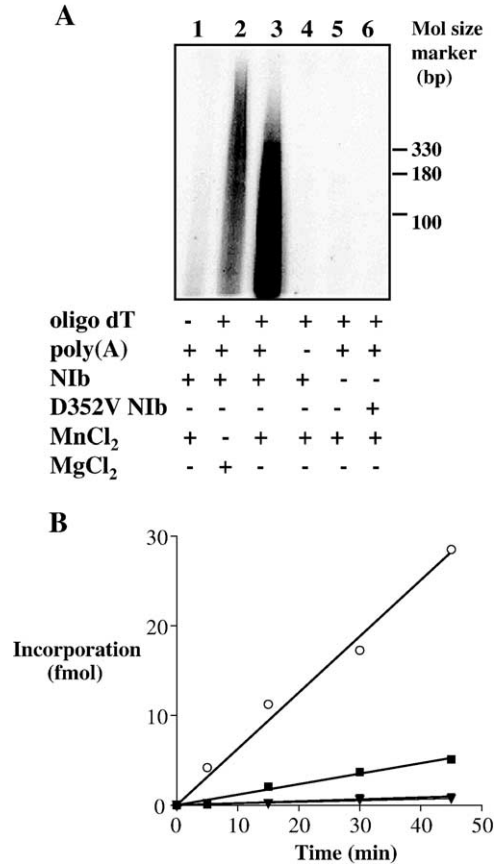


Fig. 2. Polymerase activity of PVBV Nib. (A) 2  $\mu$ g Nib (lanes 1–4) or mutant D352V Nib (lane 6), 2.5  $\mu$ g of poly(A), and 1  $\mu$ g of oligo(dT)<sub>20</sub> were incubated in reaction buffer (50 mM Tris–HCl, pH 8.0, 2 mM dithiothreitol, 0.5 mM MnCl<sub>2</sub>) containing 1  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P] UTP (3000Ci/mmol) at 30 °C for 45 min. The reaction products were extracted with Trizol and analyzed by 2% TBE agarose gel electrophoresis and autoradiography with Fuji-BAS 1000 phosphor-imager. Lane 5 denotes reaction carried out in the absence of Nib. The position of the molecular size markers (100 bp DNA ladder, Invitrogen) are as shown. The reaction components in each of the lanes are as indicated. (B) Time course of polymerase activity of Nib. Polymerase reaction was carried out as mentioned in legend to panel A and at different time points aliquots of 10  $\mu$ l were spotted on 1 cm<sup>2</sup> pieces of DE81 (Whatman) paper in triplicates, washed thrice with 5% Na<sub>2</sub>HPO<sub>4</sub> followed by water, air dried, and the retained radioactivity was quantitated by liquid scintillation counting. Counts per min (cpm) values were converted to femtomole UTP incorporated by using specific activity 6660 cpm/fmol and plotted against time in minutes. The reaction was linear up to 45 min. (O–O) and (■–■) denote reaction carried out in the presence of Mn<sup>2+</sup> and Mg<sup>2+</sup>, respectively. (▽–▽) denotes reaction carried out with D352V Nib and (▼–▼) shows reaction carried out in the absence of oligo-dT primer.

was made in the case of *Rabbit hemorrhagic disease virus* where VPg-uridylylation was completely poly(A) independent (Machin et al., 2001). The presence of Mg<sup>2+</sup> or Mn<sup>2+</sup> is crucial as no uridylylation was observed in the absence of metal ions. In order to determine the divalent metal ion requirement of PVBV Nib, MnCl<sub>2</sub> and MgCl<sub>2</sub> were used in the in vitro VPg-uridylylation reaction. When MgCl<sub>2</sub> was used instead of MnCl<sub>2</sub>, the efficiency of the uridylylation was less (Fig. 3A, lane 2) suggesting that Mn<sup>2+</sup> is better than Mg<sup>2+</sup> for this reaction. Therefore

