Identification and characterization of RNA-dependent RNA polymerase activity in recombinant Japanese encephalitis virus NS5 protein

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Summary

The complete nonstructural NS5 gene of Japanese encephalitis virus (JEV) was amplified and cloned into an expression vector. The NS5 protein was expressed in *Escherichia coli* and purified by His-tag based affinity chromatography. This recombinant NS5 protein exhibited RNA-dependent RNA polymerase (RdRp) activity *in vitro* in the absence of other viral or cellular factors. The RNA polymerase activity was dependent on divalent cations, and Mn^{2+} was found to be 20 times more effective than Mg^{2+} in coordinating the catalytic reaction of RdRp, while Ca^{2+} inhibited enzyme activity. The optimal reaction conditions for the *in vitro* RdRp reaction were established. Characterization of the RdRp reaction products demonstrated that the JEV NS5 protein can initiate RNA synthesis through a *de novo* initiation mechanism in our *in vitro* reaction system. Comparing the efficiency of different RNA templates, we found that JEV NS5 protein was more efficient in using negative-strand RNA templates, indicating that the JEV NS5 protein is involved in regulating the ratio of positive- to negative-strand RNA. Four amino acid sequence motifs crucial for RdRp activity were also identified using site-directed mutagenesis analysis. All substitutions of the conserved residues within these motifs led to a complete inactivation or severe loss of enzyme activity.

Introduction

Japanese encephalitis virus (JEV), a member of the family Flaviviridae, is transmitted by *Culex* mosquitoes and causes serious viral encephalitis in humans. The virus is found across a vast geographic area that includes China, India, Japan and virtually all of South-East Asia. Approximately 3 million people live in areas that are JEV endemic. As many as 50,000 cases of JEV infection and about 15,000 associated deaths are reported every year. A high proportion of survivors develop serious neuorologic and psychiatric sequelae [15, 26].

The complete nucleotide sequence of the JEV genome has been determined, and the structural features of the genome are similar to those of other flavivirus genomes [5, 15, 21]. The genome of JEV contains a single positive-strained RNA molecule, which has a type I cap at its 5′ end and lacks a

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poly(A) tail at its 3′ end. It contains a single open reading frame, which is translated into a large polyprotein that is subsequently processed by both host and viral protease into three structural (C, prM, E) and seven nonstructural proteins (NS1 to NS5) in the order 5′ NC-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-NC3′. The NS5 protein, with a molecular weight of 103 kD, is the largest and most highly conserved of the viral proteins [5].

Comparing the deduced amino acid sequence of the JEV NS5 protein with the polymerases of several positive-strand animal and plant RNA viruses, several short homologous regions are found that probably represent common functional domains of RNA-dependent RNA polymerases (RdRp), including the highly conserved G-D-D polymerase motif [14, 18]. This suggests that the NS5 protein is the RdRp of JEV.

JEV-infected C6/36 cell extracts have been found to have polymerase activity, and this enzymatic activity was shown to be present in the perinuclear endoplasmic reticulum membranes, where both the NS3 and NS5 proteins are located. Rabbit antiserum to NS3 or NS5 was able to inhibit the polymerase activity in cell fractions, confirming that NS3 and NS5 are involved in RNA replication [7, 22]. Uchil and Satchidanandam also characterized the RNA synthesis of JEV using virus-infected porcine kidney cell extracts [25]. These studies did not exclude the contribution of other viral or host proteins present in the infected cell extracts.

Although it has been demonstrated for other flaviviruses such as dengue virus, Kunjin virus and hepatitis C virus that recombinant NS5 protein has RdRp activity [1, 4, 8, 9, 11, 23], there has been thus far no such report for JEV. In this study, we expressed the complete JEV NS5 protein in *E. coli* and demonstrated that recombinant NS5 protein has RdRp activity in the absence of additional viral or host cell factors. The chemical and molecular characterization of Japanese encephalitis virus NS5 protein was thoroughly analyzed. The establishment of an *in vitro* RdRp assay system using recombinant JEV NS5 protein could provide a basis for further studies to identify factors involved in viral replication and offer a useful tool for the selection of effective antiviral drugs.

Materials and methods

Virus and cell culture

The JE virus strain used in this study was JaOH0566, which was isolated from the brain of a JE patient in 1966 in Osaka, Japan, using suckling mice. After isolation, the virus was passaged more than 10 times in C6/36 cells. For virus preparation, C6/36 cells were used. They were cultured in minimum essential medium with non-essential amino acids (MEM; ICN Biomedicals Inc., USA) supplemented with 10% fetal bovine serum (FBS) at 28 °C. The maintenance medium was MEM with 2% FBS.

Construction of recombinant plasmids

The JEV NS5 gene was amplified by RT-PCR. RNA extraction and RT-PCR were performed as described previously [28]. PCR amplification was carried out using the primers 5′ AGGGGATCCCCTGGGGGGCAGGA 3′ and 5′-TTAA GTCGACCTAGATGACCCTG-3′ (generated restriction sites are underlined). The 2.8-kb PCR-amplified DNA fragment was digested with *Bam*HI and *Sal*I and cloned into the corresponding restriction site of pQE30 (Qiagen, Hilden, Germany). The presence and the identity of the insert in the recombinant plasmid were confirmed by DNA sequencing; the deduced amino acid sequence of the insert was the same as JaOH0566 (GenBank accession no. AY508813). The expression construct, encompassing amino acids (aa) 1-905, the full length of JEV NS5 protein with a vector-derived His-tag (histidine hexmer) at the N-terminus, was obtained.

