RNA-DEPENDENT RNA POLYMERASE ACTIVITY OF CLASSICAL SWINE FEVER VIRUS NS5B PROTEIN EXPRESSED IN NATURAL HOST CELLS

M. XIAO[1,2], J. CHEN[2], B. LI[2*]

[1]Biology Department, College of Life and Environment Sciences, Shanghai Teachers' University, Shanghai, 200234 P.R. China;
[2]Key Laboratory for Biodiversity Science and Ecological Engineering of Ministry of Education,
The Institute of Biodiversity Science, Fudan University, Shanghai, 200433 P.R. China

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Summary. – The NS5B gene, cloned from Classical swine fever virus (CSFV) genome, was expressed in porcine kidney cells PK-15, natural host of CSFV. In purifying cytoplasmic extracts from these cells by means of different concentrations of salt, glycerol and detergent four fractions, namely crude supernatant (SC) and different purified supernatants (S1, S2 and S3) were obtained. Using Western blot analysis the NS5B protein was found in all these fractions, showing that it was soluble in both higher and lower concentrations of salt, glycerol and detergent. The NS5B protein present in the four different fractions exhibited RNA-dependent RNA polymerase (RdRp) activity, but it was unable to complete the whole process of RNA synthesis. Site-directed mutation analysis showed that Thy^{216} and Cyt^{228} were essential for RNA synthesis while Cyt^{219} was not, suggesting that CSFV RdRp was template-specific. We conclude that initiation of RNA synthesis by CSFV RdRp includes also template priming.

Key words: NS5B gene; Classical swine fever virus; RNA-dependent RNA polymerase

Introduction

The species *Classical swine fever virus* (CSFV), *Bovine viral diarrhea viruses 1* and *2* (BVDV-1, BVDV-2), and *Border disease virus* (BDV) are members of the *Pestivirus* genus of the family *Flaviviridae* (van Regenmortel *et al*., 2000). The CSFV genome consists of a positive single-stranded RNA of approximately 12.5 kb. It contains a single long ORF, flanked by untranslated regions (UTRs) at the 5'- and 3'-ends (Moormann *et al*., 1996; Ruggli *et al*., 1996; Moser *et al*., 1999). Translation of the ORF leads to a single polyprotein precursor that is both co- and post-translationally processed by cellular and viral proteases, giving rise to 5 structural (N^{pro}, C, E1, E2, and E0) and 7 non-structural (NS) viral proteins (P70, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Moormann *et al*., 1996; Ruggli *et al*., 1996; Moser *et al*., 1999). The NS5B protein is responsible for replication of the CSFV genome (Lohmann *et al*., 1999; Steffen *et al*., 1999; Xiao *et al*., 2002b). The NS3 was found to exhibit RNA-stimulated NTPase and/or RNA helicase activity (Jin and Peterson, 1995; Warrener and Collett, 1995; Wengler and Wengler, 1991). The 3'-UTR and 5'-UTR are most likely involved in regulation of genome replication of pestiviruses (Yu *et al*., 1999; Xiao *et al*., 2001, 2002a). The latter consists of two consecutive processes. The replicase first binds to the 3'-UTR and starts the genome replication, in which

*Corresponding author. E-mail: xiaoming88@263.net; xiaom88@shtu.edu.cn; fax: +8621-64322933.

Abbreviations: BDV = Border disease virus; BVDV = Bovine viral diarrhea virus; CSFV = Classical swine fever virus; DMEM = Dulbecco's Modified Eagle's Medium; DTT = dithiothreitol; HCV = hepatitis C virus; IRES = internal ribosomal entry site; NS = non-structural; PBS = phosphate-buffered saline; RdRp = RNA-dependent RNA polymerase; RT-PCR = reverse transcription – polymerase chain reaction; SC = crude supernatant; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; UTR = untranslated region

a minus RNA is produced on the infecting plus RNA as template. Then a progeny plus RNA is produced on the minus RNA as template (Deng and Brock, 1993; Hulst *et al*., 2000; Meyers *et al*., 1989, 1996; Moser *et al*., 1999; Ranjith-Kumar, 2002; Tratschin *et al*., 1998; Yu *et al*., 1998). 5'-UTR of the plus RNA is also the site of initiation of translation of the CSFV genome. An internal ribosomal entry site (IRES) is present at the 5'-UTR (Le *et al*., 1995).

