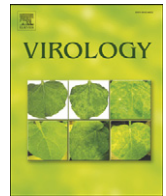




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Molecular characterization of genome segment 2 encoding RNA dependent RNA polymerase of *Antheraea mylitta* cytoplasmic polyhedrosis virus

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ABSTRACT

Genome segment 2 (S2) from *Antheraea mylitta* cypovirus (AmCPV) was converted into cDNA, cloned and sequenced. S2 consisted of 3798 nucleotides with a long ORF encoding a 1116 amino acid long protein (123 kDa). BLAST and phylogenetic analysis showed 29% sequence identity and close relatedness of AmCPV S2 with RNA dependent RNA polymerase (RdRp) of other insect cypoviruses, suggesting a common origin of all insect cypoviruses. The ORF of S2 was expressed as 123 kDa soluble His-tagged fusion protein in insect cells via baculovirus recombinants which exhibited RdRp activity in an *in vitro* RNA polymerase assay without any intrinsic terminal transferase activity. Maximum activity was observed at 37 °C at pH 6.0 in the presence of 3 mM MgCl₂. Site directed mutagenesis confirmed the importance of the conserved GDD motif. This is the first report of functional characterization of a cypoviral RdRp which may lead to the development of anti-viral agents.

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Introduction

Antheraea mylitta cytoplasmic polyhedrosis virus (AmCPV) is one of the most widespread pathogens of Indian non-mulberry silkworm, *A. mylitta*. Almost 20% crop is damaged annually due to this virus attack (Jolly et al., 1974). A large number of CPV-infected *A. mylitta* larvae develop chronic diarrhea that eventually leads to a condition known as “Grasserie” and the death of the larvae (Jolly et al., 1974). CPV belongs to the genus *Cypovirus* and family *Reoviridae* (Mertens et al., 2005; Payne and Mertens, 1983). CPV infects the midgut of the wide range of insects belonging to the order Diptera, Hymenoptera and Lepidoptera (Bellonick and Mori, 1998). Viral infection is often characterized by the production of large number of occlusion body called polyhedra in the cytoplasm of infected cells. CPV genome is composed of 10 double stranded RNA segments (S1–S10) (Payne and Mertens, 1983), although a small 11 segment (S11) has been reported in some cases such as *Trichoplusia ni* cytoplasmic polyhedrosis virus (TnCPV-15) (Rao et al., 2000) and *Bombyx mori* cytoplasmic polyhedrosis virus (BmCPV) (Arella et al., 1988). Each dsRNA segment is composed of a plus strand mRNA and its complementary minus strand in an end to end base pair configuration except for a protruding 5' cap on the plus strand. Among the viruses of the family *Reoviridae*, complete nucleotide sequences of double stranded RNA genome have been reported for the members of the genera *Orthoreovirus*, *Rotavirus*,

Orbivirus, *Phytoreovirus*, *Coltivirus*, *Oryzavirus*, *Seadornavirus*, *cypovirus* and putative members of *Fijivirus* (Attoui et al., 2005a,b; Cowled et al., 2009; Duncan, 1999; Estes and Cohen, 1989; Graham et al., 2008; Hagiwara et al., 2001, 2002; Mertens et al., 2005; Nakashima et al., 1996; Rao et al., 2000; Suzuki, 1995).

RNA dependent RNA polymerases (RdRp) of the different members of *Reoviridae* family have been characterized. Genome segment 1 of Bluetongue virus and African horse sickness virus (members of the genus *Orbivirus*), Rice gall dwarf virus (members of the *phytoreovirus*), Colorado tick fever virus (members of the genus *Coltivirus*), Fiji disease virus (member of the genus *Fiji virus*) and human rotavirus codes for VP1 protein having RdRp activity (Attoui et al., 2000; Boyce et al., 2004; Lu et al., 2008; McQualter et al., 2003; Tao et al., 2002; Vreede and Huismans, 1998; Zhang et al., 2007). In BTV, VP1 can synthesize dsRNA from a viral positive strand RNA template in the absence of any other viral protein (Roy, 2008; Urekawa et al., 1989) whereas in rotavirus VP1 catalyzes RNA synthesis with the help of core shell protein VP2 (Lu et al., 2008; Wehrfritz et al., 2007).

In case of cypovirus, RdRps encoded by genome segment 2 of BmCPV, *Lymantria dispar* CPV (LdCPV), and *Choristoneura occidentalis* CPV (CoCPV) have been cloned (Jing-Chena et al., 2003; Graham et al., 2008; Rao et al., 2003) but their sequences shows little homology and none of them have been functionally characterized.

We have previously characterized the structure of AmCPV by electron microscopy and its genome by electrophoresis which reveals that it is similar to that of a type-4 CPV (Qanungo et al., 2000) and consists of 11 dsRNA molecules. We have also reported that the genome segments 6, 7, 8, and 10 of AmCPV encodes viral structural

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proteins p68 (having ATP binding and ATPase activity), p61, p60 and polyhedrin, respectively, while segment 9 encodes a nonstructural protein, NSP38, having RNA binding property (Chavali and Ghosh, 2007; Chavali et al., 2008; Jangam et al., 2006; Qanungo et al., 2002; Sinha-Datta et al., 2005). Segment 11 of AmCPV does not contain any ORF (Jangam et al., 2006) and its function remains unclear. Other genome segments of this virus (S1–S5) have not been cloned, sequenced and characterized at molecular level. Here, we report molecular cloning, sequencing and expression of AmCPV S2 and show by functional analysis that it encodes viral RdRp which catalyzes RNA synthesis without the help of any other viral protein.

Results and discussion

Genetic analysis of AmCPV S2

AmCPV S2 RNA was isolated, converted to cDNA and cloned into pCR-XL-TOPO and the total nucleotide sequence was determined in both forward and reverse directions. Sequencing of AmCPV S2 showed that it consisted of 3798 nucleotides with a single long ORF of 3351 nucleotides, which could encode a protein of 1116 amino acids. The ORF in the cDNA started with an ATG codon at nucleotide 30 and terminates in a TGA codon at nucleotide 3380. Twenty-nine nucleotides upstream of the start codon and four hundred and eighteen nucleotides downstream of stop codon were present as untranslated sequences. The molecular mass of the encoded protein was deduced as ~123 kDa. BLAST analysis showed that nucleotide sequence of AmCPV S2 was 29% identical with RdRp of CoCPV, LdCPV and BmCPV encoded by their genome segment 2 and allows to conclude that AmCPV S2 could also encode viral RdRp. This is supported by the MotifScan result which showed the presence of characteristic signature motifs for the RdRp of members of the *Reoviridae*: the conserved GDD motif at positions 681–683 and GKQXGXXXD motif at positions 526–534 (Li et al., 2007). In addition, comparison of the sequences of AmCPV S2 encoded RdRp with those of poliovirus, rabbit hemorrhagic disease virus (RHDV), reovirus, and hepatitis C virus (HCV) showed the presence of catalytic motif A (with conserved aspartates separated by four residues), motif B (with conserved “XSG” sequence), motif C (with conserved “XDD” sequence), motif D (with conserved hydrophilic E and K residue) in the palm sub domain, as well as motif F (with submotifs, F1, F2 and F3 containing conserved positively charged, basic residues K or R) in the finger sub domain (Fig. 1). It also contained motif E (with hydrophobic residues) positioned between the palm and thumb subdomains and motif G (with conserved “SXG” sequences) to form loop and alpha-helix structure (Candress et al., 1990; Poch et al., 1989; Xu et al., 2003; O'Reilly and Kao, 1998).

