We investigated the roles and biochemical properties of recombinant murine norovirus-1 (MNV-1) 3D\textsuperscript{pol} in RNA synthesis and virus genome-linked protein (VPg) nucleotidylylation. We therefore expressed VPg and 3D\textsuperscript{pol} of MNV-1 in \textit{Escherichia coli}. MNV-1 3D\textsuperscript{pol} exhibited RNA-dependent RNA polymerase (RdRp) activity \textit{in vitro} with poly(A) RNA as a template and MnCl\textsubscript{2} as a cofactor. MNV-1 3D\textsuperscript{pol} demonstrated optimum RNA-synthesis activity at pH 7.4 and 37°C in the absence of a primer. Further, VPg was guanylylated by MNV-1 3D\textsuperscript{pol} in the presence of MnCl\textsubscript{2} in a template-independent manner. The guanylylation reaction conducted with VPg substitution mutants (Y26F, Y40F, Y45F and Y117F) and a deletion mutant (D\textsubscript{117–124}) indicated that Tyr 117 was the probable target site of guanylylation. Homopolymeric RNAs did not enhance VPg guanylylation, whereas \textit{in vitro}-transcribed (−) subgenomic (SG) and (+)SG RNA enhanced VPg guanylylation by 9.2 and 3.2 times, respectively. Within (−)SG RNA, the (−)ORF3 region played a critical role in enhancing VPg guanylylation, suggesting that the MNV-1 ORF3 region of negative-strand RNA contains a \textit{cis}-acting element that stimulates 3D\textsuperscript{pol}-mediated VPg guanylylation.

INTRODUCTION

Members of the genus \textit{Norovirus} in the family \textit{Caliciviridae} are major causes of non-bacterial acute gastroenteritis worldwide (Green, 2007). Because of the lack of an efficient cell-culture system and an animal model to study virus propagation, the molecular mechanisms of human norovirus (HuNV) replication and its pathogenetic properties remain poorly understood. Molecular characterization of HuNV replication, therefore, has mostly been focused upon the recombinant forms of non-structural proteins. Murine norovirus-1 (MNV-1) isolated from immunocompromised mice (Karst \textit{et al.}, 2003) has been adapted to grow in the macrophage cell line RAW264.7 (Wobus \textit{et al.}, 2004). Thereafter, MNV-1 has served as a surrogate model system for elucidating the molecular modes of HuNV replication and pathogenicity (Wobus \textit{et al.}, 2006).

The MNV-1 genome is a single-stranded, positive-sense RNA of approximately 7.4 kb (Karst \textit{et al.}, 2003). A virus genome-linked protein (VPg) is predicted to be attached covalently to the 5′ end of the RNA, whilst a viral gene-derived poly(A) tail resides at the 3′ end (Daughenbaugh \textit{et al.}, 2003; Karst \textit{et al.}, 2003). In addition to the three open reading frames (ORFs) within the protein-encoding region, untranslated regions of unknown function reside at the 5′ and 3′ ends of the MNV-1 genome (Karst \textit{et al.}, 2003). ORF1 encodes a polyprotein that is processed cotranslationally into six mature proteins (N-term, NTPase, p18.6, VPg, Pro and Pol) by a virus-encoded protease (3C\textsuperscript{pro}) (Sosnovtsev \textit{et al.}, 2006). ORF2 and ORF3 encode VP1 and VP2, respectively. A 2.5 kb long polyadenylated subgenomic (SG) RNA containing ORF2 and ORF3 is also produced in MNV-1-infected cells (Wobus \textit{et al.}, 2004).

