Uridylylation of the Potyvirus VPg by Viral Replicase NIb Correlates with the Nucleotide Binding Capacity of VPg*

Received for publication, March 16, 2004, and in revised form, June 24, 2004
Published, JBC Papers in Press, June 24, 2004, DOI 10.1074/jbc.M402910200

Pietti Puustinen and Kristiina Mäkinen‡
From the Department of Applied Biology, P. O. Box 27, University of Helsinki, Helsinki 00014, Finland

Poty- and picornaviruses share similar genome organizations and polyprotein processing strategies. By analogy to picornaviruses it has been proposed that the genome-linked protein VPg may serve as a primer for genome replication of potyviruses. The multifunctional VPg of potato virus A (PVA; genus Potyvirus) was found to be uridylylated by NIb, the RNA polymerase of PVA. The nucleotidylation activity of NIb is more efficient in the presence of Mn2+ than Mg2+ and does not require an RNA template. Our results suggest that the nucleotidylation reaction exhibits weak preference for UTP over other NTPs. An NTP-binding experiment with oxidized [α-32P]UTP revealed that PVA VPg contains an NTP-binding site. Deletion of a 7-amino acid-long putative NTP-binding site from VPg reduced nucleotide-binding capacity and debilitated uridylylation reaction. These results provide evidence that VPg may play a similar role in RNA synthesis of potyviruses as it does in the case of picornaviruses.

Potato virus A (PVA; genus Potyvirus; family Potyviridae) is a single-stranded, messenger polarity RNA genome of 9,565 nucleotides. The entire PVA genome is expressed as a polyprotein, which is subsequently processed into 10 mature proteins by three different viral proteinases (1). Potyviruses resemble picornaviruses in genome organization and polyprotein processing strategy (2). The genomic RNA of picornaviruses and potyviruses is covalently linked at the 5'-end to the small, virally encoded protein VPg (3–5). The N-terminal VPg domain is slowly released from Nla by the proteolytic activity of the C-terminal Nla-Pro domain in the course of potyvirus infection. It is essential for replication that proteolytic processing at the suboptimal Nla internal cleavage site occurs (6). Potyviral VPg is a multifunctional protein (as reviewed in Ref. 2), which is also exposed at one end of the virion (7). The size of potyviral VPg is substantially larger than that of poliovirus (PV) VPg, and they do not share any obvious sequence homology.

PV VPg represents the most advanced model for understanding the role of VPg as a primer for the viral replicase. PV VPg is a small peptide containing only 22 amino acids. It is genome-linked via a bond between the hydroxyl group of a tyrosine residue and the α-phosphate group of uridylic acid. In vitro uridylylation of PV VPg catalyzed by the viral polymerase resulted in a VPg-linked poly(U) (8). Replication of picornaviruses requires cis-acting RNA elements, termed cre (9). In PV this small RNA hairpin structure is within the coding region of protein 2C. Its presence in the in vitro reaction mixture stimulated uridylylation as compared with a reaction with poly(A) as a template (10). The CP cre-dependent VPg uridylylation is required only for the synthesis of new positive strands, whereas the minus strand synthesis is VPg-dependent but cre-independent (11).

VPg of tobacco vein mottling virus (TVMV; genus Potyvirus) is linked to the viral RNA via Tyr-60 and TVMV carrying the mutation Tyr-60 to Ala is not able to replicate in protoplasts (12, 13). Similarly, substitution of the corresponding Tyr with Ala in another potyvirus, tobacco etch virus (TEV), makes it amplification-defective (14). Nuclear inclusion protein NIb is the viral RNA-dependent RNA polymerase (RdRp) responsible for genome replication of potyviruses (15). It contains an amino acid triplet Gly-Asp-Asp, which is universally conserved among RdRps (16), and when Ni of TEV was mutated at this motif, the virus was unable to replicate (17). The Ni of TEV interacts specifically with the protease part of Nia (18), whereas the Ni of TVMV can interact with the Nia-VPg, as shown in vitro (19) and also in vivo with the yeast two-hybrid system (20). Taken together, these findings have supported the view that VPg may serve as a primer for genome replication of potyviruses. However, no direct evidence to support this hypothesis is available. Here we show that the recombinant PVA VPg can be uridylylated by purified recombinant PVA Ni in an in vitro reaction and that VPg is an NTP-binding protein. Deletion of a 7-amino acid-long putative NTP-binding site from VPg led to a reduced nucleotide-binding capacity and debilitated uridylylation reaction. These results increase our understanding of the mechanisms involved in potyvirus RNA synthesis.