Site-directed mutagenesis of JEV NS5 protein

Site-directed amino acid substitutions were introduced by using paired complementary oligonucleotides for the desired point mutations to generate specific mutations in the JEV NS5 protein using a QuickChange site-directed mutagenesis kit (Stratagene, USA) according to the manufacturer's instructions. Revertant mutants for all the mutants produced were constructed using the same method but with oligonucleotides to reverse the previously introduced changes. Both mutations and revertant mutations were confirmed using the dideoxy sequencing method. The mutant plasmids were introduced by transformation into *E. coli* XL1-Blue cells, and the mutant NS5 protein were expressed and purified in the same way as wild-type JEV NS5 protein. The six resultant mutant proteins were designated NS5mutA, NS5mutB, NS5mutC, NS5mutC2, NS5mutCn and NS5 mut D.

Expression and purification of the recombinant NS5 protein

To express the recombinant NS5 protein, the recombinant plasmid containing the JEV NS5 sequence was used to transform *E. coli* strain XL1- Blue. The transformed *E. coli* cells were then cultured at 30 °C in Luria-Bertani medium containing 100 µg/ml of Ampicillin. The recombinant proteins were

induced by the addition of 0.2 mM isopropyl β-D-thiogalacto-side (IPTG) when the OD600 of the culture reached 0.5. After IPTG induction at 30 °C for 3 h, the cells were harvested by centrifugation, washed in phosphate-buffered saline solution (PBS), resuspended in 20 mM Tris–HCl pH 7.5, 500 mM NaCl, and frozen at −80 °C. After freezing and thawing 3 times, the cell suspension was sonicated in 1-sec. pulses at 1-sec. intervals for 2 min. and centrifuged at 30,000 g for 15 min. at 4 °C. The supernatants were then applied to a Talon^{TM} IMAC resin column (Clontech, USA). After washing with a buffer containing 20 mM Tris–HCl, 500 mM NaCl, and 10 mM imidazole, the purified protein was then eluted with 20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 250 mM imidazole. The proteins were aliquoted and stored in 10% glycerol at −80 °C until use. The protein concentrations were determined using a Bio-Rad protein assay reagent kit (Bio-Rad, USA), and protein purity was analyzed using sodium dode-cyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blot analysis

The Western blot procedure was performed as described by Towbin et al. [24]. A semi-dry protein transfer method was used to immobilize the recombinant protein to a polyvinyli-dene difluoride (PVDF) membrane (Immobilon, Millipore, USA). Briefly, protein separated in a 10% polyacrylamide gel was transferred to a PVDF membrane using a semi-dry electroblotter (Sartorius, Germany). The membrane was initially blocked overnight at 4 °C with Blockace (Yokijirushi, Japan), then reacted with Blockace-diluted mouse anti-histi-dine (1:1000 dilution Amersham Biosciences, USA) or JEV-infected mouse immune serum(1:400) for 1 h at 37 °C, then incubated with goat anti-mouse IgG peroxidase conjugate (1:1000 dilution Biosource, CA, USA) for 1h at 37 °C. Finally, the reaction was visualized by dymethyl amino ben-zidine (DAB) staining.

RNA template synthesis

RNA templates used for the RdRp reactions were in vitro transcribed from PCR products. PCR primers were designed in which the sense or antisense strand contained a T3 pro-moter sequence. Approximately 1-kilobase (kb) PCR pro-ducts were amplified for JEV 5′ end, JEV 3′ end, dengue-2 virus (strain 16681) 5′ end and dengue-2 virus 3′ end from the respective cDNA clones. The PCR products were gel purified using a gel extraction kit (Qiagen, Germany) and used for RNA transcription using in vitro transcription kits (Ampliscribe, USA). The transcribed RNA were quantitated spectrometrically and stored at −80 °C until use. When the RNA template was to be labelled, transcription was per-formed in presence of 10 μCi of [α-^{32}P] UTP. Six RNA products were produced by in vitro transcription and used as templates for in vitro RdRp assays: 1-kb JEV 3′(−)RNA; 1-kb JEV 3′(+) RNA; 1-kb Den-2 virus 3′(−) RNA;1-kb Den-2 virus 3′(+) RNA; 1-kb HCV 3′(+)RNA and one

1.3-kb non-specific RNA produced using the control DNA supplied with the transcription kit.

RNA-dependent RNA polymerase(RdRp) assay

This assay was based on the incorporation of radiolabelled nucleotides into a newly synthesized RNA molecule [10]. The RdRp activity assay was performed at 30 °C in 50-μl reaction volumes containing 3 μg of purified NS5 protein, 50 mM HEPES, pH 7.3, 100 μM UTP, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 4 mM DTT, 3 mM $MnCl_2$ unless specif-ically indicated, 6 μM $ZnCl_2$, 5 μg of in vitro transcribed RNA template, 60 units of HPRI and 5–10 μCi of [α-^{32}P] UTP (3000Ci/mM, Amersham, USA). The reaction mixture was incubated for 4 h. A volume of 5 μl was removed hourly and spotted onto a DE81 Whatman filter (Whatman, USA), washed twice with 500 mM Na_2HPO_4, pH 7.5, and finally with 100% ethanol. The filter was air-dried, solubilized in 1 ml of scintillation fluid (Amersham, USA), and radioactivity was measured using a scintillation counter (Amersham, USA).