A virus-encoded RNA-dependent RNA polymerase (RdRp) plays a central role in the replication of the genomic RNA of RNA viruses. Recombinant forms of the uncleaved protease–polymerase (Pro–Pol) precursors of feline calicivirus (FCV) and HuNV (Belliot \textit{et al.}, 2005; Wei \textit{et al.}, 2001), along with mature RdKps of HuNV, rabbit hemorrhagic disease virus (RHDV) and sapovirus, exhibit RNA-synthesis activity \textit{in vitro} (Fukushi \textit{et al.}, 2004;
Fullerton et al., 2007; López Vázquez et al., 1998, 2001; Morales et al., 2004; Rohayem et al., 2006a, b).

As calicivirus genomic RNA does not have a cap or internal ribosome entry site at the 5' end, it has been predicted that calicivirus VPg may play a role in translation initiation. Evidence for the involvement of VPg in translation initiation has been provided by interactions between VPg and eIF3, eIF4E and eIF4GI (Chaudhry et al., 2006; Daughenbaugh et al., 2003, 2006; Goodfellow et al., 2005). Protein-primed RNA replication mediated by VPg has been described in poliovirus. A uridylylated VPg, VPg-pUpU, et al. (2005). VPg and eIF3, eIF4E and eIF4GI (Chaudhry et al., 2006; Daughenbaugh et al., 2003, 2006; Goodfellow et al., 2005). Protein-primed RNA replication mediated by VPg has been described in poliovirus. A uridylylated VPg, VPg-pUpU, produced in the reaction with UTP, 3Dpol, 3CD and cis-acting replication element (CRE) or poly(A) (Paul et al., 1998, 2000; Rieder et al., 2000), plays a critical role in the initiation of RNA replication by priming both negative- and positive-sense genomic RNA replication (Goodfellow et al., 2000; Morasco et al., 2003; Murray & Barton, 2003; van Ooj et al., 2006). Calicivirus VPg was nucleotidylylated by RHDV RdRp and HuNV Pro–Pol in a template-independent manner in the presence of MnCl2 in vitro (Belliot et al., 2008; Machin et al., 2001) whereas, in the presence of MgCl2, nucleotidylylation was only achieved after the addition of a poly(A)-tailed viral RNA template (Belliot et al., 2008). A Tyr at position 21 of RHDV VPg and a Tyr at position 27 in HuNV VPg are well-conserved; these are the target amino acids for nucleotidylylation (Belliot et al., 2001). Demonstration of nucleotidylylation of VPg led to the hypothesis that VPg could function as a primer for the initiation of RNA replication. The subsequent discovery of VPg-primed RNA synthesis in vitro by HuNV RdRp (Rohayem et al., 2006b) supports the proposed role of VPg as a primer during viral RNA replication. However, detailed information and direct evidence to support this hypothesis have not been obtained.

Although MNV-1 is a model system for elucidating the molecular mode of HuNV replication, the catalytic activity of MNV-1 3Dpol during RNA synthesis in vitro has not been characterized. In this study, we investigated the roles and biochemical properties of recombinant MNV-1 3Dpol in RNA synthesis and VPg nucleotidylylation. Our results indicated that MNV-1 3Dpol displays RdRp activity, initiates RNA synthesis with poly(A) as a template and requires MnCl2 as a cofactor. MNV-1 3Dpol guanylylated VPg Tyr117, and the reaction was stimulated greatly by the ORF3 region of MNV-1 negative-strand RNA.

**RESULTS**

**Expression and purification of recombinant MNV-1 3Dpol**

Proteolytic-cleavage sites of MNV-1 VPg and 3Dpol were mapped to E/G1178 and E/A995 and to Q/G1178 and E1687, respectively (Fig. 1a) (Sosnovtsev et al., 2006). We examined whether MNV-1 3Dpol has an essential role in RNA synthesis in vitro. To obtain large amounts of recombinant protein in *Escherichia coli*, cDNAs covering the entire coding regions of 3Dpol (Gly1178–Glu1687) and VPg (Gly871–Glu994) were cloned and expressed as a fusion protein with an N-terminal His6 tag. The His6 tag was flanked by an additional four (MGSS) and ten (SGLVRGSHM) amino acids derived from the vector required to introduce the translation-initiation codon and an Ndel site used for cloning (Fig. 1b). We placed the His6 tag at the N terminus of 3Dpol to avoid any possible interference from a C-terminal active-site cleft. His6-tagged recombinant proteins were purified with nickel–nitritolribo-acetic acid (Ni–NTA) column affinity chromatography. Analysis of the recombinant protein by SDS-PAGE indicated that 3Dpol was purified to >90 % homogeneity (Fig. 1c). The molecular masses of these proteins, estimated from the results of gel electrophoresis, were within the range of the calculated masses of the fusion proteins.