MATERIALS AND METHODS

Recombinant Protein Expression—(His)6/pQE (Qiagen) constructs for PVA VPg and NiB expressions were made as described previously (21). Both proteins were expressed in Escherichia coli strain M15[REP4] cells. VPg was purified by using Ni2+-nitrilotriacetic acid-agarose column (Qiagen). His-tagged proteins were washed with guest on July 21, 2018http://www.jbc.org/Downloaded from

This paper is available on line at http://www.jbc.org

38103
buffer B (30 mM imidazole in buffer A) and elution buffer C (500 mM imidazole in buffer A) and further dialyzed against water to remove imidazole.

**In Vitro Uridylylation Assay**—Uridylylation was measured as the incorporation of radiolabeled from [α-32P]UTP into the purified PVA VPg in an *in vitro* reaction. Reaction conditions were essentially as described previously (8). The reactions were performed at room temperature (RT = 22 °C) for 25 min in a final volume of 30 μl containing 0.75 μCi of [α-32P]UTP (3000 Ci/mmol; Amersham Biosciences), 2 μg of PVA VPg, a varying amount of Ni2+ (0–0.3 μM), 10 mM HEPES, pH 7.5, 2.5 mM MnCl2, 2 units of RNase (Promega), and 0.5 μg of poly(A) (n = 35–200; Amersham Biosciences) when required. Reactions were terminated with 5× SDS-PAGE sample buffer or they were further purified for downstream analysis. The samples were analyzed by SDS-PAGE in 12–15% gels. Radioactively labeled proteins were visualized and quantified by autoradiography with a PhosphorImager (Fuji) and Tina 2.09c software (Raytest).

**In Vitro Nucleotide Competition Assay**—PVA VPg (2 μg) was in *vitro* uridylylated essentially as described above in the presence of one of the following cold competitor NTPs: ATP, UTP, GTP, or CTP. Several concentrations of the cold competitor were tested: 0.125, 0.25, 0.5, and 0.75 μM. The final reaction volume was 20 μl containing 2 μCi of [α-32P]UTP (400 Ci/mmol; Amersham Biosciences), 10 mM HEPES, pH 7.5, 5 mM MnCl2, 2 units of RNAsin (Promega), 0.1 μg of Ni2+, and 0.5 μg of poly(A) template (Amersham Biosciences) in half of the samples. After 25 min of incubation at RT, the reaction was terminated, and proteins were separated by 12% SDS-PAGE. Dried gels were subjected to autoradiography with a PhosphorImager screen, and the [α-32P]UTP incorporated into the VPg during the nucleotide competition reaction was quantified.

**Two-dimensional Analysis of Nucleotidylated VPgs**—VPg proteins were uridylylated with [α-32P]UTP (3000 Ci/mmol) in the presence and absence of a poly(A) template in standard reactions. ZipTip C-4 columns (Millipore) were rehydrated with 50% acetonitrile in H2O and equilibrated with 0.02 mM linking mixture containing 2 μl of [α-32P]UTP (400 Ci/mmol; Amersham Biosciences), 50 mM Tris-HCl, pH 6.8, 6 M urea, 2% SDS, 0.2% bromphenol blue). Strips were applied onto the C-4 column several times to separate the labeled VPgs. The column was washed with 50% acetonitrile in buffer A and elution buffer C (500 mM acetonitrile in buffer A) and further dialyzed against water to remove acetonitrile. The eluted labeled VPg proteins were dried and washed once with H2O. The dried VPg was oxidized with 0.75 mM sodium periodate in the dark in the presence of 0.5 mM HCl at RT. The periodate was reduced with glycerol. Oxidized UTP was cross-linked to VPg by modifying the procedures used previously (23, 24). Cross-linking mixture contained 0.02 μCi of oxidized [α-32P]UTP, 10 mM HEPES, pH 7.4, 5 mM MgCl2 or MnCl2, 3 mM sodium cyanoborohydride (NaCNBH3), and 3 μg of PVA VPg in a final volume of 30 μl. Reactions were incubated at 0 °C for 60 min. The reaction products were analyzed with SDS-PAGE without any further purification. Labeled reaction products were visualized and quantified.