To check the effect of different divalent ions, similar reac-tions were performed using different solutions containing $MnCl_2$, $MgCl_2$ or $CaCl_2$. After a 4-h incubation, 5 μl of the reaction mixture was removed and spotted onto a DE81 filter membrane (Whatman, USA), which was washed, and radio-activity was measured. The effect of $CaCl_2$ was further stud-ied by increasing $CaCl_2$ concentration in the presence of 3 mM $MnCl_2$.

When analyzed by electrophoresis, the RNA products were extracted with phenol/chloroform and precipitated with ethanol. The RNA pellet was air-dried, resuspended in load-ing buffer (95% formamide, 0.5 mM EDTA, 0.025% bromo-phenol blue, 0.025% xylene cyanol), heated for 5 min at 95 °C and loaded onto a 8M-urea-denatured 3.5% polyacry-lamide gel in Tris/borate/EDTA (TBE) buffer. Electro-phoresis was carried out at a constant 20 mA until the bromophenol blue marker migrated to the bottom of the gel. The gel was then dried and autoradiographical data analysed using a Bio-Rad image developing system.

Ribonuclease A digestion of RdRp reaction product

RNase A digestion was performed at 37 °C for 30 min in 20-μl volumes of 20 mM Tris–HCl, pH 7.5, and 5 mM EDTA in the absence or presence of 0.3 M NaCl containing the RNA product of the RdRp reaction assay. Reaction products were analyzed by electrophoresis and autoradiography.

Sodium periodate treatment of RNA transcripts

The 3′-hydroxyl group of the RNA template was blocked by sodium periodate treatment as described by Ackerman et al. [1, 27]. Briefly, 10 μg of the RNA transcripts was dissolved in 200 μl 50 mM NaOAc, pH 5.0. After the addition of 50 μl $NaIO_4$(100 mM), the reaction was incubated for 1 h at 22 °C,

extracted with PCA and precipitated with propanol. The pellets were washed with 70% EtOH, dissolved in water and quantified spectrophotometrically.

Results

Expression and purification of JEV NS5 protein

The JEV JaOH0566 strain NS5 coding region was amplified using reverse transcription polymerase chain reaction (RT-PCR) and cloned into expression vector pQE30. The constructed expression plasmid spanned the entire 905 amino acids of NS5. The recombinant protein was expressed in *E. coli* and purified using Talon metal affinity resin (Clontech USA) under natural conditions. Analysis of purified recombinant NS5 protein by SDS-PAGE and Coomassie blue staining revealed a single protein band of 103 KD, as predicted (Fig. 1A). The identity of the recombinant NS5 protein was further confirmed by Western blot with mouse antiserum to both histidine and Japanese encephalitis virus (Fig. 1B, C).

Demonstration of RdRp activity in NS5

Polymerase activity of the purified recombinant NS5 protein was demonstrated by measuring the level of incorporation of $[^{32}P]$-UMP into newly

synthesized RNA strands using RNA templates. The RdRp assay was carried out as described under "Materials and Methods" except that the initial
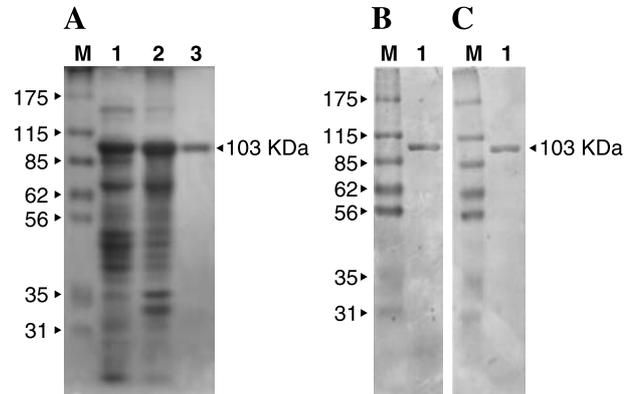
Fig. 1. SDS-PAGE and Western blot analysis of JEV NS5 protein expressed in *E. coli*. A recombinant plasmid containing the complete NS5 gene was introduced to *E. coli* strain XL1-Blue and induced with IPTG. Recombinant NS5 protein was analyzed in a 10% SDS-PAGE gel and stained with Coomassie brilliant blue (**A**) *M* molecular weight protein markers (in kilodaltons); *1* supernatant of sonicated induced cell lysate after centrifugation; *2* pellet of sonicated induced cell lysate; *3* purified NS5 protein. Western-blot analysis of purified JEV NS5 protein with anti-His-taq antibody (**B**) or JEV mouse hyperimmune serum (**C**) *M* molecular weight protein markers; *1* purified NS5 protein
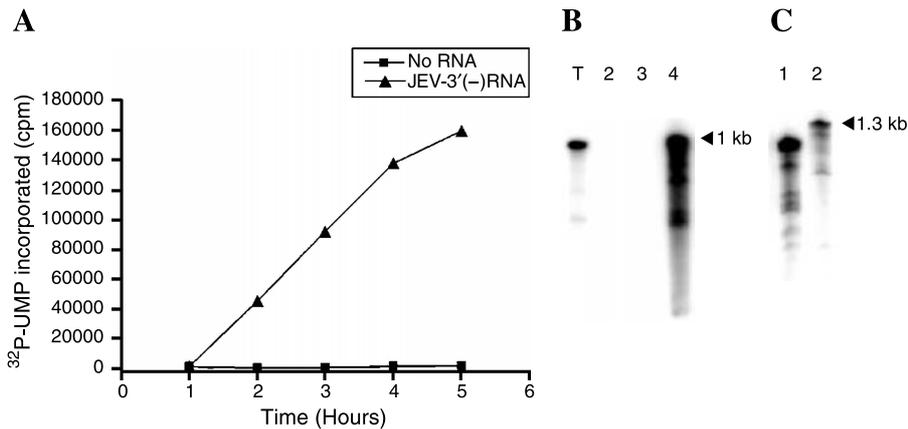

