Characterization of the N-terminal domain of classical swine fever virus RNA-dependent RNA polymerase

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To investigate RNA-dependent RNA polymerase (RdRp) further, mutational analysis of the N-terminal domain of the NS5B protein of Classical swine fever virus was performed. Results show that the N-terminal domain (positions 1–300) of the protein might be divided artificially into four different regions, N1–N4. The N1 region (positions 1–61) contained neither conserved lysine nor conserved arginine residues. NS5B protein with deletion of the N1 region has the capacity for elongative RNA synthesis, but not for de novo RNA synthesis on natural templates. All substitutions of the conserved lysines and arginines in the N2 region (positions 63–216) destroyed RdRp activity completely. Substitutions of the conserved arginines in the N3 region (positions 217–280) seriously reduced RdRp activity. However, all substitutions of the conserved lysines in this region enhanced RNA synthesis and made the mutants synthesize RNA on any template. Substitutions of the conserved arginines in the N4 region (positions 281–300) reduced elongative synthesis and destroyed de novo RNA synthesis. In contrast, substitutions of lysines in this region did not affect RdRp activity significantly. These data indicate that the N3 region might be related to the enzymic specificity for templates, and the conserved lysines and arginines in different regions have different effects on RdRp activity. In combination with the published crystal structure of bovine viral diarrhea virus NS5B, these results define the important role of the N-terminal domain of NS5B for template recognition and de novo RNA synthesis.

INTRODUCTION

Classical swine fever virus (CSFV) is a small, enveloped virus, a member of the genus Pestivirus within the family Flaviviridae (Francki et al., 1991). The other members of this genus are Bovine viral diarrhea virus 1 (BVDV-1), BVDV-2 and Border disease virus (BDV) (Becher & Thiel, 2002; Heinz et al., 2000). Hepatitis C virus (HCV), the major cause of transfusion-associated hepatitis, also belongs to this family (Cuthbert, 1994). The genome of CSFV is a single, plus-strand RNA and contains a single, large open reading frame (ORF), a 5′ untranslated region (UTR) and a 3′ UTR. The ORF encodes a polyprotein of approximately 3900 aa (Moennig & Plagemann, 1992). The 3′ UTR is probably involved in initiation of pestivirus genomic replication and contains the sequence and structure elements that constitute the binding sites of cellular host factors and viral proteins (Isken et al., 2003, 2004; Pankraz et al., 2005; Xiao et al., 2004; Yu et al., 1999). The 3′ UTR might also be involved in the coordination of viral translation and replication (Isken et al., 2004). In addition to regulation of pestivirus genomic replication, the 5′ UTR is also the site for initiating translation of the viral genomes (Fletcher & Jackson, 2002). Moreover, the 3′ UTR may contact the 5′ UTR by RNA–RNA interactions (Isken et al., 2003).

The CSFV NS5B protein contains 718 aa and has been demonstrated to possess an RNA-dependent RNA polymerase (RdRp) activity (Steffens et al., 1999; Xiao et al., 2003). Previous studies have shown that the RdRps of plus-strand RNA viruses contain motifs designated A, B, C, D and E (O’Reilly & Kao, 1998). Motifs A, B, C and D are located at the catalytic portion of the palm domain (Hansen et al., 1997). Motif C, the signature motif for RdRps, forms a ‘β-strand–turn–β-strand’ structure at which a highly conserved sequence, ‘GDD’, is located (Kamer & Argos, 1984). The GDD sequence is postulated to be involved in the catalytic activity and metal-ion regulation of the enzyme and is believed to be a hallmark of all RdRps (Jablonski et al., 1991; Lohmann et al., 1997; Lai et al., 1999).