**RESULTS**

The RNA-independent Uridylylation of PVA VPg by PVA NiB—PVA VPg and NiB were both expressed as N-terminal hexahistidine fusions in *E. coli*. PVA VPg was purified by immobilized metal affinity chromatography in denaturing conditions and was then refolded by dialysis. We have shown previously that PVA VPg obtained by using this approach takes part in several protein-protein interactions (22) and can be phosphorylated by host kinase(s) (7, 25). Purification of NiB was carried out in native conditions, because this was required for the polymerase to be active in the uridylylation reaction. After the proteins were purified with Ni2+-nitrilotriacetic acid resin, they were analyzed by 12% SDS-PAGE. One major band at the expected molecular mass of 25 kDa for VPg and 88 kDa for NiB was observed in the Coomassie-stained gels (data not shown). The purified PVA VPg was assayed for uridylylation in an *in vitro* reaction containing the purified PVA NiB and [α-32P]UTP in the presence of 5 mM MnCl2. The reaction products were analyzed by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue with autoradiography. PVA VPg was found to be uridylylated by NiB (Fig. 1A, lane 1). No labeled protein was produced in the absence of VPg (Fig. 1A, lane 2) confirming that the band identified corresponded to labeled recombinant VPg. Omission of NiB from the reaction mixture also abolished uridylylation (Fig. 1A, lane 4) indicating that the bands on the autoradiogram did not correspond to radiolabeled UTP noncovalently bound to VPg and that the affinity-purified VPg preparation did not contain any enzymatic activity capable of uridylylating VPg. Increasing amounts of NiB in reaction mixtures led to increased uridylylation (Fig. 1A, lanes 5–8). Kinetics of the uridylylation reaction remained linear for an hour at RT (data not shown).

**The NiB Activity Uridylylation PVA VPg Exhibits a Preference for Mn2+ over Mg2+**—It was well established that DNA and RNA polymerases require a divalent cation for their activity. When free Mn2+ was removed from the reaction mixture in a complex with EDTA in a 1:1 molar ratio, no uridylylation was detected (Fig. 1B). As expected, the presence of a divalent metal cation was indispensable for NiB uridylylation activity. To find out which divalent cation was required for an optimal activity, Mg2+ and Mn2+ were tested; these ions are both known to function as activators of the RNA polymerases and the nucle-
otidylation reaction. The results showed clearly that NIb has a strong preference for Mn\(^{2+}\)/H\(_{11001}\) over Mg\(^{2+}\)/H\(_{11001}\) (Fig. 1C). The capacity of Mg\(^{2+}\) to support nucleotidylation reaction was practically 10 times lower than that of Mn\(^{2+}\) in an identical concentration. Other divalent metal cations were not investigated. Mn\(^{2+}\)/H\(_{11001}\) can support the nucleotidylation reaction at concentrations ranging from 0.5 to 15 mM MnCl\(_2\) (data not shown). Tolerance for a broad concentration range for the activating cation was earlier observed for the Mg\(^{2+}\)/H\(_{11001}\)-dependent DNA polymerase of the bacteriophage PRD1 and also for PV RdRp (26, 27). We examined whether these two metal ions competed for a common binding site or whether the presence of both ions could influence the rate of nucleotidylation independently. A micromolar concentration of MgCl\(_2\) in the presence of a millimolar concentration of MnCl\(_2\) was capable of slightly increasing the nucleotidylation reaction (Fig. 1C), suggesting that Mg\(^{2+}\) and Mn\(^{2+}\) ions do not compete for the same site.

A Comparison of the in Vitro Uridylylation Reaction in the Presence and Absence of an RNA Template—The presence of an RNA template is an absolute requirement for PV VPg uridylylation, in contrast to rabbit hemorrhagic disease virus (8, 28). Our result suggests that under the experimental conditions used, the PVA system did not require a template. Addition of the poly(A) template did not stimulate the uridylylation reaction per se but induced the synthesis of a labeled high molecular weight product that did not migrate into the resolving gel (Fig. 1A, lanes 1 and 2), but free Mn\(^{2+}\) was removed from reactions by using an equimolar concentration of EDTA. The divergent cation requirement for the PVA NIb activity uridylylating PV VPg was investigated. Varying concentrations of Mg\(^{2+}\) and Mn\(^{2+}\) were used in the reactions as indicated. Radioactivity associated with uridylylated VPg was quantified with a PhosphorImager and plotted against Mg\(^{2+}\) and Mn\(^{2+}\) concentrations. The value obtained in 2.5 mM Mn\(^{2+}\) in the absence of Mg\(^{2+}\) was taken as 100%.

Fig. 1. PVA VPg is uridylylated by PVA NIb RNA polymerase. A, bacterially expressed His-tagged PVA VPg was assayed for uridylylation in the presence of [\(\alpha\)-\(^{32}\)P]UTP and PVA NIb as described under “Materials and Methods.” The specific conditions for each reaction are indicated. Proteins were separated with SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue. Autoradiograms of \(^{32}\)P-labeled reaction products indicated that uridylylated VPg had been formed. In addition, a high molecular weight product hardly able to migrate into the resolving gel was detected. B, uridylylation reactions were repeated as in A (lanes 1 and 2), but free Mn\(^{2+}\) was removed from reactions by using an equimolar concentration of EDTA. C, the divergent cation requirement for the PVA NIb activity uridylylating PV VPg was investigated. Varying concentrations of Mg\(^{2+}\) and Mn\(^{2+}\) were used in the reactions as indicated. Radioactivity associated with uridylylated VPg was quantified with a PhosphorImager and plotted against Mg\(^{2+}\) and Mn\(^{2+}\) concentrations. The value obtained in 2.5 mM Mn\(^{2+}\) in the absence of Mg\(^{2+}\) was taken as 100%.
yield of the uridylylated VPg (Fig. 2), which is most likely due to a less amount of Nib available for the uridylylation reaction in the presence of a competing reaction and more choice for the interaction partners.