**MNV-1 3Dpol exhibited RdRp activity**

RNA-synthesis activity of recombinant MNV-1 3Dpol was initially analysed with 2.5 μCi (92.5 kBq) [α-32P]UTP; newly synthesized RNA was observed in a reaction carried out in the presence of template poly(A) RNA and MnCl2 (Fig. 2a, first column). The addition of rifampicin, a potent inhibitor of bacterial DNA-dependent RNA polymerase, did not inhibit MNV-1 3Dpol RNA-synthesis activity (Fig. 2a, second column), implying that any potential cellular enzymes co-purified with recombinant 3Dpol were not responsible for the observed RNA-synthesis activity. Under reaction conditions where any one of the components – i.e. MnCl2, template poly(A) RNA or 3Dpol (Fig. 2a, third to fifth columns, respectively) – was omitted, the amount of [α-32P]UMP incorporation was reduced to an undetectable level. Taken together, these results indicate that MNV-1 3Dpol possesses RdRp activity in vitro.

**Parameters affecting the RdRp activity of MNV-1 3Dpol**

We evaluated the effects of several parameters on the RNA-synthesis activity of MNV-1 3Dpol. MNV-1 3Dpol exhibited optimum RNA-synthesis activity at pH 7.4 and at 37 °C (Fig. 2b, c). An optimum temperature of 37 °C has also been observed for sapovirus 3Dpol (Fullerton et al., 2007), whilst the RdRps of HuNV and RHDV need an optimum temperature of 30 °C (Belliot et al., 2005; López Vázquez et al., 1998; Rohayem et al., 2006a). As the divalent-cation dependence of other virus-encoded RdRp activity is well-established, we investigated the effects of divalent cations on the RdRp activity of MNV-1 3Dpol. MNV-1 3Dpol displayed RdRp activity at 0.5–10.0 mM MnCl2, with the highest level of activity being observed at 2.5 mM (Fig. 2d). RNA synthesis was not detected in the presence of MgCl2 or ZnCl2 over a wide range of concentrations (0.1–10.0 mM; Fig. 2d). In contrast, most viral RdRps can adopt MgCl2 and MnCl2 as a cofactor for their activity; for example, sapovirus 3Dpol demonstrates flexibility to MgCl2 and MnCl2 (Fullerton et al., 2007).
VPg nucleotidylylation by MNV-1 3D<sup>pol</sup>

VPgs of RHDV and HuNV are nucleotidylylated by the corresponding RdRp in vitro, and the nucleotidyldated VPgs can prime RNA replication in HuNV (Belliot et al., 2008; Machin et al., 2001). In this context, we assessed whether MNV-1 VPg could also be nucleotidyldated by 3D<sup>pol</sup> in vitro. His<sub>6</sub>-tagged recombinant wild-type and mutant VPg were purified to >60% homogenity (Fig. 1d).