Fig. 2. RdRp activity of purified NS5 protein. **A** Assayed by measuring incorporation of $[^{32}P]UMP$. RdRp reactions were performed as detailed in Materials and methods. Briefly, 5 μl was removed hourly from the reaction volume and spotted onto a DE81 Whatman filter and washed with 0.5 M Na$_2$HPO$_4$, pH7.5. Radioactivity was then measured by scintillation counting. **B** The ^{32}P-labeled RNA polymerase products were analyzed on an 8M-urea-denatured 3.5% PAGE gel. *T* ^{32}P-labeled RNA template obtained by *in vitro* transcription; *2* RdRp product with template omitted; *3* with protein omitted; *4* 1 kb JEV 3′(−)RNA as template. **C** The ^{32}P-labeled RNA polymerase products using non-specific RNA templates were analyzed on a 8M-urea-denatured 3.5% PAGE gel. *1* 1-kb HCV 3′(+) RNA as template; *2* 1.3-kb non-specific RNA as template

assays were conducted in the presence of actinomycin D ($50\,\mu g/ml$) to inhibit any possible contaminating bacterial DNA-dependent RNA polymerase. There was no difference in polymerase activity recorded in the presence or absence of actinomycin D (data not shown). Therefore it was excluded in subsequent assays. As shown in Fig. 2A, when using 1-kb JEV3$'$ ($-$) RNA as the RNA template, the incorporation of [^{32}P]-UMP into newly synthesized RNA increased linearly over a period of 4 h at 30 $^{\circ}$C, while in reactions without RNA template there was no detectable incorporation, indicating that the recombinant NS5 protein was dependent on RNA, as expected for an RdRp.

The ^{32}P-labelled RdRp products were also visualized on an 8.0 M-urea-denatured 3.5% SDS-PAGE gel, as shown in Fig. 2B. There was no synthesized RNA band in reactions without RNA template or NS5 protein (Fig. 2B, Lanes 2, 3). When using 1-kb JEV3$'$ ($-$) RNA as the RNA template, the main polymerase product was the same size as the RNA template used. In addition, the recombinant NS5 protein also synthesized several smaller polymerase products. Similar to other RNA polymerases, these smaller RNA species may represent partially or incompletely synthesized RNA products [23].