The deduced amino acid composition resulted in an isoelectric point of 8.53 and showed that this protein is rich in Leucine (9.9%), Alanine (7%), Isoleucine (6.8%) and Lysine (5.8%) residues. Four potential N-linked glycosylation sites (at positions 94–96, 123–125, 248–250, 781–783) and several phosphorylation sites were found within the protein coding region. Secondary structure prediction using PHD and GOR4 programs (Rost and Sander, 1994) showed that 45.55% of the residues are likely to form random coils, 38.47% would form α -helices and 15.98% would form extended sheets and devoid of transmembrane signal peptides. At the 5' and 3' end of AmCPV S2, AGTAAT and AGAGC sequences were found, respectively, as conserved sequences as observed at the 5' and 3' ends of other AmCPV genome segments such as S6, S7 and S10 (Chavali and Ghosh, 2007; Chavali et al., 2008; Sinha-Datta et al., 2005), indicating the genome structure of this CPV might follow the same pattern as observed in other CPVs.

Phylogenetic comparison of AmCPV RdRp sequences with cognate genes of twenty seven other viruses in the *Reoviridae* family showed its close relatedness with some members of cypovirus such as CoCPV,

BmCPV-1, DpCPV and LdCPV (Fig. 2) and indicates that all these cypoviruses may have originated from a common ancestral insect virus.

Analysis of recombinant AmCPV S2 encoded protein expressed in *Escherichia coli* and Insect cells

To produce recombinant AmCPV S2 encoded RdRp, initially the entire ORF of S2 was expressed in *E. coli* via pQE-30 vector as a 6X His-tagged fusion protein. Analysis of sonicated bacterial pellet (in PBS) and supernatant by SDS-PAGE showed expression of AmCPV S2 encoded protein in *E. coli* as insoluble form (data not shown) and may be due to improper folding of expressed protein in bacteria. Bacteria were then lysed in buffer containing 8 M urea to solubilize expressed insoluble protein, and purified through Ni-NTA affinity chromatography. Analysis of this protein by SDS-8% PAGE (Fig. 3A) showed a 123 kDa protein band. Polyclonal antibody was raised in a rabbit against this purified protein, affinity purified and the final concentration was determined as 1.5 mg/ml. The titer of purified antibody was determined by ELISA as 10,000 using 2.5 μ g of antigen, and used for immunoblot analysis. Since *E. coli* produced this protein as insoluble inclusion bodies, wild type recombinant and mutant AmCPV S2 encoded proteins were expressed in soluble form as His-tagged fusion protein in *Sf* 9 cells via baculovirus expression system and purified through Ni-NTA chromatography. The production of wild type recombinant and mutant (GDD-GAD and GDD-GAA) proteins as 123 kDa band was confirmed by immunoblot analysis using raised polyclonal antibody (Fig. 3B). This indicates that due to proper folding of expressed protein inside the insect cells, soluble protein have been produced and to our knowledge this is the first report of the production of any cypoviral RdRp in soluble form.

Primer dependent RNA synthesis by AmCPV S2 encoded protein using poly (A) RNA template

In order to determine the RdRp activity of insect cell expressed and purified AmCPV S2 encoded protein, RdRp assay was performed using homopolymeric poly (A) RNA template in the absence or presence of Oligo U₍₁₈₎ primer. As shown in Table 1, large amounts of [α ³²P] UTP were incorporated (13.48 pmol/ μ g/h) in presence of wild type AmCPV S2 encoded protein but not in the absence of the protein. This indicates RdRp activity of AmCPV S2 encoded protein which is slightly higher than that of HCV (9.41 pmol/ μ g/h) (Yamashita et al., 1998) and SARS coronavirus (10.9 pmol/ μ g/h) (Cheng et al., 2005). [α ³²P] UTP incorporation was almost undetectable in the absence of the primer and established the primer dependent polymerization activity of the AmCPV S2 encoded RdRp. No significant [α ³²P] UTP incorporation was detected when Oligo (dT) primer was used in place of Oligo U₍₁₈₎ in the reaction. Also the activity was not inhibited by Actinomycin D (50 μ g/ml), a compound which forms complex with DNA and interferes with RNA synthesis indicating that the measured activity is not caused by contaminating DNA dependent DNA polymerase activity present in insect cell extract.

Optimal conditions for RNA synthesis by AmCPV S2 encoded protein

To optimize the conditions for *in vitro* RdRp activity of expressed protein, RdRp assays were performed with different amount of insect cell expressed soluble protein. As shown in Fig. 4A, RdRp activity increased with increasing concentrations of protein up to 0.4 μ M and then attained a plateau. In another separate experiment, *in vitro* synthesized AmCPV S2 3' positive strand RNA was used as template in the assay and analyzed by gel electrophoresis. The data (band intensities of 28.72, 43.89, 54.32, 50.72, 49.8 and 49 for the enzyme concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.8 μ M, respectively) showed maximum RdRp activity up to 0.4 μ M concentration of

1

MFRYDSVKWNTFDTISAHIIIDSYENTFAFNKFGKLPENSYWKNDFKPVSYRELLDTNFRSENIIGTISECR
 RDFQFILKPRPFNRDFYPENANKTDFKLDRLPYNYLASFSLKSYAQPVPKSNSESDFEHKLFGQITALIEL
 YQFQTRGGDIWRVISSILWELDDYYNTQNHIVRHILYHILEPYTSAPFYQTHNSFKFYLEDHVDVISILPCMI
 YMLGSIGVDIIAGVLHTSTAVCAWNNYVYFNSDSIFDTYLKHKSDLTSWFEPKIASLGVQVRVPRFSLYGELI
 EAGVVDCSQDKIFAKLHKDTLDDYPETSAKCLRRLVSDCTTKEDTIRALLLCRTVSNTRLYLATLLELKGKE
 AGSGARTEGQLVIPPVPTKITISRNAIVELKFPQSVQDFPKMLAYLKNFIPQVLAAIKK-419

	423	432	442	452	462				
AmCPV	VNFNQK	FIDM	LTTRSSG	QKIRNAD	YSS	KL	VNQAS	SQVRVNA	..AITA
polio	QMCLED	AMYGTD	GLEALD	LSAGY	PPYVAM	GKKKR	DI	LNKQ
RHDV	NLNFKA	AANTLD	LSTSCG	PFV	GKKID	HV	KDGV
reovirus	PLFQA	IMRSQY	VTAR	GGSG	AA	LR	ESLYA	INVSL
HCV	LLSVEE	ACKLTP	PHSAKS	..KFGY	AKDVR	LN	SSKA

Motif G

	472	482	492	502	512				
AmCPV	DKFAHE	SIQDIAI	RMT	..RAG	VRFQI	GRRAR	QI	FITS
polio	YGIN	LPLVTY	..VKDE	ELRS	..	KTKE	VEQ	GKS
RHDV	WSVAN	SGK	ALHHI	YACGL	KDEL	RP	..	LDKV
reovirus	AL	LADT	SMGL	LRNQV	QRRP	RS
HCV	DT	V	PIDT	TTMA	KN	VE	FCVQ	PE

Motif F1 Motif F2 Motif F3

	522	532	542	552	562	572	582
AmCPV	Y AHL	..	GKQ	GGV	TDFATA	L	ATTTE
polio	PGVIT	..	GS	AVG	CD	PDL	FWSK
RHDV	ARFGP	IAV	G	VD	M	T	SRD
reovirus	MNLS	P	TS	GSA	V	IE	KVI
HCV	VMGSS	..	Y	GFQ	Y	SP	Q