We initially examined a reaction containing [γ-<sup>32</sup>P]GTP, [γ-<sup>32</sup>P]ATP, [γ-<sup>32</sup>P]CTP or [γ-<sup>32</sup>P]UTP as the substrate.
nucleotide, MnCl₂ as the cofactor and in the absence of template RNA. MNV-1 VPg was nucleotidylylated in a template-independent manner, and GTP was the preferred nucleotide (Fig. 3a). The radioactive VPg band intensity was reduced when [α-³²P]UTP was the substrate nucleotide (Fig. 3a). This preference for GTP over UTP was also reported in a nucleotidylylation reaction of VPg catalysed by the HuNV Pro–Pol precursor (Belliot et al., 2008). When [α-³²P]ATP or [α-³²P]CTP was used as substrate, the band intensity of VPg was reduced further (Fig. 3a). We also observed a band at the 3Dpol position (Fig. 3a). Poliovirus 3Dpol and HuNV Pro–Pol were nucleotidylylated in a reaction catalysed by themselves via an unidentified mechanism; the significance of this reaction, including their potential role in intermediate nucleotide delivery to the final acceptor, VPg, remains to be clarified (Richards et al., 2006). No radioactive VPg band was observed in the reaction conducted using [α-³²P]GTP instead of [α-³²P]GTP (Fig. 3b), indicating that guanylylation on VPg involved an α-phosphate group of GTP.

We also measured VPg guanylylation under various reaction conditions and in the absence of template RNA. VPg guanylylation reached its highest level at 30 °C (Fig. 4a) rather than 37 °C, which was the optimum temperature for RdRp activity found in this study (Fig. 2c). VPg guanylylation catalysed by MNV-1 3Dpol occurred over a wide range of MnCl₂ concentrations, but no VPg guanylylation was detected with MgCl₂ (Fig. 4b). These results were consistent with our observations that MNV-1 3Dpol showed no detectable RdRp activity in a reaction containing MgCl₂ over a concentration range of 0.1–10.0 mM (Fig. 2d).

**Tyr¹¹⁷ of MNV-1 VPg is a probable target site for nucleotidylylation**

Poliovirus VPg is linked to the 5’ end of the viral RNA by a bond between Tyr⁷ and the terminal uridylic acid residues
In tobacco vein mottling virus (TVMV), VPg is linked to the viral RNA via Tyr60, and substitution of Tyr60 with Ala abolishes TVMV's infectivity of protoplasts (Murphy et al., 1991, 1996). Tyr27 in HuNV (Belliot et al., 2008) and Tyr21 in RHDV (Machín et al., 2001) of VPgs are the target amino acid residues for nucleotidylylation. We therefore assumed that one (or more) of the four Tyr residues in MNV-1 VPg (Fig. 5a) might be a target for nucleotidylylation. To identify the Tyr residue(s) of the MNV-1 VPg that could be nucleotidylylated by 3Dpol, we constructed mutants with Tyr at residues 26, 40, 45 and 117 by substitution with Phe. Recombinant VPgs were purified with Ni–NTA column affinity chromatography (Fig. 1d). Despite several independent attempts, we were unable to remove a fast-migrating protein that was co-purified with the Tyr mutants (Fig. 1d). All of the VPgs except the Tyr117 mutant were guanylylated (Fig. 5b, top), suggesting that Tyr117 is the target amino acid where GMP is attached covalently to VPg. To verify VPg nucleotidylylation on Tyr117, a VPg mutant with a deletion from Tyr117 to Glu124 (D117–124) was subjected to guanylylation. As shown in Fig. 5(b) (bottom), no radioactive VPg band was detected with the D117–124 mutant, indirectly supporting Tyr117 as a target site for guanylylation. Tyr117 was not expected to be the target amino acid for guanylylation, as Tyr27 in HuNV (Belliot et al., 2008) and Tyr21 in RHDV (Machín et al., 2001) were identified as the target amino acid residues for nucleotidylylation. Moreover, Tyr residues at this position are well-conserved in calicivirus VPgs (Fig. 5a).