To determine whether JEV NS5 could use non-specific RNA templates, the enzyme were incubated with 1-kb-long HCV 3$'$(+) RNA and a 1.3-kb-long non-specific RNA transcribed from the control DNA supplied in the transcription kit (Ampliscribe,
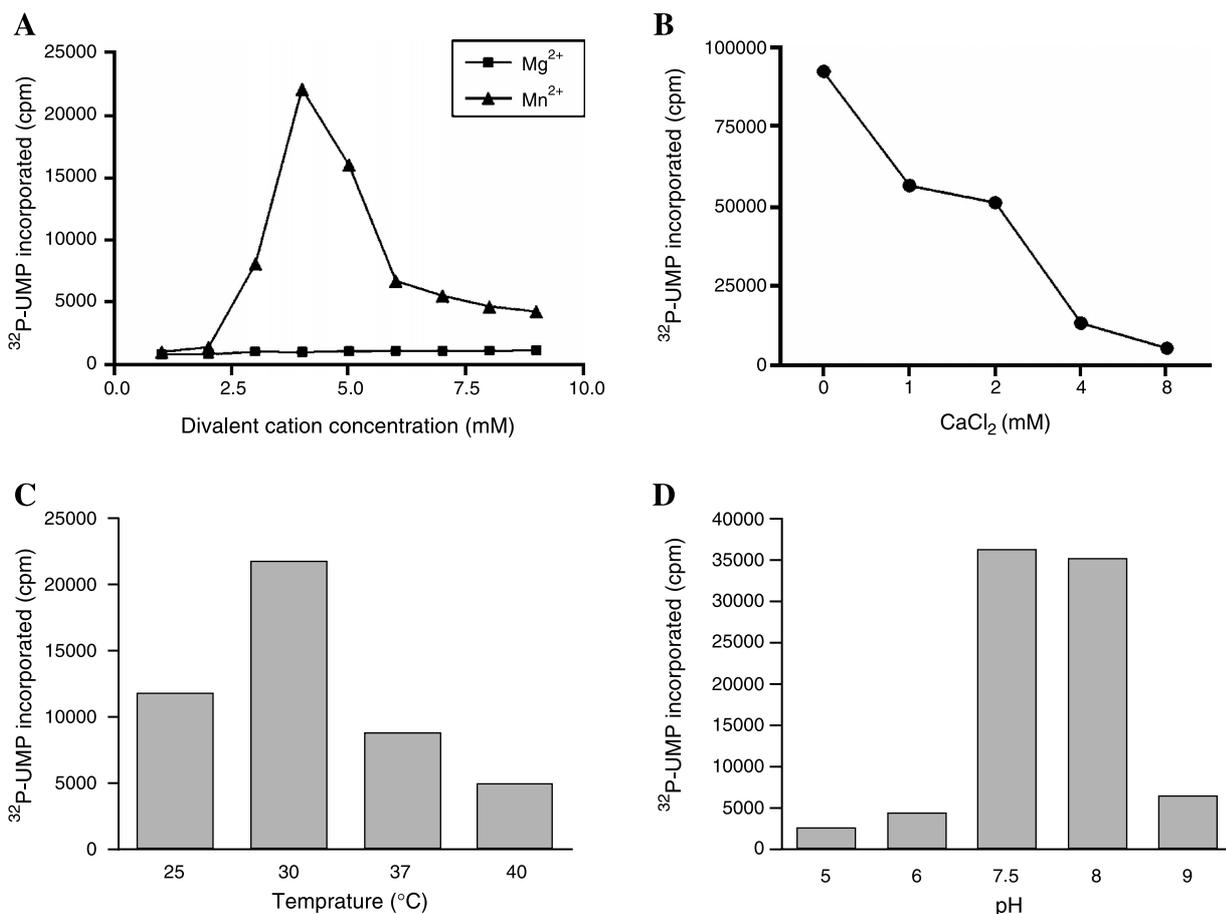
Fig. 3. Characterization of RNA polymerase of JEV. The RdRp reaction was assayed in the standard reaction mixture with $3\,\mu g$ purified JEV NS5 and $5\,\mu g$ JEV 3$'$($-$) RNA template. **A** Optimization of the concentration of $MnCl_2$ or $MgCl_2$. **B** Effect of $CaCl_2$. **C** Determination of optimal reaction temperature. **D** Optimization of reaction pH

USA). In both cases, mainly template-sized products were obtained (Fig. 2C).

Characterization of the RNA polymerase activity of JEV NS5 protein

To characterize the RNA polymerase activity of purified JEV NS5 protein, its RdRp activity was examined under various assay conditions (Fig. 3). JEV NS5 purified with buffers lacking divalent metals was used to examine the effect of an increasing concentration of exogenously provided divalent metal ions. In the absence of exogenously provided divalent metal ions, no RNA synthesis was observed, indicating that the RNA polymerase activity was dependent on divalent cations. Manganese ions were 20 times more effective than magnesium in coordinating the catalytic reaction of RdRp at optimal concentration (3 mM) (Fig. 3A). The coordinating effect of Ca^{2+} ions was tested in 3, 6 and 12 mM solutions, and in all cases, no RNA synthesis was found (data not shown). The effect of Ca^{2+} on the RdRp reaction in the presence of 3 mM Mn^{2+} was also checked. Increasing concentrations of Ca^{2+} decreased the RNA synthesis by JEV NS5, indicating that Ca^{2+} inhibits the RNA polymerase activity of JEV NS5 (Fig. 3B). The RdRp reaction was carried out at different temperatures, establishing that the optimal temperature for the reaction is 30 °C (Fig. 3C). The reaction was also tested at different pH levels; the optimal pH of JEV NS5 polymerase ranged from 7.5 to 8.0 (Fig. 3D).

Characterization of polymerase assay products

RdRp products generated by JEV NS5 in our reaction system were mainly the same size as the input template RNAs (Fig. 2B and C). To check whether the template-length products were produced through *de novo* initiation of the template RNA, 1-kb JEV 3′ minus strand and plus strand RNA was pre-treated with sodium periodate. RdRp reactions were performed using templates with a blocked 3′ hydroxyl group. As shown in Fig. 4, the sodium-periodate-treated RNA can also serve as a template for RdRp, and the reaction products were almost the same as the untreated RNA, indicating that the product
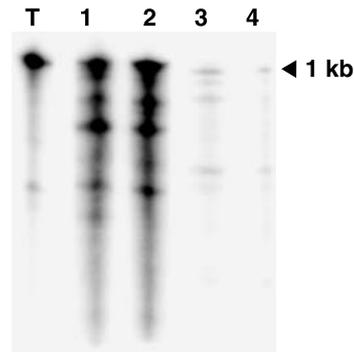

Fig. 4. NaIO$_4$ treatment of template RNA. 1-kb JEV 3′ minus-strand RNA and 1-kb JEV 3′ plus- strand RNA were treated with sodium periodate. RdRp reactions were performed using a template with a blocked 3′ hydroxyl group and compared with the corresponding RNA without treatment. *T* Labelled 1-kb JEV(−) RNA obtained by in vitro transcription; *1* 1-kb JEV(−) RNA as template; *2* NaIO$_4$-treated 1-kb JEV(−) RNA as template. *3* 1-kb JEV(+) RNA as template; *4* NaIO$_4$-treated 1-kb JEV(+) RNA as template

in this reaction system is not produced through a self-priming copy-back mechanism using the template RNA's 3′ end.