Deletion studies have revealed that the N and C termini of RdRp have different functions for enzymic activity. Deletion of 40 aa from the N terminus of the HCV NS5B protein...
abolishes RdRp activity completely. The polymerase activity of the HCV NS5B protein is reduced to about 42% of the wild-type activity when 55 aa are removed from the C terminus (Lohmann et al., 1997). For the BVDV NS5B protein, removal of up to 90 aa from the N terminus does not affect enzymic activity significantly, whereas deletion of 179 and 219 aa from the C terminus yields soluble proteins lacking RdRp activity (Lai et al., 1999). Our previous report has also shown that the activity of CSFV RdRp is lost when 82 aa are removed from the C terminus of NS5B (Xiao et al., 2003). However, the effect of the N terminus of CSFV NS5B protein on RdRp activity remains unknown. In this paper, we performed mutational analysis of the N-terminal domain of the CSFV NS5B protein and investigated the effects of the N-terminal domain on the RdRp activity of CSFV NS5B proteins.

**METHODS**

**Expression and purification of NS5B proteins.** The recombinant plasmid for expression of CSFV NS5B protein was constructed as described previously (Xiao et al., 2003). Total RNA was extracted from CSFV Shimen strain. A full-length NS5B cDNA was obtained by RT-PCR and cloned into the pET28a vector (Novagen). A methionine codon for initiating translation was added to the 5’ end of the NS5B coding sequence. Additional sequences encoding six histidines at the C terminus were engineered to facilitate the purification of the NS5B protein. The inserted regions of all clones were sequenced through dideoxynucleotide sequencing and no changes were found. A double-stranded site-directed mutagenesis kit (TaKaRa) and the oligonucleotide sites were used and allowed production of expected mutations in the cloned gene. These resulting plasmids were introduced into *Escherichia coli* strain BL21(DE3) for expression driven by bacteriophage T7 RNA polymerase. Expression was induced by addition of IPTG. Extraction and purification were performed as described by Oh et al. (1999). Briefly, the bacterial cell culture was harvested and washed with PBS. The cells from 1000 ml culture were resuspended in 20 ml buffer containing 50 mM sodium phosphate (pH 8-0), 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 10% glycerol and 1% Nonidet P-40, supplemented with 1 mM PMSF and 10 mM leupeptin. After undergoing freezing and thawing once, cells were subjected to sonication. The cleared lysate was obtained by centrifugation at 35 000 g for 15 min. The cleared lysate containing the recombinant protein was purified by using nickel-nitrilotriacetic acid (Ni–NTA)–Sepharose resin (Gibco-BRL). Briefly, CSFV NS5B with a polyhistidine tag was bound to Ni–NTA resin pre-equilibrated with the above buffer, and then washed with buffer containing 50 mM imidazole. The bound NS5B was eluted with buffer containing different concentrations of imidazole (100–500 mM). The NS5B protein was collected, combined and dialysed in buffer A ([50 mM Tris/HCl (pH 8–0), 1 mM dithiothreitol (DTT), 50 mM NaCl, 5 mM MgCl₂, 10% (v/v) glycerol]. NS5B proteins were quantified as described by Lohmann et al. (1997). In brief, NS5B protein solutions and dilutions of BSA with known concentration were subjected to SDS-PAGE. Gels with the samples were stained with Coomassie brilliant blue. The amount of NS5B protein was determined by densitometry scanning and comparing two samples on the same gel. Purified proteins were separated by SDS-PAGE.