MnCl<sub>2</sub> was used in all further experiments in the present study. When the reaction was carried out for increasing time intervals, the amount of uridylylated VPg also increased with time and after 45 min no further increase in the uridylylation was observed (Fig. 3B, lanes 1–4). Further, increase in the amount of VPg in the reaction mixture (20–300 pmol) resulted in increase in the amount of uridylylation (Fig. 3C, lanes 1–5). Quantitation of the uridylylated VPg by measuring the relative intensity of the bands corresponding to the different concentration of VPg (Fig. 3C, lanes 1–5) by Fuji MacBAS V2.4 software from the autoradiography image revealed that there is a non-linear increase in the incorporation of UMP (Fig. 3D). It appears that with the increase in the molar ratio of the VPg to UTP in the reaction mixture, efficiency of UMP incorporation also increases.

#### *Delineation of the minimum length of VPg that can be uridylylated by Nib*

PVBV VPg is a much larger (22 kDa) protein compared to poliovirus VPg (2 kDa). It was of interest to map the region in PVBV VPg that could be involved in the uridylylation reaction. Therefore, two large deletion mutants in the C-terminal half of the protein, corresponding to the C-terminal 64 and 92 residue ( $\Delta$ C64 VPg and  $\Delta$ C92 VPg), were designed. This region was reported to be involved in the interaction with HC-Pro (Yambao et al., 2003). N-terminal 21 residues do not contain any significant predicted secondary structure (Fig. 4) and deletion of these residues results in removal of only two conserved residues (Lys 13 and Ala 17). Further deletion of 17 amino acids (total 38 amino acids from the N-terminus) removes a predicted coil region located ahead of first predicted  $\alpha$ -helix from the N-terminus (Fig. 4) between amino acids Tyr 42 to Gly 49 (Tyr 40 to Gly 47 in PVY) (Plochocka et al., 1996). Therefore, two more N- and C-terminal double mutants ( $\Delta$ N21 $\Delta$ C64 VPg, with N-terminal 21 and C-terminal 64 residues deletion and  $\Delta$ N38 $\Delta$ C92 VPg, with N-terminal 38 residues and C-terminal 92 residues deletion) were also generated (Fig. 4). All these four deletion mutants were expressed with C-terminal his-tag and N-terminal GST fusion and purified by Ni-NTA affinity chromatography. When analyzed by SDS–PAGE, the purified proteins migrated at positions corresponding to the expected molecular weights (Fig. 5A, lanes 2–5). The uridylylation reactions were performed with N- and C-terminal deletion mutants of VPg in the same way as that of the wild-type VPg. The results presented in the Fig. 5B show that  $\Delta$ C64 VPg,  $\Delta$ C92 VPg, and  $\Delta$ N21 $\Delta$ C64 VPg were functionally active and could be uridylylated by PVBV Nib (Fig. 5B, lanes 2–4) suggesting that N-terminal 21 residues and C-terminal 92 residues of PVBV VPg were dispensable for the uridylylation reactions. On the contrary,  $\Delta$ N38 $\Delta$ C92 VPg protein, which lacked N-terminal 38 amino acid residues (in addition to C-terminal 92 residues) of the wild-type VPg, was not radioactively labeled in the