Motif A

	592	602	612	622	632	642
AmCPV	SFLT	KRE	PVK	DL	E	F
polio	R	V	D	Y
RHDV	E	L	T	K
reovirus	I	G	K	P	M	G
HCV	P	E	A	R

	652	662	672	682	692
AmCPV	G	S	G	L	A
polio	P	S	G	C	S
RHDV	P	S	G	M	P
reovirus	P	S	G	S	A
HCV	A	S	G	V	L

Motif B

Motif C

	702	712	722	732	742	752	762
AmCPV	N	E	L	A	L	E	Q
polio	V	D	A	S	L
RHDV	M	V	S	L	L
reovirus	E	T	I	Q	N	L	E
HCV	Q	E	D	A	S	L	R

Motif D

Motif E

763

FQLFSDLANRAIDVDRLSNFTFVKAFTSRRLTVSATPANIKKLSQSVKPDYGHACKIKILPIIERETVRRGR
 VEKKMQKVQITLPLSWLVHGGGELPSPALQRKDLTFTSPKSLYGPRGHYCNRIIFDLCDQDMELDHELLKAY
 NFDVGHALAQVNMVGFRLGRRKRIDRKEIKNMSHLLTHGDSTAI EASEKAYRQLLVNIKVPENHVYAYQT
 ENKIYEALMAPEVDKFEDVLIPTDFVSLIRKRSPI SDVNMKAHRYTINKIPDEPRMELLYPFLGECVQPN
 SKYSTSWMLMQMFGFMTSEKVERMSTLSVITGKYGLFKVGD SQFKEAMRIYRQSPDYLDVFF-1116

Fig. 1. Amino acid sequence alignment of the AmCPV S2 encoded RdRp with that of polio virus (PDB code 1RDR), RHDV (PDB code 1KHV), reovirus (PDB code 1N35), HCV (PDB Code 1QUV). Conserved motifs are highlighted with box and marked below. The alignment is done to show the equivalent position of the different signature motifs.

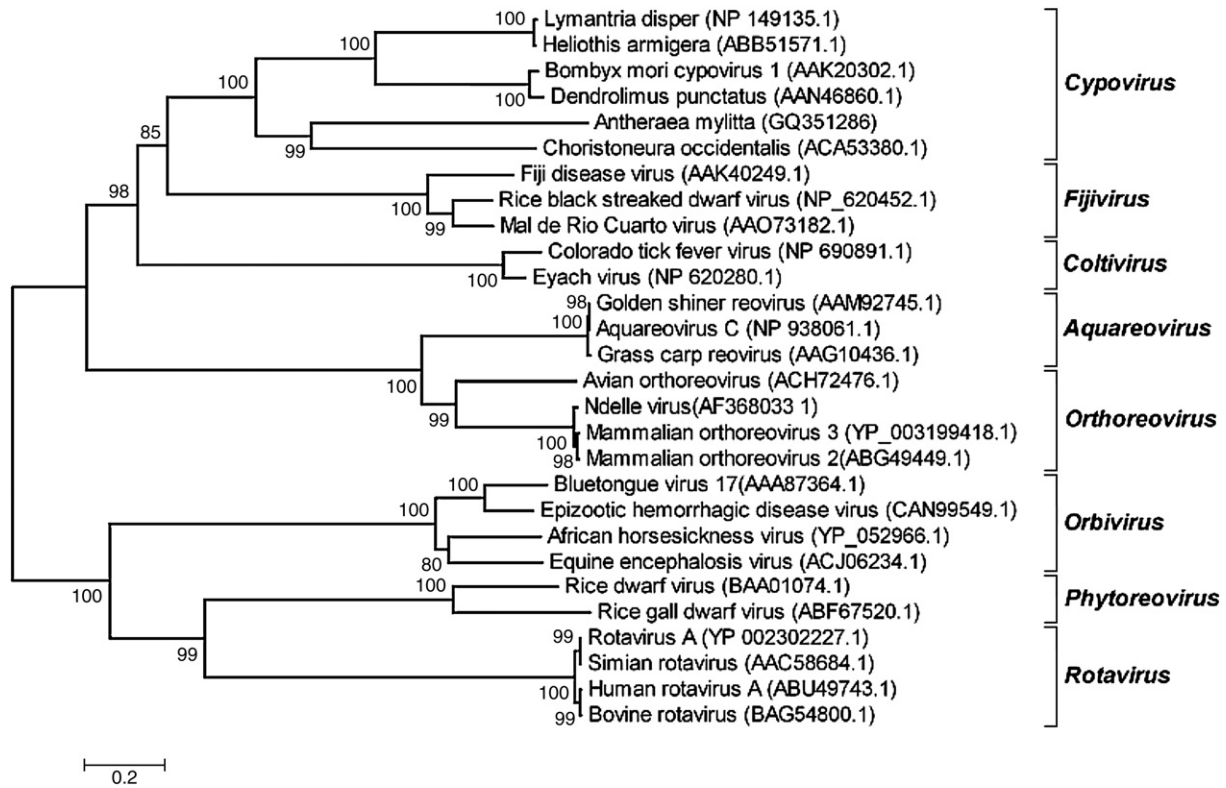


Fig. 2. Phylogenetic analysis of AmCPV RdRp with other members of the *Reoviridae* family by neighbor joining method. The number at each node represents bootstrap value of 100 replicates. GenBank accession numbers are shown in the parenthesis.

enzyme and then attained a plateau (Fig. 4A, inset). To correlate the RdRp activity of expressed protein with the template concentration, RNA synthesis was performed at 0.4 μ M purified AmCPV RdRp with varying concentrations of homopolymeric poly (A) RNA template and oligo U₍₁₈₎ primer. As shown in Fig. 4B, there was a linear correlation upto the concentration of 0.20 μ M template. The effect of pH, temperature and cation ion concentration on the RdRp activity of this protein was measured in the presence of varying concentrations of MgCl₂ and MnCl₂. Fig. 5A, showed that although some RdRp activity was detected in presence of Mn⁺⁺ but maximum RdRp activity was found in presence of Mg⁺⁺, at the concentration of 3 mM. The result was almost similar with the BTV RdRp (VP1) activity where the maximum RdRp activity was observed at 4 mM Mg⁺⁺ (Boyce et al., 2004). AmCPV RdRp activity was found to be optimal within pH range from 5.5 to 6.5 and reduced at pH below 5.0 and above 8.0 (Fig. 5B). The optimum pH for HCV RdRp was found to be 7.0 and in case of Norovirus the pH range was 6.8 to 7.5 (Fukushi et al., 2004; Lohmann et al., 1998). AmCPV RdRp activity was found to be higher at 37 °C (Fig. 5C) as observed in case of *Rotavirus* RdRp which also showed maximum activity between 30 °C–37 °C (Tortorici et al., 2003). The optimal salt concentration for AmCPV RdRp activity was found to be 20 mM KCl and 80 mM NaCl (Fig. 5D). These results were comparable with HCV RdRp where maximum RdRp activity was found to be at 10 mM KCl and 50 mM NaCl (Lohmann et al., 1998). These data indicated that AmCPV RdRp activity is dependent on protein concentration, temperature, pH, salt concentration and divalent Mg⁺⁺.