**RNA preparation.** Homopolymeric RNA templates were purchased from Pharmacia. RNA oligonucleotides (12-mer) were purchased from Promega. A 603 nt RNA fragment containing the CSFV 3’ UTR and a random coding sequence was prepared as described previously (Xiao et al., 2004). Mutated plus and minus 3’ UTRs were produced through PCR with a pair of primers at the two sides of expected mutant fragments, as applied to our previous work (Xiao et al., 2004). The expected mutated cDNA fragments were cloned into the pGEM-T vector (Promega). After plasmids were extracted and sequenced, the corresponding RNA templates were synthesized by PCR and subsequent *in vitro* transcription. DNA Vent polymerase (New England Biolabs) and a primer containing the bacteriophage T7 promoter were used in the PCR. After the sequence was verified, the resulting PCR products served as templates for the subsequent *in vitro* transcription. The *in vitro* transcription was performed in 50 µl reaction mixtures following the standard method: 20 µl transcription buffer, 2 µl Rnasin (20–40 U µl⁻¹) (Promega), 5 µl each NTP (2:5 mM), 5 µg template and 2 µl T7 RNA polymerase (10–20 U µl⁻¹) (Promega). The mixture was incubated at 37 °C for 2 h. Then, 10 µl DNase I (TaKaRa) was added to the mixture and incubated at 37 °C for 15 min. The mixture was extracted with phenol/chloroform. After ethanol precipitation, the RNA was dried and redissolved in 20 µl double-distilled H₂O. Integrity of the RNA was analysed by denaturing formaldehyde/agarose gel electrophoresis. The concentration of RNA was determined by measuring OD₂₆₀.

Oxidation of the hydroxyl group of the 3’ terminus of RNA templates was carried out with sodium metaperiodate as described by Behrens et al. (1996). In brief, 10 µg RNA transcript was dissolved in 200 µl 50 mM sodium acetate (pH 5–0). After addition of 50 µl NaIO₄ (100 mM), the reaction system was incubated for 1 h at 220 °C and extracted with phenol/chloroform. RNAs were precipitated with isopropyl alcohol, washed with 70% ethanol and passed through gel filtration to remove the NaIO₄ in the sample. The degree of oxidation was controlled by labelling the RNA templates by 3’–end labelling with [³²P]P[NTP (Amersham Biosciences) and T4 RNA ligase (Pharmacia) under the conditions suggested by the manufacturers.

**RdRp assays.** RdRp activity of the purified wild-type and mutant NS5B proteins with homopolymeric RNA template–primer pairs was performed essentially as described by Lohmann et al. (1997). RdRp activity of the NS5B protein (200 ng) was measured by using 1 µg homopolymeric RNA template in the presence of 10 pmol corresponding primer. Incorporation of radioactivity was performed in a 1 h standard reaction at 37 °C with a total volume of 25 µl containing 20 mM Tris/HCl (pH 7–5), 1 mM DTT, 25 mM NaCl, 20 U RNasin (Promega), 5 mM MgCl₂, 5 µCi (185 kBq) [³²P]T7RNA polymerase, and 25 µg actinomycin D (Sigma). The labelled products were collected on a GF/C glass filter (Whatman) after being precipitated with 5% trichloroacetic acid (TCA). Incorporation of radioactivity was measured by scintillation counting.

Total volume to determine the polymerization activity of the wild-type and mutant NS5B proteins with the viral 3’ UTR template was 50 µl containing the following supplements: 50 mM HEPES (pH 8–0), 5 mM MgCl₂, 10 µM DTT, 25 mM KCl, 1 mM EDTA, 20 U RNasin, 50 µg actinomycin D (Sigma), 200 µM each NTP (including a single radiolabelled CTP, [³²P]CTP), 1 µl RNA template (250 ng ml⁻¹) and 50 nM NS5B protein (Xiao et al., 2004). The mixture was incubated at 37 °C for 1 h and the reaction was stopped by the addition of 2 µl EDTA (200 mM). The reaction samples were extracted with phenol/chloroform and RNAs were precipitated with isopropyl alcohol. Precipitates were redissolved in 25 µl gel buffer [40 mM MOPS (pH 7–0), 10 mM sodium acetate, 1 mM EDTA, 50% (v/v) formamide, 2:2 M formaldehyde], heated to 55 °C for 15 min and chilled on ice, and 1 µl ethidium bromide (10 mg ml⁻¹) was added. After 10 min incubation at room temperature, 5 µl loading buffer [50% (v/v) glycerol, 0:25% bromophenol blue, 0:25% xylene cyanol, 1 mM EDTA] was added and samples were loaded onto 1:5% agarose gels.
Characterization of the N-terminal domain of CSFV RdRp