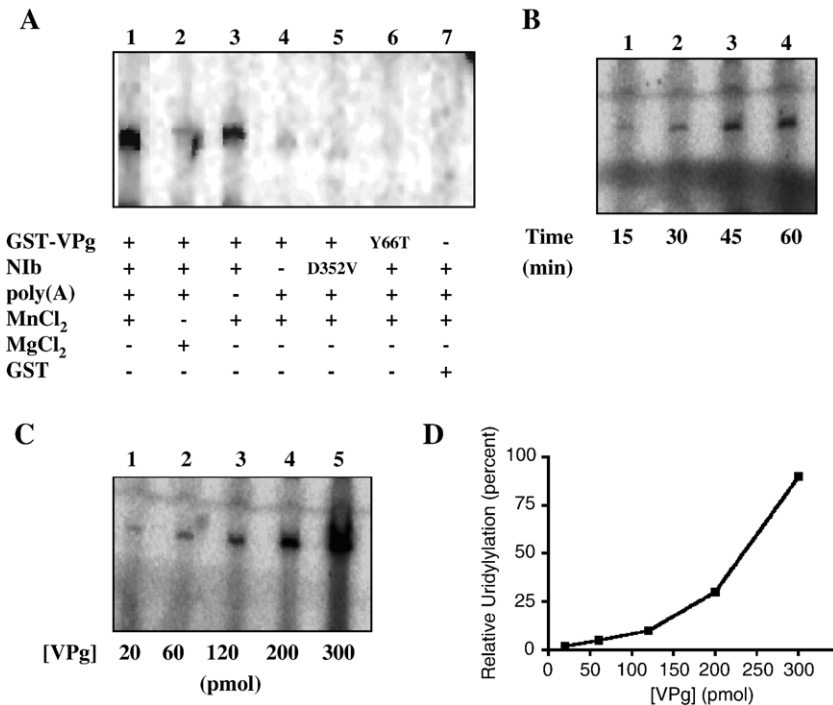


Fig. 3. In vitro uridylylation of PVBV VPg. (A) Uridylylation of PVBV VPg by PVBV Nib. The reaction carried out with purified VPg or VPg Y66T, Nib or Nib D352V, poly(A), [ $\alpha^{32}$ P] UTP, UTP, in reaction buffer containing 0.5 mM MnCl<sub>2</sub> and 10% glycerol. The assay mixture was incubated at 30 °C for 45 min and the reaction products were analyzed by electrophoresis on 12% SDS–PAGE, followed by autoradiography. The components of the reaction used are shown against the lanes as indicated. (B) Time course of VPg uridylylation. The uridylylation reaction was carried out as mentioned in legend to panel A. At different time points the reaction was stopped by adding SDS–PAGE loading buffer to the sample and the reaction products were analyzed by electrophoresis on 12% SDS–PAGE, followed by autoradiography. (C) Effect of various concentrations of VPg. The uridylylation reaction was carried out as mentioned before (A) for 45 min. Different concentrations (20–300 pmol) of PVBV VPg was used in the presence of 5  $\mu$ Ci (1.67 pmol)  $\alpha^{32}$ P-UTP and 450 pmol (15  $\mu$ M) cold UTP. (D) Quantitation of VPg uridylylation. Relative intensity of the band corresponding to the different concentrations of VPg (C, lanes 1–5) was measured by Fuji MacBAS V2.4 software from the autoradiography image and expressed as percent intensity with respect to intensity of the band of the lane 5 of panel C.

