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Catalytic Core of Alphavirus Nonstructural Protein nsP4 Possesses Terminal Adenylyltransferase Activity

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The RNA-dependent RNA polymerase nsP4 is an integral part of the alphavirus replication complex. To define the role of nsP4 in viral RNA replication and for a structure-function analysis, we expressed Sindbis virus nsP4 in *Escherichia coli*. The core catalytic domain of nsP4 (Δ97nsP4, a deletion of the N-terminal 97 amino acids), which consists of the predicted polymerase domain containing the GDD amino acid motif required for viral RNA synthesis, was stable against proteolytic degradation during expression. Therefore, the recombinant core domain and selected mutants were expressed and purified to homogeneity. We determined that Δ97nsP4 possesses terminal adenylyltransferase (TATase) activity, as it specifically catalyzed the addition of adenine to the 3′ end of an acceptor RNA in the presence of divalent cations. Furthermore, Δ97nsP4 is unable to transfer other nucleotides (UTP, CTP, GTP, and dATP) to the acceptor RNA in the absence or presence of other nucleotides. Δ97nsP4 possessing a GDD-to-GAA mutation completely inactivates the enzymatic activity. However, a GDD-to-SNN mutation did not inactivate the enzyme but reduced its activity to ~45% of that of the wild type in the presence of Mg^{2+}. Investigation of the TATase of the GDD-to-SNN mutant revealed that it had TATase equivalent to that of the wild type in the presence of Mn^{2+}. Identification of Δ97nsP4 TATase activity suggests a novel function of the alphavirus RNA-dependent RNA polymerase in the maintenance and repair of the poly(A) tail, an element required for replication of the viral genome.

Sindbis virus (SINV), the type species of the *Alphavirus* genus, is a plus-sense, single-stranded RNA virus. Its 11.7-kb genome is capped at the 5′ end and polyadenylated at the 3′ end (51). The 5′-terminal two-thirds of the genomic RNA encodes four nonstructural or replicase proteins. These nonstructural proteins are directly synthesized from the genomic RNA as two overlapping polyproteins, P123 and P1234. P1234 is produced by readthrough of a UGA termination codon present between the sequences encoding nsP3 and nsP4, which occurs 5 to 20% of the time during translation (30, 50, 51). The protease domain of nsP2 processes the polyproteins into various intermediates and individual nonstructural proteins (20). Nonstructural proteins together with an unknown host factor(s) constitute the alphavirus replication complex (51). The RNA-dependent RNA polymerase (RdRp), nsP4, which contains the signature GDD motif of viral RNA polymerases (24, 25), is activated by cleavage from nascent P1234 to form an initial P123/nsP4 replication complex (47). This complex uses the genomic RNA as a template for the synthesis of minus-strand RNA. P123 is further processed at the 1/2 junction to produce an nsP1/P23/nsP4 complex, capable of synthesizing both minus-strand and 49S genomic RNAs. Finally, polyprotein P23 is cleaved at the 2/3 junction to produce a replication complex consisting of fully processed nonstructural proteins, which is responsible for the synthesis of 26S subgenomic mRNA and genomic RNA. The synthesis of minus-strand RNA ceases at about 4 h postinfection, while the plus-strand and subgenomic RNA synthesis continues throughout the remainder of the alphavirus life cycle (28, 44, 45, 47).

The N terminus of nsP4 in all sequenced alphaviruses has a conserved Tyr residue that is destabilizing according to the N-end rule pathway in both eukaryotic and prokaryotic cells, and therefore, nsP4 is rapidly degraded in infected cells (2, 11). However, the function of nsP4 in the replication complex is dependent upon the N-terminal residue, which must be an aromatic amino acid (Tyr, Trp, or Phe) or His (48). The N-terminal ~100 amino acids of nsP4 have no sequence similarity with other viral RdRps and may be required for interactions with nonstructural proteins or unidentified host factors in the replication complex (12, 27, 49). The C-terminal region (~500 amino acids) of nsP4 is assumed to contain the polymerase catalytic core due to sequence similarities with other polymerases, the presence of conserved polymerase motifs, secondary structure predictions, and various temperature-sensitive mutations mapping in this region of nsP4 that cause defects in RNA synthesis (3, 15, 23, 37). A highly conserved GDD sequence in the nsP4 catalytic core constitutes polymerase motif C of the "palm" domain in all classes of polymerases. In other polymerases, the two conserved aspartate residues of motif C coordinate two divalent metal ions, which in turn act catalytically or bind phosphate (1, 17, 37). The GDD polymerase motif of alphavirus nsP4 is replaced by SDD in influenza virus polymerase, by GDN in the polymerases of *Mononegavirales*, and by MDD in retrovirus reverse transcriptase (4). Mutagenesis studies of poliovirus polymerase, in which GDD was replaced by GDN (22), and of coliphage Qβ polymerase, in which GDD was replaced by ADD (21), resulted in an altered metal ion requirement for polymerase activity, illustrating the importance of motif C for metal binding by viral RdRps.