Primer independent RNA synthesis using AmCPV S2 minigenome

To study the primer independent RNA synthesis by AmCPV RdRp, an AmCPV “minigenome,” containing 5′ or 3′ untranslated regions (UTRs) of AmCPV S2 RNA alongwith a portion of the coding region, was synthesized by *in vitro* transcription (Fig. 6A) and used as RNA

template in the RdRp assay. We chose these minigenome's templates based on the assumption that the 5′ and 3′ UTRs would contain the *cis* acting signals necessary for the initiation of minus strand RNA synthesis. As shown in Fig. 6B, ³²P labeled RNA products with a size similar to that of RNA substrate were obtained in reactions containing 3′ positive and 3′ negative strand RNA transcripts as template (lanes 1 and 3) but not in the reaction containing 5′ positive strand RNA fragment as template when no primer was used (lane 2). These results indicate that AmCPV RdRp can synthesize RNA *de novo* utilizing AmCPV S2 subgenomic RNA as template like those of other RNA viruses (Casey et al., 1986; Zhong et al., 1998) and without the help of any other viral proteins as observed in BTV VP1 protein (Boyce et al., 2004). The preference of 3′ positive and 3′ negative strand may depend on the specific recognition of RdRp of the special structure of RNA substrate for the initiation of RNA synthesis in a primer dependent manner. The importance of the 3′ untranslated region of RNA in the initiation of replication process has been demonstrated in several viruses including bamboo mosaic virus (Li et al., 1998), turnip crinkle virus (Rajendran et al., 2002), picorna virus (Kok and McMinn, 2009) and rotavirus (Chen et al., 2001).

But when RdRp assay was performed using 5′ positive strand RNA fragment as template with primer TF7 (5′ UAAUGAUCAUUAGUA 3′) which can binds specifically to this strand, the template size product formation was observed (lane 6), indicating primer dependent synthesis of RNA from 5′ positive strand RNA. No products were observed in the absence of enzyme (lane 5) or RNA template (lane 4) or when double stranded RNA (AmCPV S11) was used as template (lane 7). Failing to synthesize RNA *in vitro* from dsRNA template suggests the involvement of other viral proteins that may help unwinding the dsRNA template prior to transcription. In bluetongue virus, VP6 has been shown to possess nucleotide triphosphatase, RNA binding and helicase activities and to act early as part of transcriptase complex during replication (Matsuo and Roy, 2009). As shown in

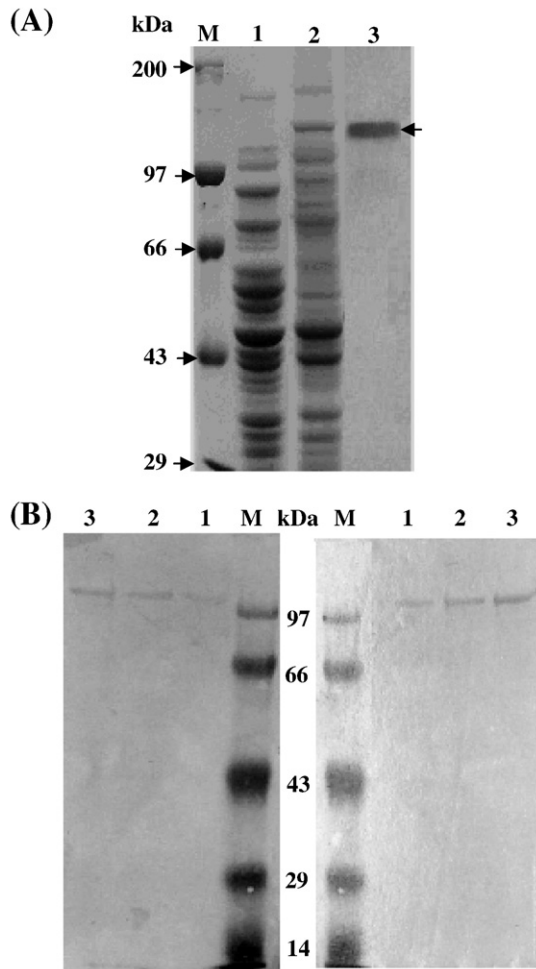


Fig. 3. (A) Analysis of *E. coli* expressed AmCPV RdRp by SDS-PAGE. Lane M, Molecular weight marker; lane 1, uninduced *E. coli* cell lysate; lane 2, induced *E. coli* cell lysate; lane 3, Ni-NTA purified AmCPV RdRp. (B) Western blot analysis of wild and mutant AmCPV RdRp expressed in insect cells. Lane M, molecular weight marker; lane 1, purified wild type AmCPV RdRp; lane 2, purified GAD mutant protein; lane 3, purified GAA mutant protein. (Right panel western blot, left panel SDS-8% PAGE).

Fig. 6C. RNA products synthesized *in vitro* during RdRp reaction were sensitive to RNase A digestion under both high and low salt conditions (lane 2 and 3) indicating that the generated RNA products were single stranded in nature. The degradation of the synthesized RNA products after RNase treatment under both high and low salt conditions suggests the synthesis of single stranded and not the double stranded RNA product *in vitro* by AmCPV RdRp. It also eliminates the possibility of the synthesis double template size RNA products by copy back

Table 1

Summary of the RdRp activity of wild and mutant AmCPV S2 encoded protein using poly (A) as a template and Oligo U18 as primer.

AmCPV S2 encoded protein	Template	Primer	Amount of enzyme (μg)	Incorporation of [^{32}P] UTP (CPM)	Specific activity (pmol/ μg of protein/h)
Wild type	Poly (rA)	Oligo U	2	135693	13.48
Wild type	Poly (rA)	–	2	954	
Wild type	–	Oligo U	2	587	
Wild type	Poly (rA)	Oligo U	0	241	
Mutant GAD AmCPV S2	Poly (rA)	Oligo U	2	1352	
Mutant GAA AmCPV S2	Poly (rA)	Oligo U	2	808	