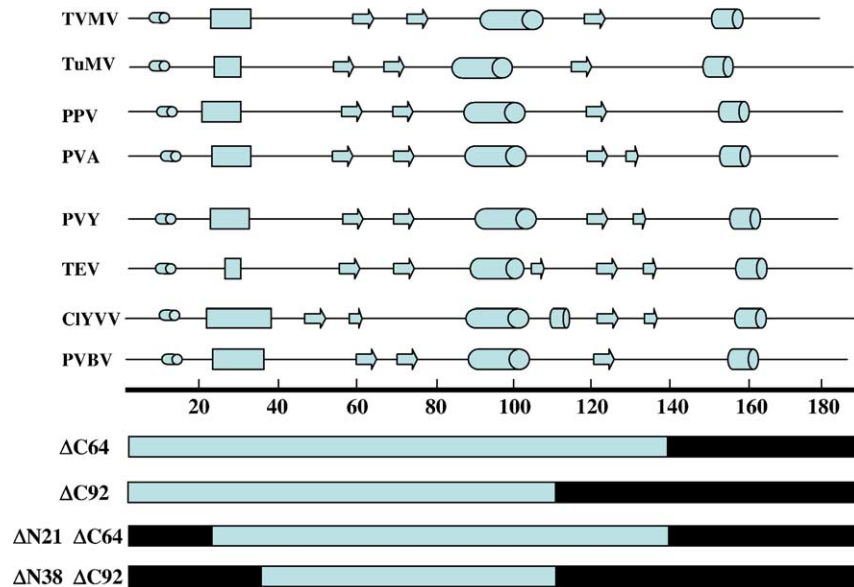


Fig. 4. Comparison of secondary structure of different potyvirus VPg. PHD protein secondary structure and coil prediction at the PBIL server (<http://pbil.univ-lyon1.fr/>) was used to generate secondary structure profile of PVBV and related potyviral VPgs. Symbols used are as follows: arrow,  $\beta$ -sheet; cylinder,  $\alpha$ -helix; and box, coil. Deletion mutants of PVBV VPg used in the present study are also shown schematically. The black boxes depict the region deleted from the N- and C-terminus of PVBV VPg.

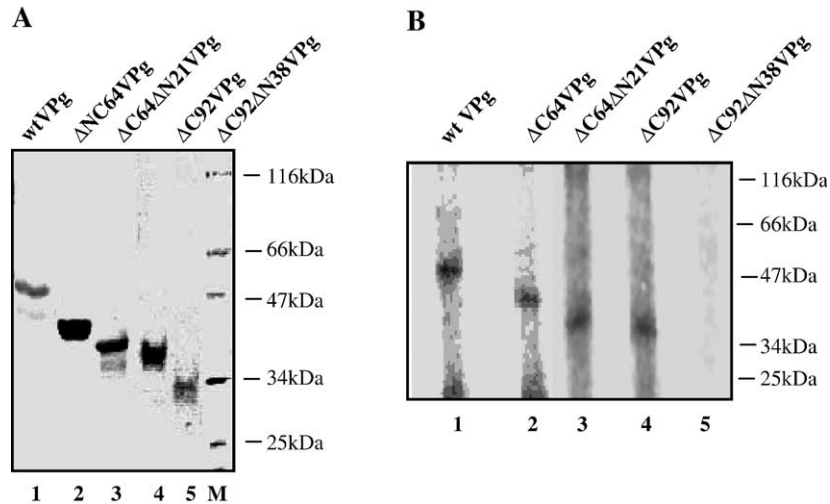


Fig. 5. Purification and characterization of deletion mutants of PVBV VPg. (A) SDS-PAGE analysis of Ni-NTA purified VPg deletion mutants (lanes 2–5). M denotes protein molecular wt. Marker. (B) In vitro uridylylation of different deletion mutants of PVBV VPg. In vitro uridylylation reactions were carried out as mentioned in Fig. 2A with wild-type (lane 1) and different deletion mutants (lanes 2–5) and analyzed.

presence of PVBV Nib under standard reaction conditions (Fig. 5B, lane 5), suggesting that the segment of VPg between amino acids residue 22–100 from the N-terminus is essential and the remaining C-terminal half of the PVBV VPg protein is dispensable for VPg uridylylation.

#### Identification of the amino acid residue of VPg that is uridylylated by Nib

It was shown earlier in TVMV that the Tyr 60 is the residue covalently linked to the viral RNA (Murphy et al., 1991). A Clustal-W analysis of VPg sequence of related potyviruses indicated that Tyr 66 of PVBV VPg corresponds Tyr 60 of TVMV and this conserved Tyr residue is present in the context of conserved NMY motif (Murphy et al., 1991). Therefore Tyr 66 of PVBV VPg was mutated to Thr in order to examine whether indeed this residue was uridylylated by Nib. The GST-Y66T VPg mutant was expressed with C-terminal His-tag and purified as described for wild-type VPg (Fig. 1A, lane 3). When the purified Y66T VPg was used in the uridylylation assay, the mutant VPg was not radioactively labeled (Fig. 3A, lane 6), revealing the specific requirement of Tyr 66 for uridylylation by PVBV-Nib.