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The 3′ end of the alphavirus genome consists of a polyadenylated tract preceded by a 19-nucleotide conserved sequence element (3′ CSE). The 3′ CSE serves as the promoter for minus-strand synthesis during alphavirus genome replication (19, 26). At least 11 adenylate residues adjacent to the 3′ CSE are required for efficient synthesis of full-length minus-strand RNA, suggesting a role for poly(A) in alphavirus viral RNA replication (19). The susceptibility of poly(A) to degradation by cellular exonucleases and its role in alphavirus replication point to the importance of maintenance and repair of the 3′ end of the genome. The existence of a 3′-end-labeled repair pathway has been proposed because a SINV genome lacking a poly(A) tail is repaired by regeneration in vivo (41). Other RNA viruses, for example, turnip crinkle virus, clover yellow vein virus, cymbidium ringspot tombus virus, and cucumber mosaic virus satellite RNA, have also developed a mechanism by which they repair the 3′ end of their viral RNA genomes (9, 10, 14, 52). In addition, a role for viral RdRp in the 3′-end-labeled repair pathway has been suggested for RNA viruses (35).

Previous studies indicated that the minus-strand RNA of alphaviruses contains a poly(U) sequence at the 5′ end (13, 43). Based on the presence of this poly(U) at the 5′ end of the minus strand, it was proposed that the poly(A) tract is synthesized in the genomic and subgenomic RNAs by direct transcription of the complementary poly(U) sequence in the minus-strand RNA (13, 43). However, recent studies demonstrate that the predominant 5′ initiation site for minus-strand RNA synthesis in alphaviruses utilizes the cytidylate residue immediately preceding the poly(A) tail (18). This finding is in conflict with the previously proposed mechanism for polyadenylation of viral RNAs and suggests that the virus requires an activity for a nontemplate-dependent specific addition of adenylate residues to the 3′ end of the plus-sense viral genome. mRNA processing and the addition of poly(A) in eukaryotes usually occur in the nucleus (6), whereas the alphavirus genome replicates in the cytoplasm of infected cells (53). Therefore, it is unlikely that the host cell polyadenylation machinery adds a poly(A) tail to the alphavirus genome. Polyadenylation seems more likely to be a function of the alphavirus replicase proteins and is supported by evidence that the poliovirus polymerase $3D^{pol}$ has terminal adenylyltransferase (TATase) activity and that the RNA polymerases of *Flaviviridae* possess 3′ terminal nucleotidyltransferase (TNTase) activities (36, 42).

In this study, we report expression and purification of a recombinant core catalytic domain of the SINV RdRp, nsP4. Divalent cation-dependent TATase was identified in nsP4 enzymatic assays using purified protein and RNA. Mutation of the GDD motif to GAA yielded an inactivated protein and confirmed that the core catalytic domain of nsP4 possesses TATase activity. The role of the GDD motif in metal ion coordination was further investigated by mutation of GDD to SNN, which reduced the TATase activity to ~45% of that of the wild type in the presence of Mg^{2+} ions, whereas activity in the presence of Mn^{2+} ions was not affected.

MATERIALS AND METHODS

Construction of expression plasmids. For all plasmid constructions, pToto64 (wild-type) SINV genomic cDNA was used as a template for DNA amplification using PCR (38). The full-length nsP4 and various N-terminal truncations (residues 1 to 48, 1 to 57, 1 to 72, 1 to 84, 1 to 97, 1 to 106, 1 to 115, 1 to 129, 1 to 140, and 1 to 148) were constructed by PCR amplification of the corresponding DNA fragments. Restriction sites NcoI and XhoI were incorporated into the 5′ end of the forward and reverse primers, respectively. PCR products were digested with NcoI and XhoI and ligated into the *Escherichia coli* expression vector pET28a (Novagen) to express nsP4 with a C-terminal histidine tag (His_6) under the control of the T7 RNA polymerase promoter. Site-specific mutations of the polymerase GDD motif in the nsP4 protein were introduced by using the QuikChange site-directed mutagenesis kit (Stratagene). Nucleotide changes resulting in amino acid substitution were introduced into synthetic oligonucleotides that were used as primers for PCR.

Production of nsP4 in *Escherichia coli*. Plasmids were transformed into *E. coli* expression strain Rosetta (DE3) (Novagen). Bacterial cultures were grown in Luria broth (LB) supplemented with 50 μg/ml kanamycin and 35 μg/ml chloramphenicol at 37°C to an optical density at 600 nm of 0.4. The temperature was then reduced to 19°C, and growth was continued until an optical density at 600 nm of 0.8, when expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were grown for ~16 h at 19°C. Cells were harvested by centrifugation at 5,000 × *g* at 4°C, and pellets were stored at −20°C.

Purification of recombinant nsP4. All purification steps were carried out at 4°C. Frozen cell pellets from a 1-liter culture were resuspended in 40 ml binding buffer (20 mM Tris-HCl, pH 7.8, 40 mM imidazole, 300 mM NaCl) and lysed by sonication (pulse, 20 s on and 20 s off; total, 10 min). The cell lysate was centrifuged at 15,000 × *g* at 4°C for 40 min. The clarified extract was loaded onto a 5-ml HiTrap chelating column (GE Healthcare) charged with $NiSO_4$ and washed with binding buffer containing 1 M NaCl. Recombinant nsP4 eluted at ~150 mM imidazole in a 40 to 500 mM imidazole gradient in 20 mM Tris-HCl (pH 7.8) and 500 mM NaCl. nsP4 was dialyzed against 20 mM Tris-HCl (pH 7.8), 25 mM NaCl, 5% glycerol, 5 mM dithiothreitol (DTT), and 2 mM EDTA, loaded onto an anion exchange (5-ml HiTrap Q HP column; GE Healthcare), and eluted with a 50-ml linear gradient of 25 mM to 500 mM NaCl in dialysis buffer. Fractions containing nsP4 were concentrated to 10 mg/ml using Amicon Ultra (Millipore) and loaded onto a size exclusion column (Superdex 200 HR 10/30; GE Healthcare) preequilibrated with 20 mM Tris-HCl (pH 7.8), 200 mM NaCl, 5% glycerol, and 5 mM DTT. Purified nsP4 was concentrated to 10 mg/ml and stored at −70°C. Protein concentrations were determined using a Bio-Rad protein assay kit.