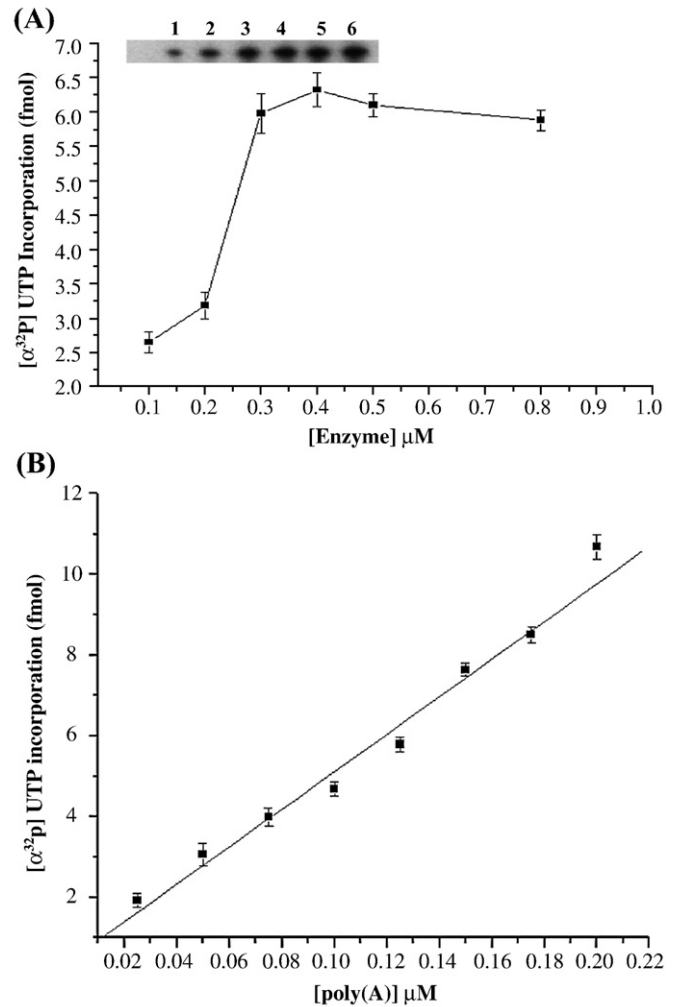


Fig. 4. (A) RdRp assay with different concentration of AmCPV S2 encoded protein. The reactions were performed at different concentrations of purified protein with fixed concentration of Oligo U₍₁₈₎ primer and Poly (A) template. Incorporation of [$\alpha^{32}\text{P}$] UTP in fmol is indicated as mean \pm standard deviation of three independent experiments. Another experiment was done using *in vitro* transcribed 3' positive strand AmCPV S2 RNA as template and the reaction products were analyzed through 1% formaldehyde agarose gel and autoradiographed (insets). Lanes 1 to 6 represent reaction products with 0.1 μM , 0.2 μM , 0.3 μM , 0.4 μM , 0.5 μM and 0.8 μM purified AmCPV S2 encoded protein, respectively. (B) RdRp assay of AmCPV S2 encoded protein (0.4 μM) with different concentration (0.02–0.22 μM) of template.

mechanism as observed in bovine viral diarrhea virus (Zhong et al., 1998). Time course of RdRp reaction was then carried out using *in vitro* transcribed 3' positive strand RNA as template under the same condition. It was observed that RNA products transcribed by AmCPV RdRp are in linear proportion to the incubation time during the first 120 min and then come to a saturation state (data not shown).

RdRp activity of the wild and the mutated AmCPV S2 encoded proteins

The GDD motif that exists in a variety of RdRp has been considered important for metal binding and as a catalytic site for enzymatic activity. In order to demonstrate the importance of GDD motif present in AmCPV RdRp, the GDD motif was changed to GAD and GAA by PCR based mutagenesis and confirmed by nucleotide sequencing. Assay of RdRp activity of these mutant proteins using both poly (A) RNA and *in vitro* transcribed 3' (–) strand of AmCPV S2 RNA as template showed that enzyme activity was reduced drastically in the GAD mutant and

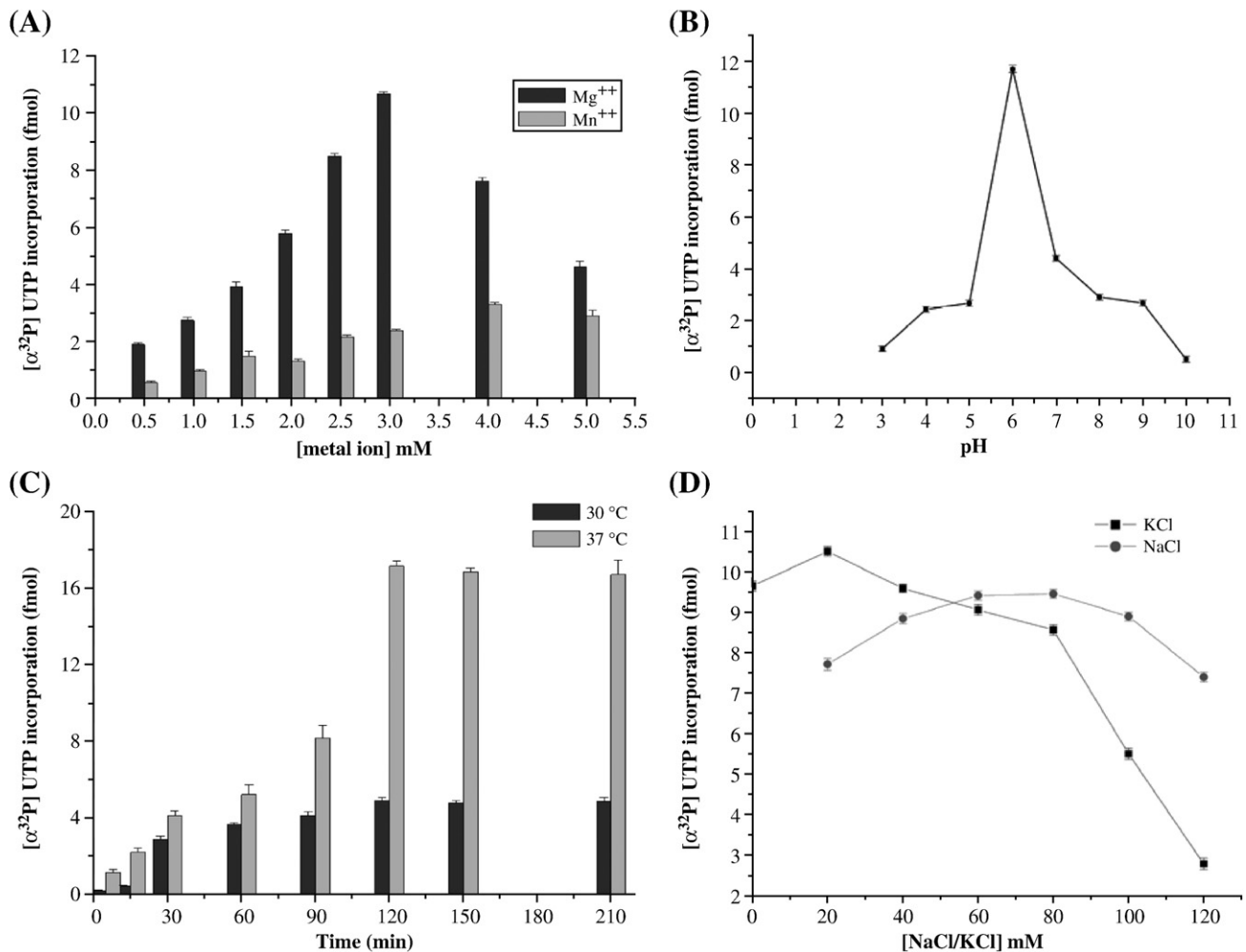


Fig. 5. Effect of divalent metal ions, pH, temperature, salts and incubation time on the RdRp activity. The reactions were carried out (A) at different concentrations of Mg⁺⁺ and Mn⁺⁺ or (B) at different pH or (C) at different time and temperature or (D) at different concentration of KCl or NaCl for the determination of maximum RdRp activity using poly (A) RNA as template (175 ng) and oligo U₍₁₈₎ primer (50 ng) in presence of 0.4 μM AmCPV S2 encoded protein. Values are mean ± standard deviation of three independent experiments.

abolished completely in the GAA mutant (Fig. 6D and Table 1) indicating its importance in the catalytic activity of the enzyme.

TNTase activity

In order to examine presence of any intrinsic TNTase activity in the expressed protein, TNTase assay was carried out because TNTase activity might confound the interpretation of the actual properties of RdRp. Since TNTase labels the 3' ends of RNA (Fukushi et al., 2004), the RNA products in our RdRp assays could result from the addition of [³²P] UTP to the 3' end of the template RNA by TNTase. To rule out this possibility we performed TNTase assays in the presence of all four NTPs and α³²P or [α³²P] UTP and cold UTP (10 μM) or in the presence of only [α³²P] UTP as a sole ribonucleotide substrate. No labeled products were detected in the presence of [α³²P] UTP only or [α³²P] UTP and cold UTP (Fig. 6E, lanes 2 and 3). These results indicate that ³²P labeled product probably represent the *de novo* RdRp activity rather than due to terminal transferase activity.