The ORF3 region of MNV-1 negative-strand RNA stimulates guanylylation of VPg

In contrast to the poliovirus uridylylation reaction, where – in addition to 3CD and 3Dpol – either CRE or poly(A) template RNA are essential, RHDV, HuNV and potyvirus (Belliot et al., 2008; Machín et al., 2001; Puustinen & Mäkinen, 2004) RdRps do not require template RNA for VPg nucleotidylylation. However, the addition of poly(A)-tailed viral RNA template enhanced VPg nucleotidylylation in a reaction with HuNV Pro–Pol (Belliot et al., 2008). These observations prompted us to determine whether the addition of heteropolymeric RNA can enhance VPg nucleotidylylation by MNV-1 3Dpol. VPg nucleotidylylation was initially assessed in the presence of one of the four homopolymeric RNA templates. We did not observe radiolabelled nucleotidylylated VPg bands, indicating that the homopolymeric RNA template did not enhance VPg nucleotidylylation by MNV-1 3Dpol (data not shown).

Whilst the analysis of 3Dpol for the nucleotidylylation of VPg with homopolymeric RNA was considerably useful, it would be more relevant to test its activity with heteropolymeric viral RNA as a template. Full-length and SG RNA enhanced VPg nucleotidylylation, whereas ORF1-containing RNA deleted at the 3′-terminal region did not enhance VPg nucleotidylylation by HuNV Pro–Pol (Belliot et al., 2008).
et al., 2008). These results imply that any cis-acting sequence stimulating VPg nucleotidylation by HuNV Pro–Pol must reside within SG RNA. We therefore investigated the presence of any cis-acting sequence that would enhance VPg nucleotidylation existing within MNV-1 (+)SG and (−)SG RNA (Figs 1a and 6a). Luciferase (employed as a negative control), MNV-1 (+)SG and (−)SG RNA were prepared from T7 RNA.

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**Fig. 4.** Optimum conditions for MNV-1 VPg nucleotidylation. (a) Temperature-dependent VPg guanylylation activity. The reaction mixture was incubated at different temperatures (20, 30, 37 or 42 °C) for 3 h with 10 μM GTP, 2.5 μCi [γ-32P]GTP, 0.9 μg 3Dpol and 2 μg VPg in the absence of template RNA. The reaction was stopped by adding protein sample buffer and the proteins were denatured at 98 °C for 10 min. Proteins were separated by SDS-PAGE (15 % gel) and visualized by Coomassie brilliant blue staining. The stained gel was dried and [γ-32P]GMP-incorporated proteins were visualized with a phosphorimager. Nucleotidylylated 3Dpol and VPg are indicated by arrows. (b) Effect of divalent cations on VPg guanylylation activity. The reaction mixture was incubated at 37 °C for 3 h with 0.9 μg 3Dpol, 2 μg VPg and 0–5 mM MnCl₂ (left) or MgCl₂ (right). The reaction was stopped and analysed as described above.