RdRps of all plus strand RNA viruses are similar in structure; they contain motifs designated A, B, C, D, and E (Ferrari *et al*., 1999; O'Reilly and Kao 1998). The CSFV NS5B gene is located at the 3'-end of the genome next to 3'-UTR, which is similar to the NS5B gene location of BVDV and Hepatitis C virus (HCV).

The NS5B protein has been expressed and shown to have the RdRp activity in HCV (Behrens *et al*., 1996; Lohmann *et al*., 1997), BVDV (Zhong *et al*., 1998) and CSFV (Lohmann *et al*., 1999; Steffen *et al*., 1999). However, expression in these studies was achieved in cells that were not natural hosts of the viruses tested. As the RdRp activity of the NS5B protein may be host-specific, for studying this phenomenon it is necessary to express the CSFV NS5B protein in the cells that are natural host of CSFV.

In this report, we expressed the CSFV NS5B protein in porcine kidney cells PK-15, natural host of CSFV and demonstrated RdRp activity of CSFV.

Materials and Methods

Vector for expression of the CSFV NS5B protein was constructed as described recently with a minor modification (Xiao *et al*., 2002). In brief, the sequence containing CSFV NS5B gene was isolated from CSFV (Shimen strain) RNA using reverse transcription–polymerase chain reaction (RT-PCR) and inserted into the pGEM-T vector (Promega). The complete NS5B cDNA sequence was isolated from the pGEM-T vector using PCR and inserted into the pcDNA-3.1 vector (Clontech). The resulting recombinant vector pcDNA-NS5B that carried a complete CSFV NS5B gene, was used for transfection of PK-15 cells.

Cell culture. PK-15 cells, the natural host of CSFV were used for expressing the CSFV NS5B protein. They were obtained from the China Center for Type Culture Collection, Wuhan. The cells were cultured in DMEM (Gibco BRL) supplemented with 10% of fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a humidified 5% CO_2 atmosphere.

Transfection. The vector pcDNA-NS5B was purified using the Wizard plus Minipreps DNA Purification System (Promega). Transfection of PK-15 cells with the pcDNA-NS5B vector was done in 16-well plates using the LipofectAMINE™2000 Reagent (Gibco-BRL) according to the manufacturer's instructions. At each well, 0.5 ml of cell suspension (3 x 10^5 cells) was transfected with 1 µg of the pcDNA-NS5B vector in 50 µl of DMEM containing 3 µl of the LF2000 reagent. After incubation at room temperature for 20 mins the DNA-LF2000 reagent complexes were directly added to the wells with cells. The plates with the complexes were further incubated at 37°C for 48 hrs and the transformants were selected using G418. All steps with cell cultures were carried out in a humidified atmosphere with 5% of CO_2.