Analysis of the Uridylylated VPg Products—Free VPg, carrying more than one covalently linked UMP (VPg pUpU), has been found in PV-infected cells (30). This precursor to elongation is most likely synthesized independently of the elongation reaction. Therefore, the question of how many uridylic acid molecules were linked to VPg during the uridylylation reaction, both in the presence and in the absence of the RNA template, was addressed. Further extensive elongation to form VPg pU₈ seemed unlikely because no VPg was detected in the high molecular weight band produced in the presence of the poly(A) template (Fig. 1A). We monitored the incorporation of [α-32P]UTP with two-dimensional gels. Uridylylated VPg forms derived from the reaction mixtures with (Fig. 3A) and without RNA (Fig. 3B) were found in the autoradiograms and silver-stained gels as extended, nearly identical labeled spots, indicating that all VPg forms were uridylylated. However, separation of nonuridylylated VPg protein through a two-dimensional gel and comparison of this silver-stained pattern to uridylylated VPg proteins revealed similar patterns (data not shown), indicating some heterogeneity within the purified VPg protein sample.

Additionally, VPg uridylylated with Nib was separated with SDS-PAGE and transferred to a membrane. VPg was trypsinized in the membrane, and released peptides were separated on TLE/TLC plates. As shown in TLE/TLC plate autoradiography (Fig. 4), two major peptides were detected. Therefore, it is possible that two uridylylated VPg forms were produced, although it is also possible that two different amino acids in two different peptides were uridylylated or a single amino acid contains the uridylic acid, but due to two alternative trypsin cleavage sites it is found in distinct peptides sharing part of the amino acid sequence. As a final attempt to detect the number of uridylic acids added by Nib, we performed a MALDI-TOF analysis of the trypsinized VPg samples to find peptides carrying covalently linked nucleotide added by Nib. However, the percent of the uridylylated VPg produced in in vitro reactions was low and unfortunately below the limits of MALDI-TOF (data not shown). Approximately only 0.3% of the input rabbit hemorrhagic disease virus VPg was uridylylated in an in vitro reaction similar to ours (28).

The identity of the amino acid residue involved in the PVA VPg-Up linkage was investigated. The 32P-labeled uridylylated PVA VPg was acid-hydrolyzed. A subsequent thin layer electrophoretic amino acid analysis revealed two 32P-labeled molecules. We assume that the faster migrating molecule represents the free label, whereas the slower one migrated to the same position with the ninhydrin-stained phosphotyrosine marker (Fig. 5), suggesting that a tyrosine residue was uridylylated in the in vitro reaction. Residue Tyr-60 forms a linkage to viral RNA in TVMV VPg, as shown in analysis of in vivo labeled virus particles (13). Consequently, we substituted the corresponding Tyr-63 with Phe-63 in PVA VPg. This point mutation did not have any obvious effect on the uridylylation reaction, indicating that another tyrosine residue was uridylylated in the in vitro reaction (data not shown).

Nucleotide Specificity of the NIB-catalyzed Reaction—To analyze further the NIB-catalyzed nucleotidylation reaction, the effect of each of the four ribonucleotides, ATP, CTP, GTP, and UTP, was tested in competition assays. The nucleotidylation reactions were carried out in the presence of constant amounts of [α-32P]UTP and increasing amounts of cold ribonucleotides. Clear specificity for [α-32P]UTP was detected in low cold NTP concentrations (0.125 μM) both in the presence (Fig. 6A) and absence (Fig. 6B) of a poly(A) template. As the competitor nucleotide concentrations increased, the specificity of the reaction for UTP decreased. In concentrations above 0.75 μM the other nucleotides were equally able to compete with [α-32P]UTP. These results suggest an RNA-independent selection for UTP at the substrate-binding site either within VPg or Nib.