## Discussion

The primary aim of the experiments reported in this paper was to understand mechanism of initiation of RNA synthesis in potyviruses. The first event in the replication of potyviruses involves uridylylation of viral genome-linked protein (VPg) which is catalyzed by viral polymerase (Nib). In vitro assay of such a reaction requires purified and soluble Nib and VPg. Earlier TVMV Nib was over expressed as a GST fusion protein and was shown to

catalyze the polymerase reaction. However, whether this protein could uridylylate the VPg was not tested (Hong and Hunt, 1996). Recently the uridylylation of PVA VPg by PVA Nib has been reported (Puustinen and Makinen, 2004). In the present study using a GST fused VPg with a C-terminal His-tag, VPg and its mutants were obtained in pure and soluble form (Figs. 1A and 5A). Further the His-tag Nib and its mutant were also purified under non-denaturing conditions (Fig. 1B). The purified recombinant Nib was functionally active as a polymerase (Figs. 2A and B) and when  $Mn^{2+}$  was used as cofactor, in presence of  $\alpha^{32}P$ -UTP, PVBV Nib successfully catalyzed the transfer of UMP from UTP to VPg resulting in the synthesis uridylylated VPg (Figs. 2 and 3A). This uridylylation reaction was specific for VPg and no radioactive products were detected when GST was used in place of VPg. Further no reaction was observed in the absence of Nib or in the presence of Nib D352V mutant, suggesting that uridylylation of VPg was an intrinsic property of PVBV Nib. Interestingly, even in the absence of poly(A) as template, uridylylated VPg was formed, suggesting that this reaction is template independent. Based on the predicted three-dimensional structure of *Potato Virus Y* VPg (Plochocka et al., 1996) and the secondary structure prediction for potyviral VPgs (Fig. 4), a set of VPg deletion mutants were constructed which lacked 21 and 38 residues from the N-terminus and 64 and 92 residues from the C-terminus, respectively. The uridylylation reaction was carried out using purified wild-type VPg and corresponding N- and C-terminal deleted forms. The results of uridylylation with VPg deletion mutants suggested that at least N-terminal 21 and C-terminal 92 residues of VPg are dispensable for uridylylation reaction. When N-terminal 38 amino acid residues were deleted VPg was unable to undergo uridylylation (Fig. 5B). However, C-terminal half of the VPg is dispensable for the uridylylation activity. This domain was shown to be important for VPg

self-interaction and VPg-HCPro interaction in the case of *Clover yellow vein virus* (Yambao et al., 2003). Such interactions were proposed to be important for virus movement and infection cycle (Rajamaki and Valkonen, 1999, 2002; Yambao et al., 2003). These results suggest that the minimal segment of VPg that is required for uridylylation is 22–100. It was shown earlier in the case of PVA that a tyrosine residue is uridylylated by N1b (Puustinen and Makinen, 2004). Within the region 22–100 there are two conserved tyrosines, Y42 and Y66, in PVBV VPg. Y42 is part of the nucleotide binding motif, AYTTKKGK, which was shown to be involved in nucleotide binding in the case of PVA VPg (Puustinen and Makinen, 2004). However, deletion of this motif did not result in complete loss of uridylylation (Puustinen and Makinen, 2004). Y42 is therefore unlikely to be the residue uridylylated by PVBV N1b. The other conserved tyrosine, Y66, is part of the NMY motif implicated to be involved in the RNA attachment. Therefore, Tyr 66 of PVBV VPg was mutated to Thr and the mutant VPg could not be uridylylated by PVBV N1b (Fig. 3A). These results suggest that even in the case of PVBV, the VPg may be linked to viral RNA via Tyr 66. It is also possible that the mutation of Tyr 66 in the vicinity of the crucial nucleotide binding site might have altered the local protein conformation leading to impaired nucleotide binding and hence the uridylylation. However, the CD spectrum of the wild-type and Y66T VPgs were nearly super imposable suggesting that there were no gross conformational changes (data not shown). It is possible that in potyviruses, replication begins with uridylylation of VPg which acts as primer for progeny RNA synthesis.