Preparation of cytoplasmic extracts from transformed PK-15 cells was carried out according to two protocols. The first protocol recently described by Xiao *et al*. (2002) was slightly modified. Approximately 3.0 x 10^8 cells were pelleted, washed three times with PBS, carefully suspended in a cell lysis buffer (200 mmol/l Tris-HCl pH 8.0, 500 mmol/l NaCl, 10 mmol/l $MgCl_2$, 2% Triton X-100, 10 mmol/l imidazole, and 50% glycerol), and incubated at 4°C for 30 mins. A crude supernatant (the CS fraction) was obtained by centrifugation at 10,000 x g at 4°C for 10 mins. Another protocol described by Lohmann *et al*. (1997) was also slightly modified. Briefly, approximately 3.0 x 10^8 cells were suspended in 5 ml of the lysis buffer I (10 mmol/l Tris-HCl pH 7.5, 10 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, and 10 mmol/l 2-mercaptoethanol) and incubated at 4°C for 30 mins. A supernatant (the S1 fraction) was obtained after centrifugation at 10,000 x g at 4°C for 10 mins. The resulting pellet was resuspended in 5 ml of the lysis buffer II (20 mmol/l Tris-HCl pH 7.5, 300 mmol/l NaCl, 10 mmol/l $MgCl_2$, 0.5% Triton X-100, 20% glycerol, and 10 mmol/l 2-mercaptoethanol) and sonicated briefly. After 10 mins of centrifugation at 10,000 x g, the resulting supernatant was saved (the S2 fraction). The pellet was resuspended in 5 ml of the lysis buffer III (200 mmol/l Tris-HCl pH 7.5, 500 mmol/l NaCl, 10 mmol/l $MgCl_2$, 2% Triton X-100, 10 mmol/l imidazole, and 50% glycerol). After sonication and centrifugation as above the supernatant (the S3 fraction) was saved. The above 4 fractions (SC, S1, S2, and S3) were subjected to RdRp activity assay.

SDS-PAGE and staining with Coomassie Blue were used to prove the authenticity of the expressed NS5B protein (Mondelli *et al*., 1994).

Western blot analysis was preformed as described by Mondelli *et al.* (1994). A rabbit polyclonal antiserum directed against the CSFV NS5B protein was raised by a standard method (Sambrook *et al*., 1989).

Preparation of RNA for RdRp assay. A 603 nucleotide-long CSFV RNA corresponding to the 3'-end of the genome was generated by *in vitro* transcription. The cDNA sequence of the fragment was cloned from the CSFV (Shimen strain) genome into the pGEM-T vector (Promega). Based on the pGEM-T vector containing the cDNA fragment, a template for the *in vitro* transcription was synthesized by PCR. As PCR primers 5'-TAATACGACTCAC TATAAGCTGGCCAAG-3' containing the T7 promoter and 5'-GGGCCGTTAGGAAATTACCTTAGT-3' were used. The resulting PCR products contained the T7 promoter in addition to original cDNA sequence, and served as template for the subsequent *in vitro* transcription.

In vitro transcription was performed in 50 µl of reaction mixture following a standard method: 20 µl of 5x transcription buffer, 2 µl of RNasin (20–40 U/µl, Promega), 5 µl of each NTP (2.5 mmol/l), 5 µg of the template, 2 µl of T7 RNA polymerase (10–20 U/µl, Promega). The mixture was incubated at 37°C for 2 hrs. Then 10 µl of DNase (Takara) was added to the mixture and the latter was incubated at 37°C for 15 mins. The mixture was extracted with phenol/chloroform/isoamylalcohol. After ethanol precipitation, the RNA was dried, and redissolved in 20 µl of bi-

distilled H_2O. The resulting RNA contained a 603 nucleotide-long sequence corresponding to the 3'-end of the CSFV genome. It was used as RNA template for the *in vitro* RdRp assay.

Site-directed mutation analysis was performed by PCR using 12 primer pairs with mutation information and by subsequent *in vitro* transcription. The sense primer was the same in all cases, it included the T7 promoter sequence (i.e. 3'-TAATACGACTCAC TATAAGCTGGCCA-5'). The antisense primers were:

5'-GGGCCGTTAGGATATTACCTT-3'
5'-GGGCCGTTAGGACATTACCTT-3'
5'-GGGCCGTTAGGAGATTACCTT-3'
5'-GGGCCGTTAGGAATTACCTTAGTCCAACT-3'
5'-GGGCCGTTATGAAATTACCTT-3'
5'-GGGCCGTTAAGAAATTACCTT-3'
5'-GGGCCGTTACGAAATTACCTT-3'
5'-GGGCCGTTAGAAATTACCTTAGTC-3'
5'-TGGCCGTTAGGAAATTACCTT-3'
5'-CGGCCGTTAGGAAATTACCTT-3'
5'-AGGCCGTTAGGAAATTACCTT-3'
5'-GGCCGTTAGGAAATTACCTT-3'

PCR products containing the T7 promoter served as templates for *in vitro* transcription with T7 polymerase. Products of *in vitro* transcription represented RNA templates with different mutations.