nsP4 polymerization assay. RNAs were synthesized chemically and high-performance liquid chromatography purified by Integrated DNA Technologies, Inc. (Coralville, IA). RNA containing 3′ dideoxycytidine (ddC3′ RNA) was synthesized chemically and polyacrylamide gel electrophoresis (PAGE) purified by Dharmacon, Inc. (Lafayette, CO). The integrity of RNAs was monitored by gel electrophoresis and autoradiography of samples that were 5′ end labeled with [γ-^{32}P]ATP. The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}), assuming that A_{260} equals 1 for 40 μg/ml (40).

The reaction was performed in a 50-μl volume with 100 pmol of purified recombinant nsP4 and 25 pmol of acceptor oligoribonucleotide in a reaction buffer containing 50 mM Tris-HCl (pH 8.5), 5.0 mM $MgCl_2$, 75 mM KCl, 10 mM DTT, 20 U of RNase inhibitor (Promega), 0.5 μg of actinomycin D, and 10.0 μCi of [α-^{32}P]ATP (400 Ci/mmol) (GE Healthcare) with or without 100 μM each of CTP, UTP, and GTP. The reaction mixtures were incubated at 25°C for 45 min and quenched by the addition of 10 mM Tris-HCl (pH 8.5) and 10 mM EDTA. RNA products were precipitated overnight at −20°C by the addition of 7.5 μg of GlycoBlue (Ambion), 1/10 volume of 1 M NaOAc (pH 5.0), and 2.5 volumes of ice-cold 95% ethanol. The RNA pellet was resuspended in loading dye containing 80% formamide, 10% glycerol, 1 mM EDTA, 0.025% bromophenol blue, and 0.25% xylene cyanol. After heat denaturation, samples were loaded and resolved on a 12.5% acrylamide, 9 M urea, and 0.5× TBE (44.5 mM Tris base, 44.5 mM boric acid, and 1 mM EDTA) gel. Radiolabeled RNA products were visualized by autoradiography. The quantification of the products was performed using a phosphorimager (Personal Molecular Imager FX; Bio-Rad) and Quantity One software.

RESULTS

Expression and purification of Δ97nsP4. Full-length SINV nsP4 was produced in *E. coli* but could not be purified due to degradation problems. Western blot analysis of the partially purified and degraded full-length nsP4, using antibodies against SINV nsP4 or against the C-terminal His tag, revealed

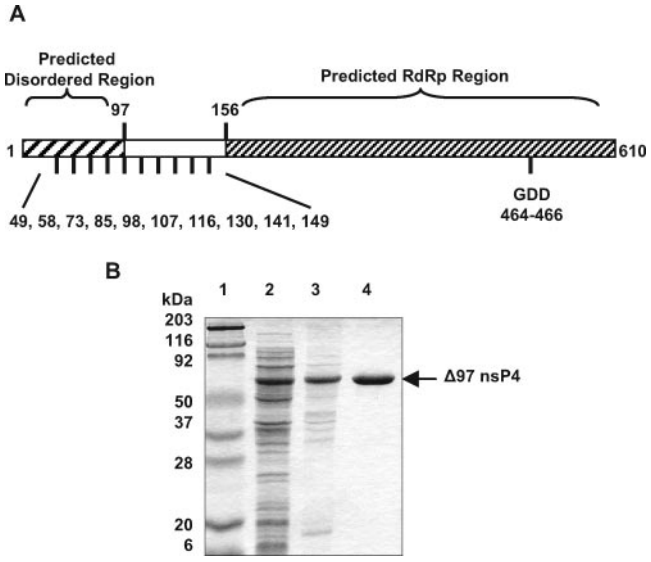

FIG. 1. Design, expression, and purification of the core domain of nsP4. (A). Schematic diagram of the nsP4 sequence, showing the disordered N-terminal domain and the core catalytic polymerase domain. The N-terminal truncation initiation sites are represented by vertical bars. (B). Sodium dodecyl sulfate-PAGE gel stained with Coomassie blue. Lane 1, molecular mass standards (kDa); lane 2, soluble fraction of *E. coli* lysate containing Δ97nsP4; lane 3, insoluble fraction of the *E. coli* lysate; lane 4, purified Δ97nsP4.