We sought to determine whether the RdRp product was in fact the plus strand formed by *de novo* initiation at the 3′ end of the RNA template. The RdRp reaction product obtained using 1-kb JEV 3′ (−) RNA as template was treated with RNase A(10 μg/ml) under conditions of high salt(0.3 M NaCl) or low salt(0.01 M NaCl) concentrations at 37 °C for 30 min. The resultant RNase A digestions showed that under high-salt conditions, which facilitate degradation of single-stranded but not double-stranded RNA species, the labelled product remained undigested (Fig. 5. Lane 3). However, under low-salt conditions, which allow digestion of all RNA species, no labelled product was detected (Fig. 5. Lane 2). These results indicate that the products of JEV NS5 protein RdRp reactions were double-stranded RNA molecules, verifying that the radioactive RNA visualized on the gel is not simply a terminally radiolabeled input template RNA, as these would be degraded by RNase A.

These results, taken together, indicate that the purified NS5 protein initiates primer-independent *de novo* RNA synthesis.
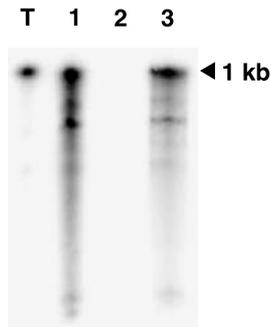
Fig. 5. The effect of ribonuclease A treatment of the polymerase reaction products. The ^{32}P-labeled RNA polymerase products obtained using 1-kb JEV 3′(−) RNA template were treated with RNase A and analyzed on an 8M-urea-containing 3.5% PAGE gel. *T* labeled 1-kb JEV (−)RNA obtained by in vitro transcription; *1* RdRp products without RNase A digestion; *2* after digestion with RNase A in the presence of 0.01 M NaCl. *3* after digestion with RNase A in the presence of 0.3 M NaCl

## Activity of JEV NS5 RdRp with different RNA templates

In flavivirus genomes, two conserved sequences within the 3′-untranslated region (3′-UTR) as well as stem-loop structures within the 3′- and 5′-UTRs are thought to be important for viral RNA replication. Hence, assuming that the two terminal regions of the JEV genome contain domains that are important for the regulation of RNA replication, we compared the efficiency of RNA synthesis with templates of the plus- or the minus-strand JEV virus RNAs. Approximately 1-kilobase(kb) RNAs corresponding to the regions of the 3′ end of the plus- or the minus-strand JEV genome were transcribed from PCR products *in vitro* and used as RNA template. As shown in Fig. 6A, both the plus-strand RNA and the minus-strand RNA were used as templates, but the incorporation of [^{32}P]-UMP was much lower when using positive-strand RNA as template than when using negative-strand RNA – an almost 10-fold difference in efficiency. A similar result was observed when using around 1 kilobase of 3′ end plus- and minus-strand RNA from the genome of another flavivirus(dengue 2 virus), which gave a difference in yield of about 5-fold. When the reaction products were analyzed on an 8M urea-denatured 3.5% PAGE gel, the synthesized RNA bands were much stronger when
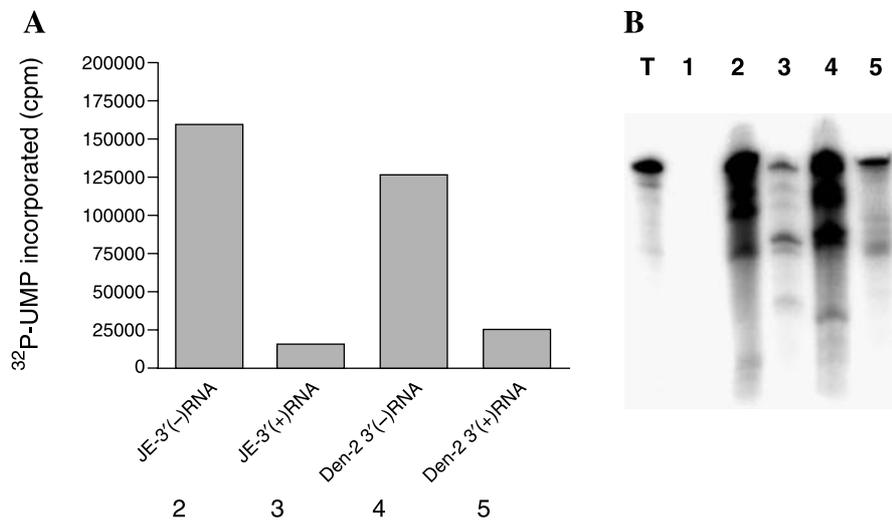
Fig. 6. Comparison of RNA synthesis by JEV NS5 using JEV and dengue-2 virus 3′ end plus- and minus-strand RNA templates. Around 1 kb plus or minus strands of JEV or dengue-2 virus 3′ end RNA were used in equal amounts (5 μg) as templates for standard RdRp reaction as mentioned in Materials and methods. The reaction products were analyzed after 4 h of reaction at 30 °C and checked by scintillation counting (**A**). **B** The reaction products (5 μl) were denatured and run on a 8M urea, 3.5% PAGE gel. *T* ^{32}P-labeled 1-kb JEV 3′(−)RNA template obtained by in vitro transcription. *1* Without RNA template. *2* 1-kb JEV 3′(−)RNA as template. *3* 1-kb JEV 3′(+) RNA as template. *4* 1-kb Den-2 virus 3′(−) RNA as template. *5* 1-kb Den-2 virus 3′(+) RNA as template