It is clear that compared to VPg of picornaviruses, potyvirus VPg has evolved uniquely and acquired two distinct domains: the C-terminal domain of the VPg is important for functions such as infection and movement, whereas the N-terminal half is important in the genome replication.

## Materials and methods

### *Construction of expression plasmids and mutants*

From the PVBV cDNA clone of pPJ15-pPV229 (Anindya et al., 2004), the VPg coding region was amplified by PCR with Deep-Vent DNA polymerase (New England Biolabs) using a set of specific primers, VPg-sen and VPg-anti (Table 1). In order to introduce His-tag at the C-terminus, the VPg-anti primer was designed with codons corresponding to six histidines. PCR product was cloned into *Sma*I site of pGEX-5x-2 vector (Amersham Pharmacia) and the clone was named as pGxVPg. Using pGxVPg as template and a corresponding set of mutant primers (Table 1; Y66A VPg-sen and Y66A VPg-anti) the mutant clone pGx-Y66A VPg was constructed by

the PCR-based method (Weiner et al., 1994). The deletion mutants were constructed using pGxVPg as template and amplification by PCR using the appropriate set of specific primers (Table 1) followed by cloning in the same way as pGxVPg. The mutants with C-terminal deletion of 64 and 92 residues were named as pGx- $\Delta$ C64 VPg and pGx- $\Delta$ C92 VPg, respectively. Two more mutants with N-terminal 21 and C-terminal 64 amino acid deletion (pGx- $\Delta$ N21 $\Delta$ C64 VPg) and N-terminal 38 and C-terminal 92 amino acid deletion (pGx- $\Delta$ N38 $\Delta$ C92 VPg) were also constructed similarly.

By using the same PVBV cDNA clone as template and specific pair of primers (Table 1) N1b coding regions were amplified by PCR and cloned into *Pvu*II site of pRSET-C vector (Invitrogen) and the recombinant clone was named pRN1b. In order to generate mutant N1b, Asp 352 of N1b was mutated to Val by using pRN1b as template and specific primers (D352V N1b-sen and D352V N1b-anti, Table 1) as before and the mutant construct was named pR-D352V N1b.

### *Overexpression and purification of GST-VPg and its mutants*

*E. coli* BL21(DE3) cells transformed with VPg or its mutant constructs were grown and induced for protein expression as previously described (Studier and Moffatt, 1986; Studier et al., 1990). A single colony was inoculated in 50 ml of Luria–Bertani (LB) medium containing 50  $\mu$ g/ml ampicillin and grown overnight at 30 °C. This overnight culture was inoculated into 500 ml of LB medium containing 100  $\mu$ g/ml ampicillin. After 2 h of growth at 37 °C, the cells were induced with 0.3 mM (final concentration) isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) (Sigma) and further grown for 6 h at 25 °C. The cells were harvested by centrifugation and resuspended in extraction buffer (10 mM Tris–HCl, pH 8.0, 200 mM NaCl) and sonicated to disrupt the cells. 30  $\mu$ l of this sample was analyzed by SDS–PAGE analysis (Laemmli, 1970) in 12% gel and the proteins were stained with Coomassie brilliant blue. Since the PVBV VPg and its mutants had the C-terminal his-tag, they were purified by Ni-NTA affinity chromatography as described by the manufacturer protocol (Qiagen handbook).