that the N terminus was not stable due to protease sensitivity (data not shown). Bioinformatics analysis of nsP4 was consistent with the observed N-terminal degradation as a disordered region and was predicted in the N terminus using the FoldIndex server (39). Few secondary structures were predicted for this region using the JPRED server (8), and the predicted RdRp domain begins at approximately residue 156 (31). Ten N-terminal truncations of nsP4 were designed based on our observation of degradation and these predictions (Fig. 1A). The engineered N-terminal truncations of nsP4 had a histidine tag (His_6) at the C terminus. Expression of the truncated constructs and solubility of the engineered proteins were tested by culturing *E. coli* containing the expression plasmid at various temperatures (37°C, 25°C, and 19°C) and were induced with 0.4 mM IPTG. All 10 constructs were expressed, but Δ97nsP4 (deletion of first 97 residues) was the only protein produced in soluble form (Fig. 1B, lanes 2 and 3). Recombinant Δ97nsP4 was purified in three steps, with yields of ~15 mg per liter of *E. coli* culture. Elution profiles from the gel filtration column demonstrated that Δ97nsP4 exists as a monomer. The purified protein exhibited a single band in a sodium dodecyl sulfate-PAGE gel (Fig. 1B, lane 4). The identity of the preparation was confirmed by N-terminal amino acid sequencing.

Identification of TATase activity of Δ97nsP4. In an attempt to characterize the activity of the core catalytic domain of nsP4, we performed an RNA synthesis assay using purified Δ97nsP4 and a 45-nucleotide (nt) template RNA corresponding to the 3′ end of the SINV genome adjacent to the poly(A) tail (3′ RNA) (Table 1). Products migrating slower than the input RNA were generated in the assay (Fig. 2A, lane 5). These products are 47, 48, and 49 nt in length compared to a 45-nt RNA ladder used as a marker. The possibility that the observed activity was due to a contaminating bacterial protein was eliminated by mutation of the conserved RdRp GDD motif of Δ97nsP4 to GAA. No products were produced by the GAA variant of Δ97nsP4 (Fig. 2A, lane 6), confirming that the observed activity is an inherent property of Δ97nsP4.

The reaction products could be due to the addition of nontemplated terminal nucleotides to the 3′ end of the input RNA or to newly synthesized RNA produced as a result of polymerase activity by de novo initiation. To distinguish de novo polymerase activity from TNTase activity, the incorporation of each α-^{32}P-labeled nucleotide (UTP, CTP, GTP, and ATP) in the presence of all other nucleotides was examined. De novo polymerase activity is expected to incorporate nucleotides from all four input nucleoside triphosphates (NTPs), given the sequence of the 45-nt input template, 3′ RNA. However, no radiolabeled products were detected from α-^{32}P-labeled UTP, CTP, GTP, and dATP (Fig. 2B, lanes 3, 4, 5, and 6). Products higher in molecular weight than the input RNA were detected only in the presence of ^{32}P-labeled ATP (Fig. 2B, lane 2). Thus, we concluded that the products are not the result of a copying activity but that these higher-molecular-weight products could be a result of terminal nucleotide transferase activity. Another important conclusion from the above-described experiment was that Δ97nsP4 preferentially adds ATP and not UTP, CTP, or GTP.

We performed the assay in the absence of cold NTPs and in the presence of a single species of [α-^{32}P]NTP to confirm the TNTase activity of Δ97nsP4. Only [α-^{32}P]ATP was added to the input acceptor RNA, whereas [α-^{32}P]UTP, [α-^{32}P]CTP, or [α-^{32}P]GTP was not incorporated into the acceptor RNA (Fig. 2C). Additions of higher concentrations (up to 10 μM) of unlabeled NTPs also did not show transferase activity for these nucleotides. The use of higher concentrations of unlabeled ATP in the reaction had no effect on the length of products. Thus, the TATase activity of Δ97nsP4 was identified because α-^{32}P-labeled adenylate residues were specifically added to the input RNA. [α-^{32}P]dATP was also tested for incorporation, but no products were detected (Fig. 2C).

TABLE 1. Sequences of oligonucleotides used in the TATase assay

Template	Sequence (5′ to 3′)	G+C content (%)
3′ RNA	UAUUAUUUCUUUUAUUAAUCAACAAAAUUUUGUUUUUAACAUUUC	13.3
ddC3′ RNA	UAUUAUUUCUUUUAUUAAUCAACAAAAUUUUGUUUUUAACAUUUddC	13.3
U3′ RNA	UAUUAUUUCUUUUAUUAAUCAACAAAAUUUUGUUUUUAACAUUUU	11.1
5′ RNA	AUUGACGGCGUAGUACACACUAUUGAAUCAAACAGCCGACCAAUU	42.0
Rdm3′ RNA	UUUUGAUAUUACUAUUUUCUCUUAAAAACAUUAUUCUUUUAUUAA	13.3
3′ DNA	TATTATTTCTTTTATTAATCAACAAAATTTTGTTTTTAACATTTC	13.3