using the minus-strand RNA as template (Fig. 6B, Lanes 2 and 4) than when using the plus-strand RNA template (Fig. 6B, Lanes 3 and 5).
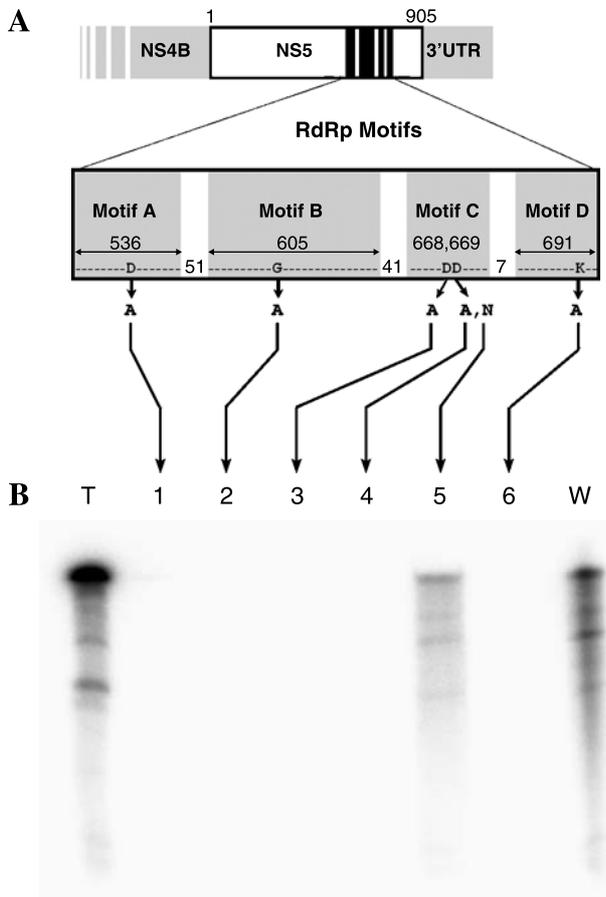
Fig. 7. Mutation analysis of JEV NS5 protein. Site-directed amino acid substitutions were introduced to the four motifs of JEV NS5 protein. **A** Diagram of NS5 protein and the four conserved motifs. Bold capital letters refer to amino acid residues that are highly conserved among all viral polymerases. Numbers above the letters refer to the amino acid position of NS5. Arrows below the letters point to the substitutions made. **B** RNA synthesis by mutated JEV NS5 proteins. The mutant proteins were expressed and purified in the same way as wild-type NS5. Standard RdRp reactions were conducted using these mutant NS5 proteins and 1-kb JEV 3′(−) RNA as template. The reaction products were extracted and analyzed on an 8M-urea-denatured 3.5% PAGE gel. *T* labeled 1-kb JEV 3′(−) RNA template obtained by in vitro transcription; *1* RdRp products obtained using NS5mutA; *2* RdRp products obtained using NS5mutB; *3* RdRp products obtained using NS5mutC; *4* RdRp products obtained using NS5mutC2; *5* RdRp products obtained using NS5mut Cn; *6* RdRp products obtained using NS5 mut D; *w* RdRp products obtained using wild-type NS5

Mutation analysis of the RNA polymerase of JEV NS5 protein

By comparing the sequences of various reverse transcriptases and various viral RdRps, Poch et al. identified four sequence motifs for RdRps [18]. By sequence alignments of JEV NS5 with other viral RdRps, the same four sequence motifs could be identified.

To identify the importance of these motifs for the JEV NS5 RdRp activity, single amino acid substitutions were introduced into the JEV NS5 gene by site-directed mutagenesis. The exact substitution were: Asp536 to Ala in Motif A, Gly605 to Ala in Motif B, Asp668 to Ala, Asp669 to Ala and Asp669 to Asn in Motif C, Lys691 to Ala in Motif D (illustrated in Fig. 7A). The mutant proteins were expressed in *E. coli* and purified as described for the parental JEV NS5. The mutations were confirmed by DNA sequencing. For all proteins, the expression level and purity were comparable to those of the wild type (data not shown). To measure the RdRp activities of all of the mutant NS5 proteins, standard reactions were performed with JEV 3′-end 1-kb (−) RNA as template, and 3 µg of each purified NS5 protein mutant was used for each reaction. Of the 6 mutants in the 4 motifs we produced, 5 substitutions led to complete inactivation of the enzyme. Only one mutant, NS5 mutCn, the Asp-to-Asn substitution at position 669 in Motif C showed a reduction in activity to about 5% relative to the parental NS5 (Fig. 7B). Revertant mutation of each mutant was performed in the same way, and polymerase activity was recovered in all the revertants (data not shown). These results also confirm that the RNA polymerase activity is due to the wild JEV NS5 protein rather than contaminating proteins derived from bacterial cell lysates.