RdRp assay was performed essentially as described previously (Xiao *et al*., 2002; Lohmann *et al*., 1999). The reaction mixture (50 µl) consisted of 20 µl of cytoplasmic fraction to be tested with the following supplements: 50 mmol/l Hepes pH 8.0, 5mmol/l $MgCl_2$, 10 µmol/l dithiothreitol (DTT), 25 mmol/l KCl, 1 mmol/l EDTA, 20 U of RNasin, 50 µg of actinomycin D (Sigma), 10 or 200 µmol/l each NTP including [alpha-^{32}P]CTP, and 1 µl of 250 ng/ml RNA template. The mixture was incubated at 37°C for 2 hrs, and the reaction was stopped by addition of 2 µl of 200 mmol/l EDTA. The reaction sample was extracted with phenol/chloroform and RNA was precipitated with isopropyl alcohol. The precipitate was dissolved in 25 µl of the gel buffer (40 mmol/l MOPS pH 7.0, 10 mmol/l sodium acetate, 1 mmol/l EDTA, 50% (v/v) formamide, and 2.2 mol/l formaldehyde), heated to 55°C for 15 mins, chilled on ice, and 1 µl of 10 mg/ml ethidium bromide was added. After 10 mins of incubation at room temperature, 5 µl of the loading buffer (50% glycerol, 0.25% Bromphenol Blue, 0.25% xylene/cyanol, and 1 mmol/l EDTA) was added, and samples were subjected to electrophoresis in 1.5% agarose gel with a buffer consisting of 2.2 mol/l formaldehyde, 40 mmol/l MOPS pH 7.0, 10 mmol/l sodium acetate, and 1 mmol/l EDTA. The electrophoresis was run at 5 V/cm. The gels were dried and autoradiographed (Eastman Kodak).

Oxidation of hydroxyl groups of RNA templates was carried out with sodium metaperiodate as described by Behrens *et al*. (1996). In brief, 10 µg of an RNA transcript was dissolved in 200 µl of 50 mmol/l sodium acetate pH 5.0. Then 50 µl of 100 mmol/l sodium periodate was added and the mixture was incubated at 22°C for 1 hr and extracted with phenol/chloroform. RNA was precipitated with isopropyl alcohol, washed with 70% ethanol and sodium periodate was removed by gel filtration. The degree of oxidation of template RNA was checked by 3'-end labeling with [^{32}P]pCp (Amersham) using T4 RNA ligase (Pharmacia) under the conditions suggested by the manufacturer.

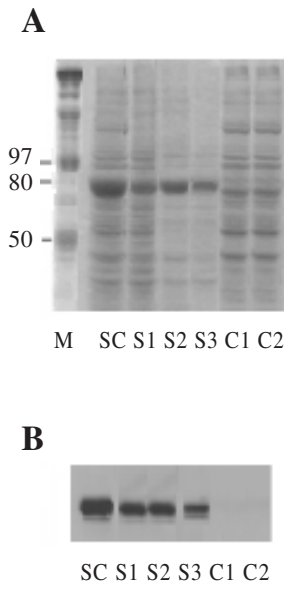
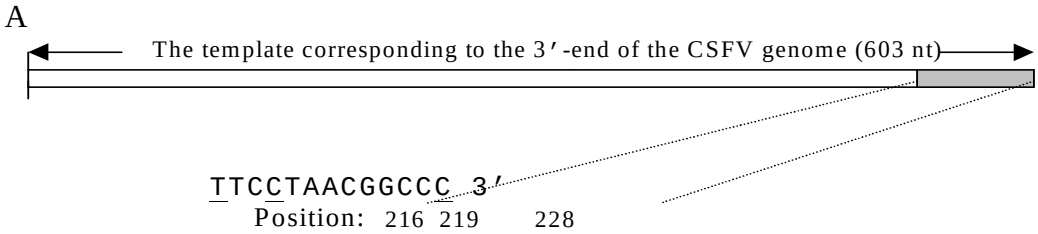