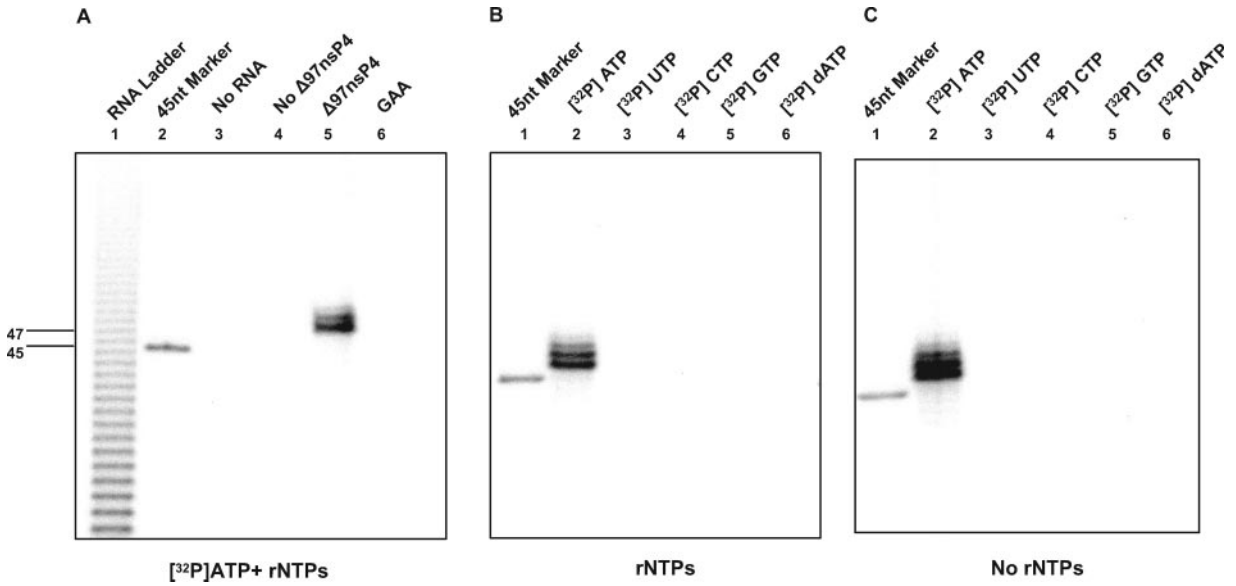

FIG. 2. TATase activity of Δ97nsP4. The 45-nt 3′ RNA oligonucleotide was used in the assay, and products were resolved on a 12.5% polyacrylamide-9 M urea-TBE gel. (A) Δ97nsP4-specific activity. Reactions were performed in the presence of [α-^{32}P]ATP and rNTPs. Lane 1, RNA ladder made by alkaline hydrolysis of a poly(A) homopolymer 5′ labeled with [γ-^{32}P]ATP using polynucleotide kinase; lane 2, 45-nt marker made by labeling 3′ RNA oligonucleotide with [γ-^{32}P]ATP using polynucleotide kinase. In lane 3, no products were produced in the absence of input RNA. In lane 4, no products were produced without the addition of Δ97nsP4, and in lane 5, Δ97nsP4 produced products with estimated sizes of 47 nt, 48 nt, and 49 nt that migrated slower than the input 3′ RNA (45 nt). In lane 6, Δ97nsP4 with the GDD-to-GAA mutation did not produce products. (B) Requirement of ATP. Each reaction was done using a single radiolabeled nucleotide (0.5 μM), which is shown above the autoradiogram, in the presence of other nucleotides (100 μM). Lane 1, 45-nt marker. Lane 2 contains the products produced in the presence of [α-^{32}P]ATP and rNTPs. In lanes 3, 4, 5, and 6, no products were produced in the reactions with [α-^{32}P]UTP, [α-^{32}P]CTP, [α-^{32}P]GTP, or [α-^{32}P]dATP, respectively, in the presence of all other nucleotides. (C) Adenylation-only activity. The reactions were done using only radiolabeled nucleotides (0.5 μM) in the absence of other nucleotides. Lane 1, 45-nt marker. In lane 2, the products produced in the reaction with only [α-^{32}P]ATP. In lanes 3, 4, 5, and 6, no products were produced in the reactions with only [α-^{32}P]UTP, [α-^{32}P]CTP, [α-^{32}P]GTP, or [α-^{32}P]dATP, respectively.

Determination of optimal conditions for TATase activity. Various parameters, such as pH, temperature, ionic strength, and divalent metal ion, were investigated to determine optimal conditions for the TATase activity of Δ97nsP4. The pH of the reaction was varied from 4.5 to 10.5. The optimal pH for nsP4 TATase activity is between pH 8.0 and 9.0, with maximum activity at pH 8.5 (Fig. 3A). Temperature was varied from 0°C to 40°C, with optimal activity at 25°C (Fig. 3B). Ionic strength was investigated by varying the concentration of KCl from 0 mM to 300 mM. KCl was not absolutely required, but the TATase activity was greatest at ~75 mM KCl (Fig. 3D). In contrast, divalent cation is absolutely required for the activity, with maximal activity at Mg^{2+} concentrations above 2 mM (Fig. 3C). To test whether Mg^{2+} could be substituted by another divalent cation, assays were performed in the presence of 5 mM and 10 mM concentrations of the following divalent cations: Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+}. Maximum activity was observed with the Mg^{2+} ion. The Mn^{2+} ion supported catalysis at ~60% of the levels observed with Mg^{2+}. Catalytic turnover with Ca^{2+} was about fourfold lower than that with Mg^{2+}. The other metals were much less effective (Table 2).