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**Fig. 5.** Guanylylation of mutant VPgs. (a) Alignment of amino acid sequences of VPgs from viruses belonging to the family Caliciviridae (NCBI Entrez accession numbers: FCV, AAA79323; sapovirus, O6XDK8; RHDV, P27410; MNV-1, NC008311; HuNV, AAK50354). Four tyrosine residues of MNV-1 VPg (shaded), essential for nucleotidylylation and RNA replication in FCV Tyr²⁴, RHDV Tyr²¹ and HuNV Tyr²⁷ (asterisks), and the conserved Tyr residue (box) are indicated. Eight amino acids (Tyr₁₁⁷ – Glu₁₂⁴) deleted in the Δ₁₁⁷–₁₂⁴ mutant are underlined. (b) Guanylylation of mutant VPgs. The reaction mixture was incubated at 37 °C for 3 h with 2 μg wild-type or substitution mutant (Y26F, Y40F, Y45F or Y117F) VPgs (top) or with 2 μg deletion mutant (Δ₁₁⁷–₁₂⁴) VPg (bottom). The reaction was stopped by adding protein sample buffer and the proteins were denatured at 98 °C for 10 min. Proteins were separated by SDS-PAGE (15 % gel) and visualized by Coomassie brilliant blue staining. The stained gel was dried and [γ-32P]GMP-incorporated proteins were visualized by a phosphorimager.
polymerase in vitro transcription. The addition of (−)SG RNA greatly enhanced VPg guanylylation; (+)SG RNA also stimulated VPg guanylylation, but to a much lesser extent, whilst luciferase RNA did not stimulate any VPg guanylylation (Fig. 6b, lanes ‘G’; Fig. 6c). This result suggests that the observed enhancement was sequence-specific (Fig. 6b). To determine whether the type of NTP could affect the stimulation of VPg nucleotidylylation in the presence of (−)SG RNA, we performed identical experiments using only one of the four [α-32P]NTPs as a substrate. VPg uridylylation was enhanced to a lesser degree than guanylylation (Fig. 6b, lanes ‘U’); VPg adenylylation and cytidylylation were not enhanced to a detectable level (Fig. 6b, lanes ‘A’ and ‘C’). To locate the VPg guanylylation-stimulating site within (−)SG RNA, ORF2- or ORF3-deleted (−)SG RNA (Fig. 6a) was subjected to the VPg guanylylation reaction. VPg guanylylation was enhanced by the addition of (−)ORF3-3'UTR-poly(A), whereas (−)3'UTR-poly(A) did not enhance the reaction (Fig. 6d). Taken together, these results suggest that the ORF3 region of MNV-1 negative-strand RNA may contain a cis-acting element that stimulates 3Dpol-mediated VPg guanylylation.

**DISCUSSION**

Studies on norovirus RNA replication have focused on the functions of non-structural proteins in vitro, due to the lack of a tissue-culture system and a small-animal model. MNV-1 propagated in the macrophage-derived RAW293.7 cell line (Wobus et al., 2004) is a surrogate system for investigating norovirus replication. As both Pol (3D) and its uncleaved precursor Pro–Pol (3CD) of HuNV MD145-2 exhibit RdRp activity (Belliot et al., 2005) and 3Dpol is the predominantly observed form in MNV-1-infected cells...
(Sosnovtsev et al., 2006), we cloned and expressed 3DPoL to investigate its in vitro RNA-synthesis activity. We cloned a His6 tag at the N terminus of the recombinant 3DPoL because, in norovirus, the C terminus of the protein is located within the active-site cleft (Ng et al., 2004). To exclude the possible interaction of a C-terminal His tag with the active-site cleft, the His tag was placed at the N terminus of the recombinant sapovirus 3DPoL (Fullerton et al., 2007). It should be noted, however, that the histidine residues cloned at the N terminus of MD145-2 Pol had an inhibitory effect on its RdRp activity (Belliot et al., 2005).

We only observed MNV-1 3DPoL-dependent RNA synthesis in the presence of a poly(A) template and MnCl2. To exclude the possible interaction of a C-terminal His tag with the active-site cleft, the His tag was placed at the N terminus of the recombinant sapovirus 3DPoL (Fullerton et al., 2007). It should be noted, however, that the histidine residues cloned at the N terminus of MD145-2 Pol had an inhibitory effect on its RdRp activity (Belliot et al., 2005). We only observed MNV-1 3DPoL-dependent RNA synthesis in the presence of a poly(A) template and MnCl2.