Fig. 1
NS5B protein in different fractions of cytoplasmic extract
SDS-PAGE (A) and Western blot analysis (B). C1 and C2 are controls, i.e. cytoplasmic extract untransfected with the pcDNA-NS5B vector.

Results and Discussion

Expression of CSFV NS5B protein in PK-15 cells

To characterize the CSFV RdRp activity in PK-15 cells, the CSFV NS5B protein was fused with a green fluorescent protein (GFP). RNA polymerization was detected in the presence of the expression product and the 3'-UTR of CSFV RNA. For correct depiction of the CSFV RdRp activity, expression of the authentic CSFV NS5B protein was performed in PK-15 cells. We first attempted to express the authentic CSFV NS5B protein in PK-15 cells transfected with the expression vector, but little expression and poor RNA polymerization were observed (data not shown). To obtain a stronger expression in the cells only transformants selected with G418 were used. Cytoplasmic lysates of these cells were used for preparation of fractions SC, S1, S2 and S3. SDS-PAGE (Fig. 1A) and Western blot analysis (Fig. 1B) showed the presence of the NS5B protein in all these fractions. For SC, S2 and S3 fractions our results were consistent with the earlier finding that the NS5B protein was efficiently extracted from cell lysates with the lysis buffers containing high concentrations of salt, glycerol and detergent (Lohmann *et al*., 1999). It is worth noting that the NS5B protein was also present in the S1 fraction. However, earlier studies have shown that the NS5B protein expressed in insect or *Escherichia coli* cells was poorly soluble under



B

RNA 1 **A**TCCTAACGGCCC 3′ (T216A)
RNA 2 **G**TCCTAACGGCCC 3′ (T216G)
RNA 3 **C**TCCTAACGGCCC 3′ (T216C)
RNA 4 **-**TCCTAACGGCCC 3′ (T216-)
RNA 5 TTC**A**TAACGGCCC 3′ (C219A)
RNA 6 TTC**T**TAACGGCCC 3′ (C219T)
RNA 7 TTC**G**TAACGGCCC 3′ (C219G)
RNA 8 TTC**-**TAACGGCCC 3′ (C219-)
RNA 9 TTCCTAACGGCC**A** 3′ (C228A)
RNA 10 TTCCTAACGGCC**G** 3′ (C228G)
RNA 11 TTCCTAACGGCC**T** 3′ (C228T)
RNA 12 TTCCTAACGGCC**-**3′ (C228-)

Fig. 2
Scheme of RNA templates for RdRp assays

The 603 nucleotide-long CSFV RNA template corresponding to the 3'-end of the genome. The sequence of the 3'-end of the RNA template. The nucleotides to be mutated are underlined. Their positions are given below the sequence (A).
The sequences of twelve RNA templates (RNA 1–12) with mutation. Replaced nucleotides are in bold, deleted nucleotides are marked with dashes. Symbols in brackets specify mutations (B).

physiological conditions (Lohmann *et al*., 1997; Ronald *et al*., 1998; Ferrari *et al*., 1999), which might indicate that the solubility of the NS5B protein was host-specific. Evidently, the NS5B protein obtained from different expression systems has different solubility. The NS5B protein obtained from the expression system originating from the natural CSFV host was soluble in both high and low concentrations of salt, glycerol and detergent.