A Putative Nucleotide-binding Site within PVA VPg—A PVA VPg sequence motif, which contains a putative nucleotide-binding site, was identified by using the program Prosite (us.expasy.org). To verify that VPg is indeed an NTP-binding protein, we carried out a cross-linking experiment with oxidized [α-32P]UTP in the presence of a reducing agent, NaCNBH₃, and either Mg²⁺ or Mn²⁺ in similar 5 mM concentrations that were used in the nucleotidylation reactions. The results indicated that wild type VPg binds oxidized UTP in the presence of divalent metal ions (Fig. 7A). Again, Mn²⁺ was clearly preferred over Mg²⁺ as in the uridylylation reaction. It has been proposed that under the reaction conditions we used, the cross-link is formed between the oxidized nucleotide and a lysine residue (31). The predicted nucleotide-binding sequence of PVA VPg ([39AYTKKGK44]) contains three lysine residues. Deletion of this 7-amino acid-long region in VPg (Δ38–44) affected nucleotide binding (Fig. 7A), decreasing it by ~80% as compared with the wild type VPg. Because chemical cross-linking of the oxidized nucleotide to a protein may occur nonspecifically via exposed lysine, some residual binding was expected (24).

To understand the influence of the putative NTP-binding site on the NIB-catalyzed uridylylation reaction, the deletion mutant VPg Δ38–44 was assayed in the in vitro uridylylation reaction. The efficiency of uridylylation of VPg Δ38–44 was only 30% of that of wild type VPg (Fig. 7B). We conclude that the VPg region containing the amino acids 38–44 has some role in the uridylylation reaction. The deleted peptide contained one tyrosine residue in a sequence context resembling that surrounding the uridylylated tyrosine in PV VPg. However, a point mutation Tyr-39 to Phe-39 did not have any detectable effect on PVA VPg uridylylation (data not shown). The reason for decreased uridylylation must therefore be either the requirement of an intact NTP-binding domain or a conformational change in the three-dimensional structure of PVA VPg.

The three-dimensional structure of PVA VPg is not known. Therefore, it is not possible to predict the effect of VPg Δ38–44 on uridylylation activity.

Fig. 2. Uridylylation activity of PVA NIB is RNA template-independent. The uridylylation assay was carried out in the presence and in the absence of a poly(A) template. An increasing amount of NIB RNA polymerase was added to the reactions. Reaction products were separated with SDS-PAGE, blotted onto a nylon membrane, and visualized with Ponceau S. An autoradiogram is shown together with the stained membrane.
deletion to the overall structure of VPg. To investigate the possibility that the deletion mutation exerted its effect through alteration of the VPg conformation, we compared the secondary structure of the mutant with that of the wild type PVA VPg by using circular dichroism spectroscopy. As indicated in the Fig. 7 the obtained CD spectra were nearly identical in both cases. Some variation is seen at the 215 nm area. However, we conclude that the overall structure of PVA VPg is intact in the VPg/H9004–44 mutant.

The Majority of the Uridylylated PVA VPg Is in Monomeric Form—The multifunctional protein 3AB of PV, domain 3B being the VPg, has been shown to oligomerize (32). Also PVA VPg forms dimers (5). A chemical cross-linking experiment was carried out to see whether the dimerization PVA VPg form is uridylylated. After the normal nucleotidylation reaction, increasing amounts of glutaraldehyde were added to the reaction mix. In fact, a similar amount of dimerized form was detected in the sample not treated with glutaraldehyde and in the cross-linked samples (Fig. 8A), indicating that the noncross-linked dimers were strong enough to last in denaturing gel electrophoresis conditions. Separation of the chemically cross-linked reaction mixtures in SDS-PAGE and the subsequent autoradiography revealed that most of the uridylylated VPg was in a monomeric form (Fig. 8B). The amount of labeled dimers was very low. Dimers and higher form oligomers were apparently not capable of interacting with NiB in such a way that it catalyzed the nucleotidylation reaction. Alternatively, incorporation of the uridylic acid into VPg decreased the self-interaction capability of PVA VPg.

DISCUSSION

Virally encoded RdRp is the major enzyme responsible for replicating the genomic RNA of positive-strand RNA viruses. Their replication consists of two steps as follows: synthesis of a (−)-strand RNA using the (+)-strand viral RNA genome as template and synthesis of the (+)-strand viral RNA using the newly synthesized (−)-strand RNA as template. In the group of picornaviruses initiation of RNA synthesis is dependent on a VPgpU or VPgpUpU primer. The present study revealed that this is possibly also the case with potyviruses, because the multifunctional VPg of PVA was found to be uridylylated by NiB, the viral polymerase. It was unexpected, however, that the point mutation Tyr-63 to Phe-63 within the recombinant PVA VPg did not have any effect on the uridylylation reaction, because evidence exists that this residue links VPg to the genomic RNA in TVMV and TEV (12–14). The importance of this particular site in linking the genomic RNA of PVA to VPg is not proven, and therefore it is not ruled out that another tyrosine in PVA VPg may form the link. Adding support to this possibility is that the 5-amino acid insertion adjacent to Tyr-63 of VPg in the infectious cDNA of PVA had no influence on infectivity (33). However, other explanations seem more likely. For example, the (−)-strand synthesis may require a different priming mechanism from that of the (+)-strand synthesis or the in vitro system may not faithfully recapitulate in vivo events. All potyviral proteins are essential for virus propagation at a single cell level (33), and it therefore seems that the potyviral replication complex is formed via a complicated set of protein-protein interactions. The 6K2 protein directs the rep-
licase to the endoplasmic reticulum (34, 35). Most likely the correct membranous environment together with the whole replication complex provides the platform for the putative in vivo uridylylation reaction. These surroundings obviously provide more optimal and stringently regulated reaction conditions than those prevailing in vitro.