Determination of elongation rate

The rate of *de novo* synthesis of RNA by AmCPV S2 encoded protein was determined by analyzing the size of synthesized RNA at different time period in a formaldehyde agarose gel (Fig. 7A). From the plot linear increase in the size of RdRp product over time was found and the elongation rate was calculated from the slope of the curve as approx-

imately 120 nucleotides per minute (Fig. 7B). This rate of RNA synthesis by AmCPV RdRp (120 nt/min) is comparable with that of poliovirus, encephalomyocarditis virus and rhinovirus (150–200 nt/min), (Neufeld et al., 1991; Tuschall et al., 1982; Van Dyke et al., 1982).

Conclusion

Viral polymerases are essential for viral genome replication and are attractive target for anti-viral drug development. Since to our knowledge no other cytoviral RdRp has been functionally characterized so far, expression, purification and functional analysis of a recombinant soluble and active AmCPV RdRp will not only provide a tool for future detailed structural studies of this enzyme but also will facilitate to develop antiviral compounds. In addition, it will be useful for identification of other factors involved in viral replication.

Materials and methods

Silkworm, virus and cell lines

The CPV infected Indian non-mulberry silkworms, *A. mylitta*, were collected from different tasar farms of West Bengal state of India. The *Spodoptera frugiperda* cell lines, Sf 9 (Invitrogen) was maintained on Grace Insect media supplemented with 10% foetal bovine serum, lactalbumin hydrolysate and yeastolate at 27 °C.

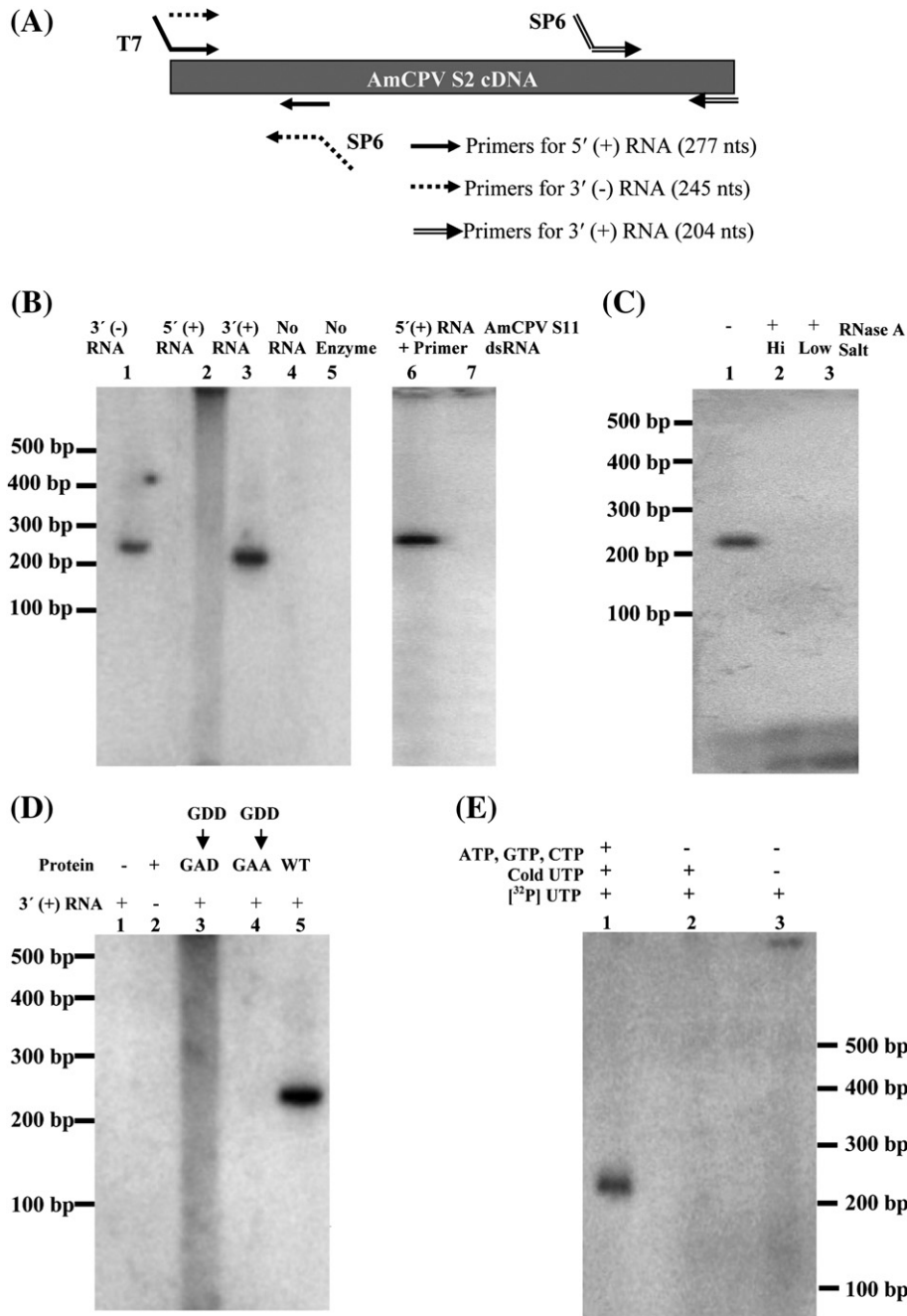


Fig. 6. (A) Schematic diagram of the positions of three pairs of primers that were used to generate a promoter (T7 or SP6) containing cDNA fragments by PCR. The amplified cDNA fragments of AmCPV were then used to produce the 5' positive strand RNA (277 nt), the 3' positive strand (204 nt), and 3' negative strand RNA (245 nt) by *in vitro* transcription. (B) RdRp assay using various *in vitro* synthesized RNA templates (indicated above the gel) in presence or absence of AmCPV RdRp. Sizes of the RNA markers (Ambion) are on the left. (C) Analysis of RNase A treated RdRp product under high (250 mM NaCl) and low salt (50 mM NaCl) conditions. (D) Effect of mutation in the GDD motif on RdRp activity of AmCPV S2 encoded protein using *in vitro* transcribed 3' (-) strand of AmCPV S2 RNA as template. (E) Assay of TNTase activity in AmCPV S2 encoded protein.

Purification of polyhedral bodies, isolation of total genomic RNA and extraction of AmCPV S2 RNA

Polyhedra were isolated from the CPV infected midgut of *A. mylitta*, by sucrose density gradient centrifugation according to a method of Hayashi and Bird (1970) with some modification (Qanungo et al., 2000). Genomic RNA was isolated from the purified Polyhedra following a standard guanidium isothiocyanate method (Ausubel et al., 1995). The isolated RNA was then resolved through 8% polyacrylamide gel electrophoresis, S2 RNA was excised and eluted from the gel by crush and soak method (Sambrook et al., 1989).