Verification of RdRp activity of CSFV NS5B protein expressed in PK-15 cells

To test whether the different cytoplasmic fractions have the RdRp activity they were subjected to the RdRp assay in which the natural CSFV genomic RNA was used as template. A 603 nucleotide-long CSFV RNA corresponding to the 3'-end of the genome with the 3'-UTR was generated by *in vitro* transcription (Fig. 2A). It was incubated with each of the four cytoplasmic fraction (i.e. SC, S1, S2, and S3) samples and two controls in the presence of other necessary components. With the SC fraction but not with the two controls a newly synthesized RNA was detected.. A series of RNA molecules of different length, shorter or longer than the template, was detected (Fig. 3). The CSFV NS5B protein present in the SC fraction exerted RdRp activity.

With the fractions S1, S2 and S3 no newly synthesized RNA was detected (Fig. 3), indicating that the expression product present in these fractions was unable to synthesize RNA. This fact was inconsistent with the previous finding that a purified NS5B could still synthesize RNA (Lohmann *et al*., 1997; Zhong *et al*., 1998).

It is well known that besides polymerases also other viral and host cell proteins may be involved in viral genome replication, and that the polymerases, other viral proteins

and host cell proteins constitute a replicase complex responsible for the whole process of genome replication.

Here we face a question whether some of essential cellular proteins were absent in the fractions S1, S2 and S3 so that the RNA synthesis could not proceed or their concentration was too low to complete the whole process of RNA synthesis. To answer this question we mixed all these fractions together and tested this mixture for the RdRp activity. However, in this case we detected no RNA products (data not shown). It is very likely that some cellular proteins essential for RNA synthesis in the S1, S2 or S3 fraction, e.g. cellular RNA helicase etc., were lost or denatured in the process of obtaining these fractions. It indicated that in the case of CSFV the NS5B protein obtained from the expression system alone might not be enough to complete the whole process of RNA synthesis. Since the SC fraction could synthesize RNA, the cellular proteins essential for the synthesis of RNA might be present in the SC fraction. Therefore, only the SC fraction was used in further experiments for characterizing the RdRp activity of the CSFV NS5B protein.

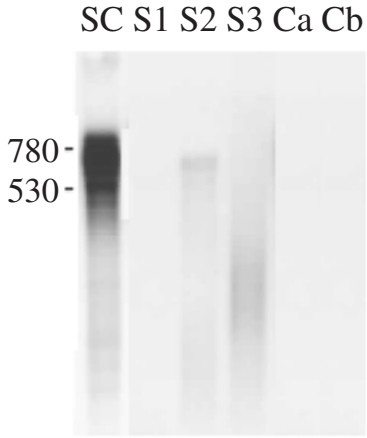


Fig. 3
RdRp activity of different fractions of cytoplasmic extract
Negative controls: no template (Ca), extracts from untransfected cells (Cb).

Characterization of template specificity of CSFV RdRp

To characterize the template specificity of CSFV RdRp, the site-directed mutation analysis of the 603 nucleotide-long CSFV RNA corresponding to the 3'-end of the genome was performed using PCR and *in vitro* transcription. The analysis focused on the nucleotides at positions 216, 219 and 228, suspected to be sensitive to initiation of RNA synthesis (Xiao *et al.*, 2001, 2002). The nucleotides of concern were Thy^{216}, Cyt^{219} and Cyt^{228} (Fig. 2A). The primers were designed for mutations at positions 216, 219 and 228 (see Materials and Methods). Four mutations at each position were made, namely the nucleotide at each position was replaced by other 3 nucleotides or removed. Twelve mutated RNA templates (RNA 1–12) were prepared (Fig. 2B).

Polymerization assays were performed in the presence of the mutated RNA templates. No newly synthesized RNA was detectable in the assays with any mutations at positions 216 or 228, while a newly synthesized RNA was found in the assays with any mutations at the position 219 (Fig. 4). Thus both Thy^{216} and Cyt^{228} were essential for initiation of RNA synthesis, while Cyt^{219} was not. Why did the same Cyt have different functions in RNA template in the RdRp assay?