Substrate requirement for TATase activity. We expected that nontemplated nucleotides were added to the 3′ end of the input RNA. This was tested by use of a substrate with a dideoxycytidine (ddC3′ RNA) (Table 1) at its 3′ end in place of a cytidine nucleotide at the 3′ terminus in the parent RNA substrate, 3′ RNA. This RNA with a 3′ dideoxycytidylate is not capable of acting as a substrate for adenylation. ddC3′ RNA did not produce radiolabeled products, confirming that nucleotide incorporation is at the 3′ end of the input RNA (Fig. 4A, lane 3). The sequence specificity of the TATase substrate was tested with several input nucleic acid 45-mers (Table 1): U3′ RNA in which cytidine at the 3′ end was changed to uridine; 5′ RNA with a sequence corresponding to the 5′ end of the SINV genome; and Rdm3′ RNA, which has a random RNA sequence and the same nucleotide content as in the 3′ RNA. Adenine was incorporated into all of these input RNAs irrespective of sequence (Fig. 4B). Therefore, we concluded that Δ97nsP4 TATase activity is not sequence specific but does require RNA because no activity was detected with a single-stranded DNA substrate (3′ DNA) (Table 1) having a sequence identical to the 3′ end of genome RNA (Fig. 4B, lane 7). The low activity observed for the 5′ RNA could be due to its higher G+C content, which may confer greater stability on secondary structures of 5′ RNA (Table 1) than other RNA substrates.

Altered metal ion requirement of Δ97nsP4 with GDD motif mutation. The GDD polymerase motif is postulated to be involved in divalent metal ion coordination, and mutations of this motif in other RdRps modify their divalent cation preferences (21, 22, 37). Mutation of GDD to GAA abolishes Δ97nsP4 TATase activity (Fig. 2A and 5). We hypothesized that the mutations at the GDD motif may change the levels of

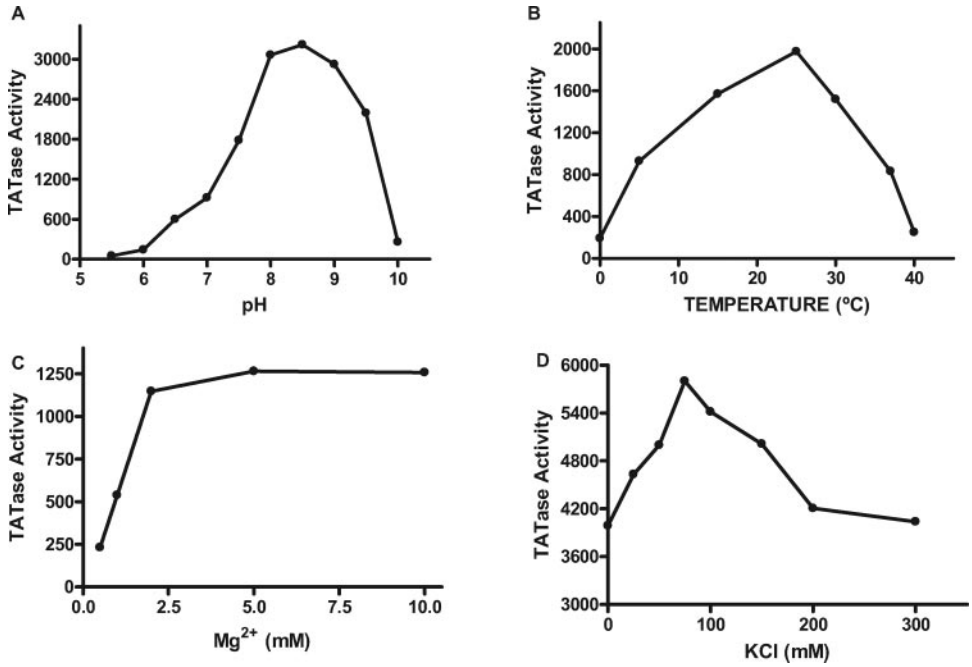

FIG. 3. Determination of optimal conditions for the TATase activity of Δ97nsP4. Incorporation of radiolabel is plotted as a function of (A) pH in the range of 5.5 to 10.0, (B) temperature in the range of 0°C to 40°C, (C) Mg^{2+} ion concentration in the range of 1 to 10 mM, and (D) KCl concentration in the range of 1 to 300 mM.

TATase activity or alter the divalent cation preferences. Substitution of GDD with SNN reduced TATase activity by ~55% in the presence of 5 mM Mg^{2+} compared to the activity of the wild-type protein (Fig. 5). Higher concentrations of Mg^{2+} did not increase the activity of the mutant enzyme.

In contrast to Mg^{2+}-supported catalysis, the SNN mutant of Δ97nsP4 in the presence of 5 mM Mn^{2+} ion had TATase activity almost equivalent to that of the wild type (Fig. 5). These data demonstrate that the GDD-to-SNN mutation alters the TATase divalent metal ion requirement. The SNN mutation decreased TATase activity in the presence of magnesium but had no effect in the presence of manganese.

DISCUSSION

In this study, we report the expression and purification of a recombinant catalytic core of the alphavirus nonstructural pro-

TABLE 2. Quantification of Δ97nsP4 TATase activity in the presence of various divalent cations[a]

Metal	% Activity at ion concn	
	5 mM	10 mM
None	0	0
Mg^{2+}	100	98
Mn^{2+}	60	58
Ca^{2+}	25	27
Co^{2+}	<5	<5
Ni^{2+}	<5	<5
Cu^{2+}	<5	<5
Zn^{2+}	<5	<5
Fe^{2+}	<5	<5

[a] Values are percentages of the activity at 5 mM Mg^{2+}, which is set at 100%.