As the RdRps of caliciviruses require divalent cations to express their catalytic activity (Belliot et al., 2005; Fullerton et al., 2007; López Vázquez et al., 2001; Rohayem et al., 2006a, b; Wei et al., 2001), the activity of RdRp in other caliciviruses relies on Mg2+ or Mn2+ (Belliot et al., 2005; Fullerton et al., 2007; López Vázquez et al., 2001; Rohayem et al., 2006a; Wei et al., 2001); moreover, the RdRps of RHDV and HuNV exhibit a higher efficiency in the presence of Mg2+ (López Vázquez et al., 2001; Rohayem et al., 2006a). An RHDV RdRp mutant within the YGDD of motif C, in which the first D is replaced with E, has been noted to exhibit enzyme activity in the presence of MnCl2 under conditions in which no activity was observed with magnesium acetate (López Vázquez et al., 2000). Polioviruses containing a mutation at Asn297 of RdRp are dependent on Mn2+ for RNA replication and growth (Crotty et al., 2003). Hepatitis C virus RdRp requires Mn2+ for de novo initiation, but requires Mg2+ for primer extension (Ranjith-Kumar et al., 2002). These observations suggest that, although most RNA polymerases require Mg2+ as a cofactor, there is some flexibility in their requirement for divalent metal ions and, occasionally, they prefer Mn2+ over Mg2+. Plants generally contain significant intracellular stores of Mn2+, and it has therefore been suggested that certain RdRps of plant viruses would possess a requirement for Mn2+ rather than Mg2+ (Crotty et al., 2003).

The Pro–Pol precursor of HuNV catalyses VPg nucleotidylylation (Belliot et al., 2008), and both Pro–Pol and Pol of RHDV can nucleotidylylate VPg (Machin et al., 2001, 2009). In order to address whether MNV-1 VPg is also nucleotidylylated by 3DPoL in vitro, His6-tagged recombinant VPg was expressed in E. coli. MNV-1 VPg was nucleotidylylated in a template-independent manner and GTP was the preferred nucleotide (Fig. 3a, lane 1). The optimum temperature was 37 °C for RNA synthesis (Fig. 2c) and 30 °C for VPg nucleotidylylation (Fig. 4a).

Comparison of the amino acid sequences of MNV-1 and HuNV MD145-2 VPg demonstrated that the four Tyr residues in MNV-1 VPg are conserved in HuNVs. Alignment of the amino acid sequences of calicivirus VPgs revealed that MNV-1 Tyr26, RHDV Tyr21, HuNV Tyr27, and FCV Tyr24 are well-conserved and are located at the same position (Fig. 5a). Nucleotidylylation studies on amino acid substitution and deletion mutants of VPg indicated that RHDV Tyr21 (Machin et al., 2001), HuNV Tyr27 (Belliot et al., 2008) and FCV Tyr24 (Mirata et al., 2004) play essential roles in this reaction. The four Tyr residues of MNV-1 VPg were replaced with Phe and their nucleotidylylation by 3DPoL was examined. Unlike RHDV and HuNV, the Tyr26 mutant continued to be nucleotidylylated, but the Tyr117 mutant was not. This result was supported further by nucleotidylylation performed with Δ117–124 VPg, where Tyr117–Glu224 was deleted (Fig. 5b). These data indicated that Tyr117 is essential for the incorporation of radioactive GMP into MNV-1 VPg.

The VPg uridylylation reaction of poliovirus is known to require a CRE sequence in the 5′ region of genomic RNA; further, VPg uridylylation is essential for viral RNA replication (Goodfellow et al., 2000). No enhancement of RHDV VPg uridylylation was observed after poly(A), genomic RNA, antisense genomic RNA or SG RNA was added to the reaction (Machin et al., 2001). However, VPg uridylylation catalysed by the HuNV Pro–Pol precursor was stimulated by the addition of the ORF3-3′-NTR-poly(A) template (Belliot et al., 2008). This observation led us to investigate whether the ORF3-3′-NTR-poly(A) sequence, carrying (+)SG or (−)SG RNA, stimulated the VPg nucleotidylylation reaction catalysed by MNV-1 3DPoL. Therefore, (+)SG and (−)SG RNAs were prepared by T7 RNA polymerase-mediated in vitro transcription by using PCR products as template DNA. We demonstrated that VPg nucleotidylylation was enhanced by the addition of negative-strand viral RNA. VPg guanylylation by MNV-1 3DPoL was stimulated greatly by the addition of (−)SG RNA and (−)ORF3-3′UTR-poly(A) RNA, but not by (−)3′UTR-poly(A) RNA (Fig. 6b–d). Our results suggest strongly that cis-acting sequences residing within the ORF3 region of negative-strand RNA are involved in the nucleotidylylation process catalysed by MNV-1 3DPoL in vitro.