Neither an artificial poly(A) molecule nor the full-length PVA RNA was required as templates to stabilize the incorporation of the first nucleotide in vitro. In the case of PV, the A1 residue of the cre (2C) RNA sequence (A1A2A3CA) is shown to be the primary template for VPg uridylylation and to enhance the reaction efficiency severalfold (36). This signal within PV RNA directs the uridylylation exclusively to the viral genome. It is possible that such a signal resides in potyvirus RNA, but this study did not reveal it. The correct recognition of the viral genome may occur by some other means than via an RNA structure. No elongation initiated from the uridylylated VPgpU was detected. It may require either other cellular and viral factors not present in our in vitro reaction mixtures or alternatively elongation is not initiated due to some structural property of NIb. It was recently reported that T7 RNA polymerase undergoes major structural changes during the transition from initiation to the elongation phase of transcription (37). The question whether a structural change is required in PVA NIb to meet the requirements of template-dependent elongation needs to be solved.

RdRps require divalent cations for their activity. Divalent cations act in general as catalytic ligands of the phosphate group of the NTP substrate. The 2 Å resolution x-ray structure of the 6 RdRp revealed an additional cation-binding site ~6 Å from the expected catalytic position (38). Our results demonstrate that PVA NIb requires Mn2+ for optimal in vitro VPg nucleotidylation activity, but additional further activation, although slight, was achieved by adding Mg2+ simultaneously. Plants generally contain significant intracellular stores of Mn2+, and it has been speculated that certain plant virus
RdRps have a requirement for Mn\(^{2+}\) rather than Mg\(^{2+}\) (27). The function of NiB may also depend on Mg\(^{2+}\) for RNA synthesis in vivo. For example, Mg\(^{2+}\) could be required in changing the activity from a protein-primed initiation to the RNA synthesis phase as initially proposed (27). The use of Mn\(^{2+}\) as the metal activator of the PRD DNA-dependent DNA polymerase stimulated the protein-primed initiation, but it was not capable of activating the overall replication of the phage genome (26).

The competition assay revealed that UTP is preferred over the other three nucleotides in the relatively low nucleotide concentrations used in our uridylylation assays. The preference for UTP did not depend on a template RNA. However, the specificity for formation of the VPg-UMP complex was abolished when the concentration of any of the three remaining competitive cold nucleotides was increased over a certain threshold. This suggests that the transfer of UTP to the reactive hydroxyl group within VPg is to some extent easier than with the other nucleotides. In fact, we can only speculate how the link between the first nucleotide and the priming aromatic ring of tyrosine is mechanistically formed. Either NiB binds the nucleotide and by interacting with VPg brings the substrate to the vicinity of the correct tyrosine for catalysis or alternatively the initial binding of the NTP is to VPg, and the VPg/NiB interaction is required for placing the catalytic NiB correctly over the substrate. Because VPg appeared to be an NTP-binding protein even in the absence of NiB, the direct role of VPg in nucleotide selection seems quite likely.

The NTP-binding capacity of PVA VPg was observed in an experiment with a cross-linker specifically making a bond between the nucleotide and an adjacent lysine. Mn\(^{2+}\) stimulated the NTP-binding activity of wt VPg, and this may reflect that the metal cation is needed for coordination and stabilization of the nucleotide bound to VPg. Deletion of Lys-41, Lys-42, and Lys-44 residues in the VPg\(_{38-44}\) mutant abolished the NTP-binding capacity. Because the deleted region was 7 amino acids long, it was not possible to know the exact role of this region for the overall structure of VPg. CD spectra of the mutant VPg, by SDS-PAGE, and the incorporated label was visualized and quantified by using a PhosphorImager. Deletion of the NTP-binding site decreased the uridylylation of PVA VPg. C, structural comparison of PVA VPg and VPg\(_{38-44}\) (0.2 µg/ml in water) was performed by using CD spectroscopy. CD spectra on 260 to 185-nm wavelength range were recorded at 22°C. Each spectrum represents an average of 20 scans. Background spectra were subtracted. The raw ellipticity data in millidegrees are presented. Only minor structural changes are associated with the deletion mutant.