### *Overexpression and purification of N1b and its mutant*

A single colony of *E. coli* BL21(DE3) cells transformed with either pRN1b or pRN1b D352V was inoculated into 50 ml LB and grown overnight at 37 °C. This pre-inoculum was inoculated into 1 L of LB and grown at 37 °C till the  $A_{600}$  reached 0.9. The cells were then induced with 0.3 mM (final concentration) IPTG and further grown for 10 h at 10 °C. The cells were harvested by centrifugation and resuspended in 50 ml extraction buffer (50 mM Tris–HCl, pH 8.0, 1 M NaCl, and 500 mM L-

Arg) and sonicated to disrupt the cells. Insoluble debris was removed by centrifugation and the soluble fraction was bound to phenyl-sepharose (Sigma) column equilibrated with the extraction buffer. The column was sequentially washed with 50 mM Tris-HCl, pH 8.0 buffer, containing 500 mM, 400 mM, 250 mM, and 100 mM NaCl. The bound hydrophobic proteins were finally eluted with 10 mM Tris-HCl, pH 8.0 buffer, and the purity was checked by SDS-PAGE. This phenyl-sepharose eluate was further allowed to bind to Ni-NTA agarose and the his-tagged N1b or D352V N1b was further purified by Ni-NTA affinity chromatography as described above and the purity was checked by 12% SDS-PAGE.

#### *RNA-dependent RNA polymerase assay*

Polymerase assay was carried out as described by Neufeld et al. (1991), with some modification. The assay mixture (50  $\mu$ l) contained 2  $\mu$ g N1b or mutant D352V N1b, 2.5  $\mu$ g of poly(A) (Sigma), and 1  $\mu$ g of oligo(dT)<sub>20</sub> (Genei, Bangalore) in reaction buffer (50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 0.5 mM MnCl<sub>2</sub>) containing 1  $\mu$ Ci of  $\alpha$ 32P-UTP (3000 Ci/mmol). The reaction was carried out at 30 °C for 45 min. For agarose gel analysis, the reaction was stopped by addition of 250  $\mu$ l of Tri-reagent (Sigma) and chloroform and RNA was extracted, ethanol precipitated, and dissolved in RNase-free water. The sample was heated at 70 °C in presence of 50% (v/v) formamide and electrophoresed on 2% TBE agarose gel (Sambrook et al., 1989). After electrophoresis, the gel was dried under vacuum with a whatman-1 filter support and analyzed by Fuji-BAS 1000 phosphor-imager. For quantitation, 10  $\mu$ l of extracted samples were spotted on 1 cm<sup>2</sup> pieces of DE81 (Whatman) paper in triplicates, washed thrice with 5% Na<sub>2</sub>HPO<sub>4</sub> followed by water, air dried, and the retained radioactivity was quantitated by liquid scintillation counting.

The VPg uridylylation assay was done as described previously for *Rabbit hemorrhagic disease virus* (RHDV) (Machin et al., 2001) with minor modification. The assay mixture (30  $\mu$ l) contained 6  $\mu$ g of purified VPg (4  $\mu$ M) or VPg Y66T or the deletion mutants, 1  $\mu$ g N1b (0.5  $\mu$ M) or mutant N1b D352V, 0.5  $\mu$ g of poly(A), in reaction buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM MnCl<sub>2</sub>) containing 5  $\mu$ Ci (3000 Ci/mmol, 0.02  $\mu$ M or 1.67 pmol) of  $\alpha$ 32P-UTP, 15  $\mu$ M UTP (450 pmol), and 10% glycerol (where indicated 0.5 mM MgCl<sub>2</sub> was added to the reaction mixture instead of MnCl<sub>2</sub>). The reaction was carried out at 30 °C for 45 min and stopped by addition of SDS-PAGE sample-loading dye. After heating for 5 min at 95 °C, the reaction products were analyzed by electrophoresis on 12% SDS-PAGE, containing 20 mM vanadyl ribonucleoside complex (Sigma). The gel was dried without fixing under vacuum with a whatman-1 filter support. The dried gel was exposed to phosphor-imager cassette for 6 h and analyzed by Fuji-BAS 1000 phosphor-imager.

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