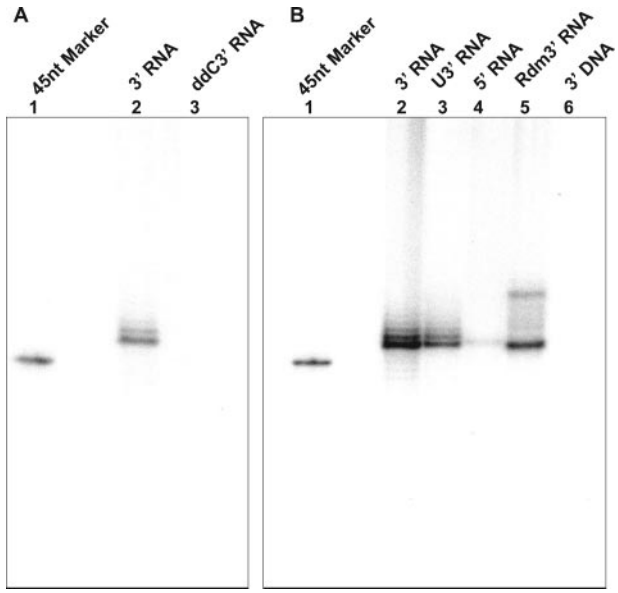

FIG. 4. Template requirements for TATase activity. The acceptor RNAs used in the reaction are shown above the autoradiogram. (A) 3′-specific polyadenylation. Lane 1, 45-nt marker made by labeling 3′ RNA oligonucleotide with [γ-^{32}P]ATP using polynucleotide kinase; lane 2, reaction performed using 3′ RNA; lane 3, reaction performed using ddC3′ RNA with a 3′ end blocked with dideoxycytidine. (B) Lack of sequence specificity for Δ97nsP4 TATase. Lane 1, 45-nt marker made by labeling 3′ RNA oligonucleotide with [γ-^{32}P]ATP using polynucleotide kinase; lanes 2, 3, 4, 5, and 6, autoradiogram of products produced in the reactions performed using the oligonucleotides 3′ RNA, U3′ RNA, 5′ RNA, Rdm3′ RNA, and 3′ DNA, respectively.

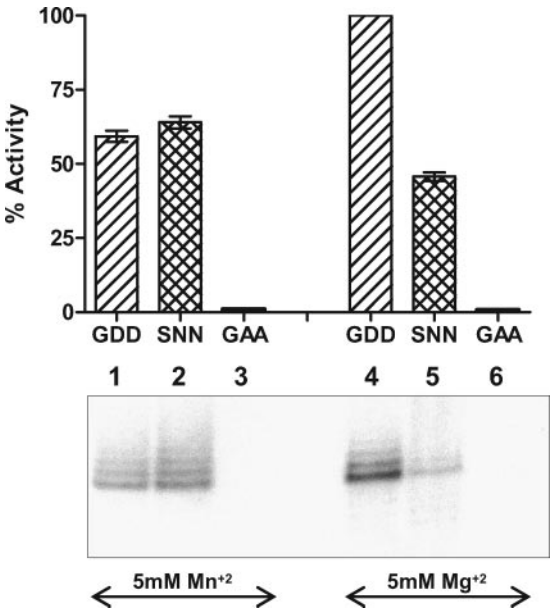

FIG. 5. SNN Δ97nsP4 variant with an altered metal ion requirement. TATase activity of wild-type and mutant enzymes in the presence of 5 mM Mn^{2+} (lanes 1 to 3) and 5 mM Mg^{2+} (lanes 4 to 6). Quantitation of the reactions in each lane of the autoradiogram is shown above the individual lanes. Activities of the wild type and mutants are relative to the activity of the wild type in the presence of 5 mM Mg^{2+} (100%). The plot represents the average of three measurements. Lanes 1 and 5, wild-type Δ97nsP4 (GDD); lanes 2 and 6, Δ97nsP4 GDD-to-SNN mutation (SNN); lanes 3 and 7, Δ97nsP4 GDD-to-GAA mutation (GAA).

tein, nsP4. To our knowledge, this is the first report of the purification and characterization of enzymatic activity of recombinant nsP4. We demonstrate that Δ97nsP4 specifically adds nontemplated adenylate residues at the 3′ end of input RNA and, therefore, possesses TATase activity. While we cannot rule out the possibility that the TATase activity observed in vitro does not occur in vivo, the adenine specificity of the reaction argues that it is not simply a reflection of the polymerase activity of nsP4.

The biological relevance of the observed Δ97nsP4 TATase activity is consistent with the fact that many viral RdRps exhibit a nontemplated addition of nucleotides to acceptor RNAs, and these activities have been proposed to play a role in the maintenance or repair of the 3′ end of viral genomes (14). Similar to alphaviruses, the poliovirus genome carries a 3′ poly(A) tail and the poliovirus polymerase ($3D^{pol}$) also manifests TATase activity, which is proposed to play a role in the initiation of poliovirus plus-strand RNA synthesis (36). In contrast, flaviviruses, which lack a poly(A) tail, have been reported to exhibit TNTase activity instead of TATase activity (42).