Discussion

We successfully expressed JEV NS5 protein in *E. coli* and purified the recombinant protein to near homogeneity. The purified recombinant protein was enzymatically active, and we were able to demonstrate polymerase activity in the absence of either viral or host cofactors. This indicates that the product of the viral NS5 gene is an RdRp, which is involved in the replication of the virus. This is the

first direct confirmation of JEV's NS5 as a viral RdRp. As with other enzymes of this class, the RdRp activity of JEV was found to be dependent on divalent ions, Mn^{2+} or Mg^{2+}. We found that Mn^{2+} is much more effective than Mg^{2+} in the RdRp reactions, similar to reports for HCV NS5B [8, 16]. Taken together, this would suggest that Mn^{2+} is more favoured by flaviviral RdRps. Ca^{2+} inhibits polymerase activity. The optimal reaction conditions for *in vitro* RdRp assay were determined. Like the report for Polio virus RdRp, the optimum reaction temperature is $30\,^{\circ}C$ [10]. A possible reason for a low optimal reaction temperature may be the fact that the rate of UMP incorporation at lower temperature was linear over a longer time period. This *in vitro* RdRp reaction system would be a good system for studying the viral and host factors involved in viral replication and also for assessing antiviral drugs targeting viral replication.

RNA viruses initiate RNA synthesis mainly by two mechanisms: a primer extension mechanism and a *de novo* initiation mechanism [12, 13]. In this report, RNA synthesis occurred without adding primers; the RdRp products were mainly the same size as the input template when analysed on 8M-urea-denatured PAGE gel, no products twice the size of template were found. Sodium periodate treatment of the template, which blocked the $3'$ end of the template to prevent "copy-back" synthesis [1, 27], does not otherwise change the RdRP reaction products (Fig. 4), and RNase A treatment did not digest the synthesised RNA under high-salt conditions (Fig. 5). All of these results indicates that JEV NS5 RdRp initiate RNA synthesis on RNA templates by a *de novo* mechanism.

Ranjith-Kumar et al. reported that Mn^{2+} cations significantly increased the *de novo* initiation products of HCV RdRp [19]. Ackermann et al. reported that at low initiation temperature, more *de novo* initiation products were produced [1]. In our reaction system, we use Mn^{2+} instead of Mg^{2+}, and the reaction temperature we use is low. This may partially explain why in our reaction system, no primer extension products were found. This may also be related to the RNA template used and the RdRp characteristics of each virus. In RdRp of dengue 1 virus and Kunjin virus, only template-sized products were produced [11, 23].

The NS5-mediated reaction was found not to be limited to JEV RNAs. RNA synthesis was also observed with HCV and unrelated input RNA templates. This is a common phenomenon among RNA polymerases [4, 23]. This suggests that NS5 is necessary but not solely sufficient to direct the replication of the JEV genome as other viral and/or cellular factors must by necessity be involved in this process in order to restrict the RNA synthesis exclusively to viral templates.

During the plus-strand RNA virus replication cycle, the genomic RNA serves as a template for synthesis of negative-strand RNA, and the negative-strand RNA serves as a template for synthesis of further positive-strand genomic RNA. The synthesis of positive-strand RNA versus negative-strand RNA must be regulated because a ratio of positive-to negative-strand RNA of 10:1 to 100:1 has been observed in virus-infected cells [2, 3, 5, 6, 20]. When we compared the efficiency of RNA synthesis of JEV NS5 protein using JEV and dengue-2 virus minus-strand RNAs templates with that of the corresponding plus-strand templates, a 5–10 fold efficiency difference was observed (Fig. 6). JEV NS5 protein used the minus-strand RNA to synthesize the plus-strand product much more efficiently than it uses the plus-strand template to synthesize the minus RNA *in vitro*. This correlates with findings in virus-infected cells in which 10–100 times more plus-strand RNA was synthesized than minus-strand RNA. Our results indicate that the RNA ratio in infected cells is, at least partially, regulated by the viral RdRp.

In a recent report, Filomatori et al. suggest that the $5'$ UTR of dengue 2 virus genome functions as a promoter for RdRp activity [9]. Comparing RNA molecules carrying the $5'$-terminal or the $3'$-terminal sequences of the dengue 2 virus genome RNA, they observed a remarkable preference (100 fold) for the template carrying the $5'$-terminal sequences ($5'$DV RNA) over that of the RNA template containing the $3'$ end sequences[9]. Similar to their report, we observed a much lower RNA synthesis efficiency using the $3'$-end plus-strand RNA template with the NS5 protein, which may be due to the lack of a $5'$ end promoter sequence in the template.

Numerous studies have shown that nucleic acid polymerases have fundamental structural and mech-

anistic similarities, which are reflected by four distinct sequence motifs, Motif A, Motif B, Motif C and Motif D [14, 17, 18]. Using linear sequence alignments of JEV NS5 with other RdRps as a basis for site-directed mutagenesis experiments, we also identified four amino acid motifs which appear to play a vital role in the polymerase activity for JEV. Substitutions of almost all these 4 motifs led to a complete inactivation of the enzyme, except for the Asp-to-Asn substitution at position 669 in Motif C, which markedly reduced the activity to about 5% that of parental NS5. This GDN motif was the RdRp motif for some of the negative-strand RNA viruses in the alignment comparison, and this higher tolerance of the second Asp residue of the GDD motif for substitutions has also been reported for other RNA polymerases [17, 18]. These mutagenesis results also confirm that the RNA polymerase activity is due to the wild JEV NS5 protein rather than contaminating proteins derived from the bacterial cell lysates.

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