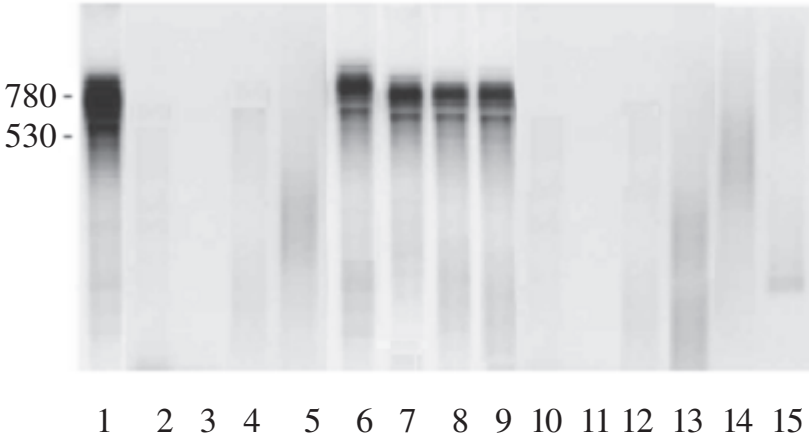


Fig. 4
Characterization of template-specificity of CSFV RdRp
RdRp assay. Non-mutated template (lane 1). Template mutated at the position 216 (lanes 2–5), 219 (lanes 6–9) and 228 (lanes 10–13). Template with oxidized hydroxyl group at the 3'-end (lane 14). No template (lane 15).

The reason may be that the same Cyt was located at different positions. Cyt^{228} was located at the 3'-end of the 3'-UTR, which has been demonstrated to be essential for the initiation of RNA synthesis in Hepatitis C virus (HCV, Behrens *et al*., 1996; Lohmann *et al*., 1997) and BVDV (Zhong *et al*., 1998). The fact that only a change of single nucleotide in natural RNA template resulted in no RNA synthesis shows that the initiation of RNA synthesis by CSFV RdRp needs a specific template, which is different from the previous finding obtained from the expression of NS5B protein in cells that are not natural host of CSFV, in which the RdRp activity of the NS5B protein was not template-specific (Lohmann *et al*., 1997; Steffen *et al*., 1999; Zhong *et al*., 1998). It was very likely that the NS5B protein obtained from different expression systems were different to some extent in exhibition of RdRp activity. PK-15 cells used for expression in this work were natural host for CSFV. Therefore, the template specificity might be a natural property of the NS5B protein in exhibiting the RdRp activity.

Characterization of initiation of RNA synthesis by CSFV RdRp

Previous studies have shown that synthesis of a nucleic acid by RdRp included a template-priming, divided into at least four consecutive steps: binding of RdRp to the template, initiation of RNA polymerization, polynucleotide elongation and termination (Lohmann *et al*., 1999). In this context minus strand RNAs are synthesized, then they are used as templates for synthesis of plus strand RNAs (Zhong *et al*., 1998; Kao *et al*., 1999; Lohmann *et al*., 1999). It is the hydroxyl groups at the 3'-ends of RNA templates that were the place to which the first nucleotide of the newly synthesized minus strand is connected. To examine whether the RdRp expressed in natural host cells possesses the same specificity, the hydroxyl groups of 3'-ends of RNA templates were oxidated and incubated with the SC fraction. No newly synthesized RNAs were observed in the reaction system (Fig. 4, lane 14). Therefore, it is suggested that initiation of RNA synthesis by the NS5B protein expressed in natural host cells included also template-priming, in agreement with the copy-back priming mechanism described earlier (Kao *et al*., 1999; Lohmann *et al*., 1997, 1999). Furthermore, the fact that the RNA products obtained from RdRp assays were longer than the templates supports the copy-back priming mechanism.

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