![Fig. 7. PVA VPg contains an NTP-binding site. A, wt PVA VPg and VPg\(_{38-44}\), having the putative NTP-binding site AYTKKGK deleted, were incubated in the presence of periodate-oxidized [α\(^{32}\)P]UTP and NaCNBH\(_3\) as a reducing agent. wt PVA VPg binds UTP, and the binding is enhanced in the presence of Mn\(^{2+}\) instead of Mg\(^{2+}\). The deletion mutant does not bind UTP. B, efficiency of wt PVA VPg was compared with that of VPg\(_{38-44}\).](#)

![Fig. 8. The uridylated PVA VPg is mainly in monomeric form. PVA VPg was cross-linked after the nucleotidylation reaction with increasing amounts of glutaraldehyde. Monomers, dimers, and higher order oligomers were separated by SDS-PAGE and blotted onto a nylon membrane. Proteins were detected by Western blot using anti-VPg antibody to detect all the VPg-specific bands. Autoradiogram indicates that the majority of the label is associated with the monomeric VPg form.](#)
amino acids with double asterisks. Identical amino acids are indicated with an asterisk and similar amino acids with double or single dots.

however, suggests that no major rearrangements took place in the VPg structure due to the deletion. More careful point mutations must be done in the future to fully characterize this NTP-binding site.

Most interestingly, the VPg Δ38–44 deletion mutant exhibited only about one-third of the wt VPg in vitro uridylylation activity. Because the point mutation Tyr-39 to Phe-39 did not affect uridylylation, this region must have some other important function either in VPg-Nb interaction or in assisting nucleotidylation. Our NTP-binding deletion overlaps with the region that is reported to be the nuclear localization signal of TEV VPg. A mutational analysis of the nuclear localization signal region within TEV VPg revealed that mutations resulting in deletion of Nls nucleic translocation destabilized also to genome amplification (14). More specifically, TEV containing a double mutation of the residues corresponding to PVA VPg residues Lys-42 and Lys-44 resulted in a 10-fold reduction in amplification of the virus in tobacco protoplasts. The authors speculated that the RNA attachment activity of VPg possibly was affected by the nuclear localization signal site mutations. The in vitro assay used in this study indeed revealed that the homologous region in PVA VPg is essential for VPg activity. We also found a limited homology between PV VPg and the NTP-binding region in PVA VPg (Fig. 9). Most interestingly, the PV VPg in vivo and in vitro uridylylation function is also sensitive to the double mutation Lys-9 to Ala-9, Lys-10 to Ala-10 resulting in a nonviable virus phenotype (39). In addition, an NTP binding function was predicted by the same authors for the amino acids Tyr-3, Gly-5, Lys-9, Lys-10, and Arg-17 in PV VPg. Taken together, we propose that the NTP-binding site of VPg has an essential function in potyvirus infection. We also propose that the picorna-like superfAMILY of viruses share mechanistically similar stages in the initiation phase of replication.

Acknowledgments—We thank Dr. Roman Tuma for assistance with CD spectroscopy, Dr. Konstantin Ivanov for advice in protein purification and help with the figures in the manuscript, and Dr. Mikko Frilander for valuable discussions and shearing labeled nucleotides. We also thank Drs. Minna Rajamaki, Lesley Torrance, and Jari Valkonen for critical reading of the manuscript.