Molecular cloning and sequencing of AmCPV S2 RNA

AmCPV S2 RNA was converted to cDNA following a sequence independent RT method (Lambden et al., 1992) using two primers AG1 and AG2 as discussed by Chavali et al. (2008). cDNA was then cloned into pCR-XL-TOPO vector to make plasmid pCR-XL-TOPO/AmCPV S2. After transforming *E. coli* Top 10 cells, plasmids were isolated and characterized by *EcoRI* digestion. Recombinant plasmids having the proper size insert were then sequenced using BigDye Terminator V 3.1 cycle sequencing kit (ABI) with M13 forward and reverse primers and a set of internal primers designed from the

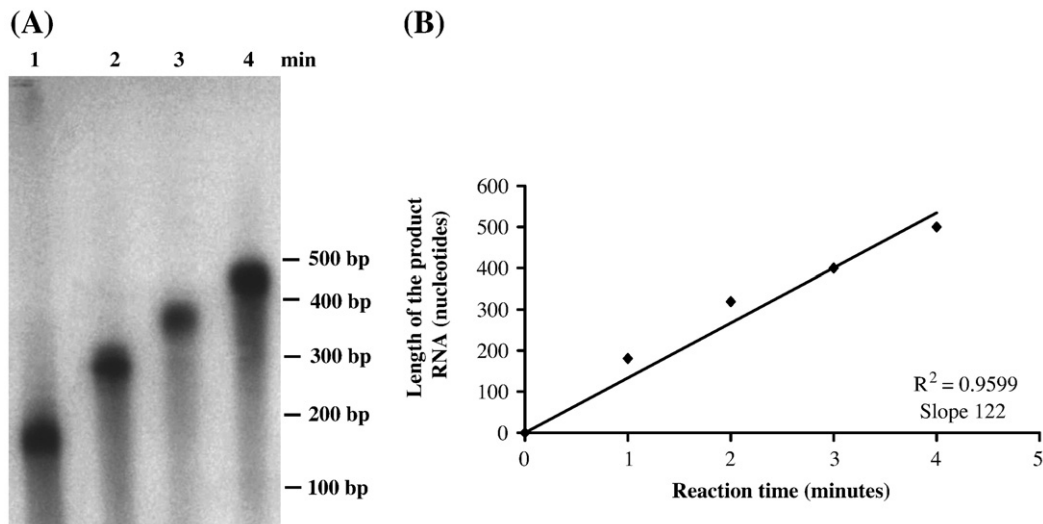


Fig. 7. (A) Synthesis of product RNA using AmCPV RdRp using *in vitro* transcribed 500 nt long 3' positive strand AmCPV S2 RNA as template in respect of time. At the times indicated, aliquots were removed from the reaction mixture and the size of labeled product RNA was analyzed by formaldehyde agarose gel electrophoresis, and autoradiographed. (B) Plot showing the size of product RNA vs. reaction time. The straight line was obtained by linear regression.

deduced nucleotide sequences in an automated DNA sequencer (ABI). The sequences were then analyzed using Sequencher (Gene Codes Corporation) and secondary structure was predicted following the methods of Rost and Sander (1994).

In order to obtain an initial idea about the structural and functional features of AmCPV S2 encoded protein, available nucleic acid and protein databases were searched using BLAST. AmCPV S2 amino acid sequences were then aligned with the amino acid sequence of RdRp from polio virus, rabbit hemorrhagic disease virus (RHDV), reovirus, hepatitis C virus and using the ESPript 2.2 program (<http://espript.fr/ESPrIPT/ESPrIPT/>) to obtain and compare different conserved functional motifs. Finally to evaluate the evolutionary relationship between AmCPV and other members in the family *Reoviridae*, the amino acid sequences of AmCPV RdRp were compared with that of twenty seven viruses in the family *Reoviridae* and phylogenetic tree was generated by the neighbour-joining method with the program MEGA (<http://www.megasoftware.net/index.html>) (Li et al., 2007; Saitou and Nei, 1987; Tamura et al., 2007). Tree drawing was performed with the help of TREEVIEW program (Kumar et al., 2004).

Expression of AmCPV S2 in *E. coli*

The entire open reading frame of S2 cDNA was amplified by PCR from the plasmid pCR-XL-TOPO/AmCPV S2 by using *Accuzyme* DNA polymerase (Biolone) and two synthetic primers, AGCPV 143 F (5' GTAATCATCCCGGGAAGAGACATG 3', forward primer) and AGCPV 144 R (5' AGATTGCTGCTGCAGAAAAAACGTC 3', reverse primer) containing *Pst*I (in forward primer) and *Sma*I (in reverse primer) restriction sites (underlined). PCR amplified product (3.35 kb) was digested with *Sma*I and *Pst*I, and ligated to *Sma*I/*Pst*I digested pQE-30 vector (Qiagen), in frame with a sequence encoding six histidine residues at the N-terminus. The resulting recombinant plasmid, pQE-30/AmCPV S2 was then transformed into M15 *E. coli* cells and colonies were screened following *Sma*I/*Pst*I digestion of isolated plasmids. In order to check the protein expression, recombinant bacteria were grown in 5 ml LB media at 37 °C until the O.D at 600 nm reached to 0.6. The culture was then induced with 1 mM IPTG and allowed to grow for another 4 hrs at the same temperature. Bacterial cells were harvested by centrifugation, and then analyzed by SDS-8% PAGE (Laemmli, 1970).

Purification of His-tagged protein

Recombinant *E. coli* containing pQE-30/AmCPV S2 were grown in 1 L LB medium, induced with 1 mM IPTG and after solubilizing the insoluble 6x His-tagged fusion protein with 8 M urea, it was purified by Ni-NTA affinity chromatography (Qiagen). The amount of the purified protein was determined by the method of Bradford (Bradford, 1976) using BSA as standard and the purity was checked by SDS-8 % PAGE.

Rabbit immunization and production of polyclonal antibodies

One rabbit was immunized with the purified recombinant AmCPV RdRp by standard method (Harlow and Lane, 1988) and as discussed by Chavali et al., 2008. The antibody titer in the immunized serum was determined by ELISA using 2.5 µg of antigen. Specific antibody was purified by antigen affinity chromatography (Harlow and Lane, 1988; Sambrook et al., 1989).

Expression and purification of wild and mutant AmCPV RdRp in insect cells

The entire ORF of AmCPV S2 was amplified from pCR-XL-TOPO/AmCPV S2 by PCR by using two primers, AGCPV 153 F (5' GTAATCACTCGAGCTAAGAGAAGCTG 3', forward primer) and AGCPV 144 R (5' AGATTGCTGCTGCAGAAAAAACGTC 3', reverse primer), containing *Xho*I and *Pst*I sites in the forward and reverse primer, respectively (underlined). PCR amplified product was digested with *Xho*I and *Pst*I, and cloned into pBluebacHis2A baculovirus transfer vector (Invitrogen) downstream of the baculovirus polyhedrin promoter to generate pBluebacHis2A/AmCPV S2. Overlapping extension PCR based site directed mutagenesis (Ho et al., 1989) was done to mutate the conserved GDD motif to GAD and GAA, (located at amino acid residues 681 to 683) cloned into pBluebacHis2A and confirmed by sequencing of the positive clones. The resultant recombinant baculovirus transfer vectors (4 µg) and *Bsu*I digested linearized *Autographa californica* nuclear polyhedrosis viral DNA (0.5 µg) were cotransfected into Sf9 (10⁶) cells using insectin plus according to the manufacturer's protocol (Invitrogen). Culture medium was collected 72 h post infection and after infecting fresh Sf9 cells with this culture supernatant, recombinant baculovirus were isolated by

plaque purification. To produce recombinant AmCPV S2 encoded protein, Sf9 cells were cultured in a 1 L spinner flask (2×10^7 cells) and infected with recombinant baculovirus at an m.o.i of five. The cells were harvested 72 h postinfection, washed twice with phosphate buffer saline and a cytosolic extract was prepared by the method of Behrens et al. (1996). Baculovirus expressed His-tagged RdRp was then purified by Ni-NTA affinity chromatography.

Immunoblot analysis of baculovirus expressed wild and mutant AmCPV RdRp in insect cells

In order to verify the expression of wild and mutant AmCPV RdRp in Sf9 cell, Ni-NTA purified recombinant proteins from Sf9 cells were resolved in a SDS-8% PAGE and transferred electrophoretically onto a Duralose membrane (Towbin et al., 1979) and treated with purified anti-RdRp polyclonal antibodies as described by Chavali et al. (2008).