**METHODS**

**Plasmid construction.** MNV-1 RNA was extracted from RAW264.7 cells infected with MNV-1 (kindly provided by Herbert W. Virgin, IV, Washington University School of Medicine, St Louis, MO, USA) as described by Hsu et al. (2005) with minor modifications. MNV-1 cDNA was synthesized by Moloney murine leukemia virus reverse...

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transcriptase (Bioneer). The cDNAs encoding VPg (nt 2616–2987) and 3Dpol (nt 3537–5069) were amplified by PCR using the cDNA synthesis reaction product as a template, primer sets for VPg (M-VPg-F and M-VPg-R) or 3Dpol (M-Pol-F and M-Pol-R) (see Supplementary Table S1, available in JGV Online) and AccuPower HF PCR PreMix (Bioneer), according to the manufacturer’s instructions. The PCR products were then cloned into the pCR2.1-TOPO vector (Invitrogen). The resulting recombinant pCR2.1-TOPO vectors were digested with NdeI and BamHI and cloned into the E. coli expression vector pET-14b (Novagen) previously digested with the corresponding restriction enzymes. The recombinant plasmids were designated pET-VPg-WT and pET-Pol-WT primers for the N-terminal half or M-VPg-R and VPg-Y26F-R primers for the C-terminal half (Supplementary Table S1), and Pfu polymerase (Vivagen). After agarose gel electrophoresis, both fragments were isolated by gel extraction. cDNA spanning the entire coding sequence of the VPg Y26F mutant was amplified by PCR using both N- and C-terminal DNA fragments, M-VPg-F and M-VPg-R primers and AccuPower HF PCR PreMix (Bioneer), and cloned into pET-14b as described above. The plasmid was designated pET-VPg-Y26F. The other VPg mutants were generated in a similar manner with specific primers (Supplementary Table S1) and cloned into pET-14b.

**In vitro transcription.** Template DNA, used for *in vitro* transcription of MNV-1 RNAs, was amplified by PCR using pMNV-SG as a template, T7-SG-F and MNV-R primers for the UTR-poly(A) (Supplementary Table S1), and AccuPower HF PCR PreMix (Bioneer). The reaction was stopped by adding an equal volume of 200 mM EDTA (pH 8.0); then, 8 µl reaction mixture was spotted onto DE81 filter paper (Whatman). The filter paper was dried at room temperature for 10 min and washed three times with 2 ml 2 × SSC solution for 10 min. Finally, the filter paper was dehydrated with 2 ml absolute ethanol and dried at 80 ºC. The radioactivity of incorporated [γ-32P]NMP was measured with a liquid scintillation counter (Wallac 1407).

**VPg nucleotidylylation assay.** The nucleotidylylation assay was performed as described by Belliot *et al.* (2008) with some modifications. The reaction was carried out for 3 h at 37 ºC in a 20 µl reaction mixture containing 50 mM HEPES (pH 7.4), 2.5 mM MnCl2, 10 mM DTT, 1 µg template RNA, 10 µM GTP, 2.5 µCi [γ-32P]GTP (3000 Ci mmol−1, 10 mCi ml−1), 2 µg VPg and 1.8 µg 3Dpol. The divalent-cation concentrations, template RNAs, type of VPg, temperature and type of radioisotope used are indicated in the figure legends. The reaction was stopped by adding 5 µl 5 × protein sample buffer, and the reaction products were separated by SDS-PAGE (15% gel) and visualized by Coomassie brilliant blue staining. The gel was dried and nucleotidylylated VPg bands were visualized with a phosphorimagery (BAS1000). To quantify [γ-32P]NMP incorporated into VPg, VPg bands were excised from the gel and radioactivity was measured in a liquid scintillation counter.

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