REFERENCES

1. Merits, A., Rajamaki, M.-L., Lindholm, P., Ruzenberg-Roos, P., Kekarojuhala, T., Puustinen, P., Makelainen, K., Valkonen, J. P. T., and Saarma, M. (2002) J. Gen. Virol. 83, 1211–1221
2. Sadowy, E., Milner, M., and Hanni, A. L. (2001) Adv. Virus Res. 57, 185–262
3. Ambros, V., and Baltimore, D. (1978) J. Biol. Chem. 253, 5263–5266
4. Rothberg, P. G., Harris, T. J. R., Nomoto, A., and Wimmer, E. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4868–4872
5. Orensuzbebarria, I., Guo, D., Merits, A., Makinen, K., Saarma, M., and Valkonen, J. P. T. (2003) Virology 312, 163–172
6. Carrington, J. C., Hadelman, R., Dolja, V. V., and Restrepo-Hartwig, M. A. (1995) J. Virol. 69, 6995–7000
7. Puustinen, P., Rajamaki, M.-L., Ivanov, K., Valkonen, J. P. T., and Makinen, K. (2002) J. Virol. 76, 12703–12711
8. Paul, V. A., Boom, J., Filipov, D., and Wimmer, E. (1998) Nature 393, 280–284
9. McKnight, K. L., and Lemon, S. M. (1996) J. Virol. 70, 1941–1952
10. Paul, V. A., Rieder, E., Kim, D. W., Boom, J. H., and Wimmer, E. (2000) J. Virol. 74, 10359–10370
11. Murray, K. E., and Barton, D. J. (2003) J. Virol. 77, 4729–4750
12. Murphy, J. F., Klein, P. G., Hunt, A. G., and Shaw, J. G. (1996) Virology 220, 535–538
13. Murphy, J. F., Ryvkhlik, W., Bhoads, R. A., Hunt, A. G., and Shaw, J. G. (1991) J. Virol. 65, 511–513
14. Schaad, M. C., Haldeman-Cahill, R., Cromin, S., and Carrington, J. C. (1996) J. Virol. 70, 7038–7048
15. Hong, Y., and Hunt, A. (1996) Virology 226, 146–151
16. Kamer, G., and Argos, P. (1984) Nucleic Acids Res. 12, 7269–7282
17. Li, X. H., and Carrington, J. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 457–461
18. Li, X. H., Valdez, P., Olivera, R. E., and Carrington, J. C. (1997) J. Virol. 71, 1598–1607
19. Fellers, J., Wan, J., Hong, Y., Collins, G. B., and Hunt, A. G. (1998) J. Gen. Virol. 79, 2043–2049
20. Hong, Y., Levay, K., Murphy, J. F., Klein, P. G., Shaw, J. G., and Hunt, A. G. (1995) Virology 214, 159–166
21. Merits, A., Guo, D., and Saarma, M. (1998) J. Gen. Virol. 79, 3123–3127
22. Merits, A., Guo, D., Jarvekel, L., and Saarma, M. (1999) Virology 263, 15–22
23. Cleriant, P., and Cuzin, F. (1982) J. Biol. Chem. 257, 6300–6305
24. Richards, O. C., Hanson, J. L., Schultz, S., and Ehrenfeld, E. (1995) Biochemistry 34, 6288–6295
25. Ivanov, K. I., Puustinen, P., Merits, A., Saarma, M., and Makinen, K. (2001) J. Biol. Chem. 276, 13530–13540
26. Caldentey, J., Blanco, L., Savilahti, H., Bamford, D. H., and Salas, M. (1992) Nucleic Acids Res. 20, 3971–3976
27. Crotty, S., Gohara, D., Gilligan, D. K., Karelisky, S., Cameron, C. E., and Andino, R. (2003) J. Virol. 77, 5378–5388
28. Machin, A., Martin Alonso, J. M., and Parra, M. (2001) J. Biol. Chem. 276, 27787–27792
29. Potash, S. J., Palant, O., and Gluzman, Y. (1989) J. Virol. 63, 216–225
30. Crawford, N. M., and Baltimore, D. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 7452–7455
31. Richards, O. C., and Ehrenfeld, E. (1997) J. Biol. Chem. 272, 23261–23264
32. Xiang, W., Cucunati, A., Hope, D., Kirkegaard, K., and Wimmer, E. (1998) J. Virol. 72, 6732–6741
33. Kekarojuhala, T., Savilahti, H., and Valkonen, J. P. T. (2002) Genome Res. 12, 584–594
34. Martin, M. T., Cervera, M. T., and Garcia, J. A. (1995) Virus Res. 37, 127–137
35. Restrepo-Hartwig, M. A., and Carrington, J. C. (1994) J. Virol. 68, 2388–2397
36. Rieder, E., Paul, V. A., Kim, D. W., van Boom, J. H., and Wimmer, E. (2000) J. Virol. 74, 10371–10380
37. Yin, Y. W., and Steitz, T. A. (2002) Science 298, 1387–1395
38. Butcher, S. J., Grimes, J. M., Makeyev, E. V., Bamford, D. H., and Stuart, D. I. (2001) Nature 410, 235–240
39. Paul, V. A., Peters, J., Muggrov, J., Yin, J., van Boom, J. H., and Wimmer, E. (2003) J. Virol. 77, 891–904
40. Notredame, C., Higgins, D. G., and Heringa, J. (2000) J. Mol. Biol. 302, 205–217

Fig. 9. Sequence homology between the NTP-binding region of PVA VPg and PV VPg. The NTP-binding region of PVA VPg containing amino acids 36–38 and the 22-amino acid-long VP VPg share a limited amino acid homology as was found by using the T-coffee method (www.ch.embnet.org/software/TCoffee.html (40)). Underlined residues within PVA VPg amino acid sequence form the VPg Δ38–44 deletion area. Identical amino acids are indicated with an asterisk and similar amino acids with double or single dots.
Uridylylation of the Potyvirus VPg by Viral Replicase N1b Correlates with the Nucleotide Binding Capacity of VPg

Pietri Puustinen and Kristiina Mäkinen

J. Biol. Chem. 2004, 279:38103-38110.
doi: 10.1074/jbc.M402910200 originally published online June 24, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402910200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 24 of which can be accessed free at http://www.jbc.org/content/279/37/38103.full.html#ref-list-1