Preparation of RNA template by *in vitro* transcription

In order to establish the *de novo* synthesis of RNA by AmCPV RdRp, 5' and 3' terminal fragments of AmCPV S2 genomic RNA were synthesized as "mini genome" by *in vitro* transcription using T7 and Sp6 polymerase. To prepare the 277 nucleotide long 5' terminal fragment of the positive strand RNA of the AmCPV S2, a DNA fragment was amplified first from the cDNA clone of the AmCPV S2 by PCR with primers TF1 (5' GCTCTAGATAATACGACTCACTATAATTACTAGTAATCATCCTTG 3') and TF2 (5' AATCGCGTAATCTGCACTCAC 3'). The bold underlined sequences in TF1 represent T7 promoter followed by nucleotide sequence 1 to 20, and TF2 primer is complementary to positive strand AmCPV S2 cDNA sequence from the nucleotide 232 to 252. Similarly, primers TF5 (5' GATTTAGGTGACACTATAGCTTCAGCC-CAGGAGTACGCG 3') and TF6 (5' GCTCTATACCATCCGATCGCT 3') were used as forward and reverse primers, respectively, to generate a 204 nucleotide long 3' terminal fragment of the positive strand RNA of the AmCPV S2. The bold underlined sequences in TF5 represent Sp6 promoter sequence followed by nucleotide sequence 3614 to 3633 and TF6 is complementary to positive strand AmCPV2 cDNA sequence from nucleotides 3778 to 3798. Primers TF3 (5' ATTACTAGTAATCATCCTTG 3') and TF4 (5' GATTTAGGTGACACTATAGGGTACCATAATATTCTCGC 3') were used as forward and reverse primers, respectively, to amplify a 245 nucleotide long 3' terminal fragment of the negative strand RNA of the AmCPV S2. The bold underlined sequences in TF4 primer represents the Sp6 promoter sequence preceded by nucleotide sequence 208 to 228, and TF3 primer is complementary to negative strand AmCPV S2 cDNA from nucleotides 1 to 20. The amplified products were analyzed on a 1% agarose gel and correct sized bands were eluted using gel extraction kit (Qiagen).

In vitro transcription was carried out to generate RNA templates from the gel eluted PCR products by using MAXIscript® *in vitro* transcription kit (Ambion). The reaction mixture containing 1 µg of each PCR product corresponding to 5'(+), 3'(+), or 3'(-) strand RNA as template, transcription reaction buffer, 0.5 mM of each rNTP and 2 µl of T7 and Sp6 RNA polymerase cocktail (Ambion) in a total volume of 20 µl was incubated at 37 °C for 1 h and then DNA was destroyed by treating with TURBO DNase (2 U) (Ambion) for 5 min. Finally the reaction was stopped by adding 1 µl of 0.5 M EDTA and the products were analyzed by formaldehyde agarose gel.

RNA dependent RNA polymerase (RdRp) assay

RNA dependent RNA polymerase activity of the AmCPV S2 encoded protein was assayed by filter binding technique following a modified protocol used for the assay of Hepatitis C virus HCV RdRp (Oh et al., 1999; Yamashita et al., 1998). In brief, in a 50 µl reaction mixture [containing 50 mM Tris-HCl, pH 8.0, 5 mM KCl, 5 mM MgCl₂, 10 mM DTT, 1% BSA, 3.5 µl of 1 mM UTP, 0.4 µl (4 µCi) of [α^{32} P] UTP

(specific activity 3500 Ci/mmol) (BARC, India), 175 ng Poly(A) template (Amarsham), 50 ng Oligo U₍₁₈₎ as primer (Amarsham), 20 units of RNase inhibitor and 50 µg/ml actinomycin D], 5 µl (2 µg) of purified protein was added and incubated at 37 °C for 2 hrs. After 2 hrs, the reaction was stopped by adding 10 µl of cold 5% TCA containing 20 mM sodium pyrophosphate. The reaction mixture was then filtered through a GF/C filter paper (Whatman), washed five times with cold 20 mM sodium pyrophosphate buffer (pH 7.0) and once with 75% ethanol. The filter paper was then dried and the incorporated [α^{32} P] UTP was measured in a liquid scintillation counter (Beckman). In order to find out the optimum condition for the enzyme reaction, the polymerase reaction was carried out with different concentrations of the enzyme, RNA template, at different incubation temperature and pH. To understand the divalent cation requirement of the enzyme, the reaction was also carried out at different concentrations of Mg⁺⁺ and Mn⁺⁺. Similarly, in order to determine optimum salt concentration, assay was performed with different concentration of KCl and NaCl. RdRp assay was also performed with *in vitro* transcribed AmCPV S2 subgenomic RNA (single stranded) (1 µg) or AmCPV S11 dsRNA (1 µg) as templates without any primer in presence of 0.4 µM AmCPV RdRp in the same manner as described above for Poly (A) template/ Oligo U₍₁₈₎ primer in presence of 1 mM each of NTPs (Ambion) and 4 µCi of [α^{32} P] UTP. To determine the primer-dependent initiation of RNA synthesis, the *in vitro* transcribed subgenomic 5' positive strand of S2 RNA was used as template in RdRp reaction with primer which can bind specifically to that strand. Reaction products were then separated in formaldehyde agarose gel and exposed to X ray film. The intensity of band (density) was determined by scanning the autoradiogram in KODAK 1D image analysis software (Kodak, EDAS 290).

To further characterize the product RNA synthesized by RdRp assay, it was treated with RNase A under high salt (250 mM NaCl) and low salt (50 mM NaCl) conditions at 37 °C for 30 min. After the incubation, the reaction mixture was extracted with phenol-chloroform and then precipitated with ethanol. The pellet was dissolved in electrophoresis sample buffer and analyzed in formaldehyde agarose gel.

Terminal transferase (TNTase) activity assay

TNTase assay of purified protein using *in vitro* transcribed 245 nt 3' negative strand AmCPV S2 RNA segment as template was performed in the same buffer as RdRp assay with specified nucleoside triphosphates such as, ATP, GTP, CTP, cold UTP and [α^{32} P] UTP or without ATP, GTP, CTP but in the presence of [α^{32} P] UTP and cold UTP (10 µM) or in the presence of [α^{32} P] UTP as a sole ribonucleotide substrate. Reactions were carried out at 37 °C for 90 min and terminated by the addition of 60 µl of stop solution (10 mM Tris-HCl, [pH 7.5], 10 mM EDTA and 100 mM NaCl). Products were dissolved in RNA sample buffer containing 80% formamide, 1 mM EDTA and 0.1% bromophenol blue. After heat denaturation, the RNA products were separated in 1% formaldehyde agarose gel, exposed to the X ray film and autoradiographed.

Determination of elongation rate of AmCPV S2 encoded RdRp

The elongation rate of the polymerization reaction was measured by determining the change in size of the product RNA as a function of time. An *in vitro* transcribed 3' positive strand RNA (500 nt) was synthesized and used as template in the RdRp assay under optimized conditions. Reaction was stopped by removing aliquots at different time intervals and by adding 1 M EDTA. Reaction products were then analyzed by 1% formaldehyde agarose gel and the size of the largest product RNA at each time point (detected in the "stepladder" gel) was plotted as a function of time. The elongation rate was calculated from the slope of the curve.

Nucleotide sequence accession number

The nucleotide sequence of AmCPV S2 has been deposited in the Genbank database under Accession no: GQ351286.

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