The 19-nt conserved sequence element (3′ CSE) at the 3′ end of the alphavirus genome serves as the promoter for minus-strand RNA synthesis (19, 26, 29). Previous studies also suggest a role for the 3′ CSE in the interaction of host factors to regulate minus-strand synthesis (26). Viral genome replication requires the 3′ CSE and at least 11 adenylate residues in the poly(A) tail for efficient synthesis of full-length minus-strand RNA (19). Thus, the poly(A) tail, which is postulated to play a role in the initiation of translation and the stability of cellular mRNA, has an integral role in alphavirus viral RNA replication (2, 16, 19, 33). Recent findings indicate that the 5′ end of the minus strand does not possess an extensive poly(U) sequence (18), which was previously proposed to serve as a template for synthesis of the poly(A) tail at the 3′ end of the plus-strand genomic and subgenomic RNAs (13, 43). The new result implies that an additional step in viral RNA replication is required to produce polyadenylated plus-strand genomic RNA. The mechanism of the addition of a poly(A) tail to the alphavirus genome is likely to be different from that for cellular poly(A) addition, based on the evidence that the average length of the alphavirus poly(A) tract is ~70 nt (13) compared to the 200 to 250 nt in cellular mRNAs (34, 46) and the absence of a *cis*-acting polyadenylation signal (AAUAAA) in the 3′ noncoding region essential for binding of polyadenylation factors and subsequent polyadenylation (16). The present study demonstrates that nsP4 has the necessary specific TATase activity to specifically add adenylate residues at the 3′ end of the RNA genome.

Additional evidence for the biological relevance of the nsP4-associated TATase activity is suggested by the requirement for the 3′ end repair mechanism that was proposed by Raju et al. (41). Those investigators found that the poly(A) tail was rapidly regenerated in vivo when a genomic RNA without a poly(A) tail was transfected into cells. Additionally, rubella virus, another member of the *Togaviridae* family, also regenerates its poly(A) tract in vivo, indicating that the virus has a mechanism to repair and maintain the 3′ end of its genome (7). Identification of a core domain of SINV nsP4 possessing TATase activity indicates a role for nsP4 in the establishment and restoration of a poly(A) tail in alphavirus RNAs.

nsP4 specifically incorporated adenylate residues at the 3′ end of the RNA, and the products formed were 2 to 4 nt longer than the 45-nt RNA substrate. The addition of more than one adenylate residue supports the hypothesis that polyadenylation of the genomic and subgenomic RNAs is one function of the alphavirus polymerase. The function of the RdRp in the polyadenylation of RNA is not unexpected because the poly(A) polymerases responsible for the addition of a poly(A) tail to mRNAs are structurally homologous to the pol X superfamily that includes DNA polymerase β and nucleotidyltransferases (3, 5, 32, 33). Although the average length of poly(A) in alphaviruses is ~70 nt (13), the purified Δ97nsP4 in the TATase adds only two to four adenylate residues, suggesting a requirement for another viral or host factor(s).

In the in vitro assays, the Δ97nsP4 TATase also lacks sequence specificity for the input RNA as it adds adenylate to several RNA oligonucleotides. The lack of sequence specificity suggests that additional viral or cellular factors that are present in the replication complex or residues 1 to 97 of nsP4 may be required for specific recognition of a polyadenylation signal in the genomic and subgenomic RNAs.

TATase activity is an inherent property of Δ97nsP4, as was confirmed by testing the enzymatic activity of purified Δ97nsP4 containing mutations in the conserved polymerase GDD motif. Mutation of both aspartate residues in the GDD motif to alanine resulted in a complete loss of TATase activity. However, mutation of GDD to SNN did not result in complete loss of enzymatic activity. Instead it was reduced to ~45% of the

wild-type level in the presence of Mg^{2+} ions. This result can be explained by the diversity of the "GDD" motif among viral RNA polymerases. The motif is GDN in *Mononegavirales* polymerases, SDD in influenza virus polymerase, and MDD in retrovirus reverse transcriptase (4). The aspartate residues of the polymerase GDD motif have been shown to be involved in metal ion coordination. Poliovirus polymerase with GDN substituted for GDD resulted in a polymerase that was active in the presence of Mn^{2+} (22). Therefore, we tested the TATase activity of the SINV SNN mutant in the presence of Mn^{2+} and, as expected, it was active. SNN Δ97nsP4 had no difference in Mn^{2+}-supported activity compared to the wild-type activity. Moreover, SNN Δ97nsP4 had activity comparable with that of either Mn^{2+} or Mg^{2+}. This suggests a role for the GDD motif in alphavirus RdRps in metal ion coordination similar to that reported for other RNA polymerases.

We performed enzymatic assays to characterize the de novo and polymerase activities of Δ97nsP4 but were unable to detect the expected products. One possibility could be that other viral replicase proteins, which together with nsP4 form the replication complex, are required for initiation and template-dependent RNA synthesis. In addition, the conserved N-terminal tyrosine residue, which is required for virus viability, is missing in Δ97nsP4 and might be required for polymerase activity. The apparently disordered N-terminal domain may also play a role in this activity.

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