RESULTS

Expression of wild-type and N-terminally truncated NS5B proteins

To analyse the importance of the N-terminal domain of the NS5B protein for enzymic activity, a series of deletions was introduced into the N-terminal domain of the CSFV NS5B protein. Additional sequences encoding polyhistidine peptides at the C terminus were engineered to facilitate the purification of the NS5B protein. The resulting plasmid was used to transform E. coli BL21 (DE3). Expression was induced by addition of IPTG. The truncated NS5B proteins were purified by using Ni–NTA resin. SDS-PAGE analysis showed that several N-terminally truncated NS5B proteins were expressed and purified efficiently (Fig. 1). The products, NS5BΔN45, NS5BΔN62, NS5BΔN73 and NS5BΔN100, were NS5B mutants lacking 45, 62, 73 and 100 N-terminal amino acids, respectively. As a comparison, the full-length NS5B protein was expressed and purified in the same way.

Optimal conditions for RNA synthesis

Template preference was analysed by using four homopolymeric RNA template–primer pairs: poly(A)–oligo(U)12, poly(C)–oligo(G)12, poly(G)–oligo(C)12 and poly(U)–oligo(A)12. The polymerase assays of the full-length NS5B protein were performed with the above four homopolymeric RNA template–primer pairs. It was found that the poly(C)–oligo(G)12 template–primer pair was preferred by the CSFV RdRp. A similar result was observed previously in HCV (Lohmann et al., 1997). Therefore, the poly(C)–oligo(G)12 template–primer pair was applied in the following experiments for further analysis of NS5B proteins.

In almost all previous RdRp assays for plus-strand RNA viruses, the reaction temperature used was 22, 25, 30 or 37°C. The reaction time was usually 1, 2 or 3 h (Ferrari et al., 1999; Lai et al., 1999; Oh et al., 2002; Zhong et al., 1998). Generally, both the reaction temperature and time have significant effects on the results of the RdRp assay. In order to identify the optimal conditions for our assays, several temperature–time combinations (i.e. 37°C–1 h, 37°C–2 h, 30°C–1 h, 30°C–2 h, 25°C–2 h and 22°C–3 h) were used in our CSFV RdRp assays, which included the poly(C)–oligo(G)12 template–primer pair and the full-length NS5B protein. Our data show that the optimal temperature–time combination was 30°C–2 h, followed by 37°C–1 h, 30°C–1 h, 37°C–2 h, 22°C–3 h and 25°C–2 h. The 37°C–1 h combination was only slightly less effective than the 30°C–2 h combination. Because 37°C is closer to the natural temperature for CSFV replication, the 37°C–1 h temperature–time combination was used in all of the following polymerase assays. Moreover, we found that the CSFV RdRp still had higher activities within a rather broad range of pH (6.5–8.0), being similar to its BVDV counterparts in this sense (Lai et al., 1999).

Previous reports have shown that magnesium (Mg2+) or manganese (Mn2+) ions are able to regulate NS5B enzymic activities (Lohmann et al., 1997; López Vázquez et al., 2000; Reigadas et al., 2001; Xiao et al., 2003). However, several studies (Ferrari et al., 1999; Lai et al., 1999) have demonstrated that, whilst Mg2+ ions have only weak effects on RdRp activities, Mn2+ ions are several times more effective than Mg2+ ions in regulating the activities (Ferrari et al., 1999; Lai et al., 1999). Our data show that Mg2+ ions are still effective in the CSFV RdRp assays and that Mn2+ and Mg2+ ions are equally effective. Our results are similar to those of other studies of CSFV RdRp, in which Mn2+ and Mg2+ ions support RNA synthesis almost equally (Yi et al., 2003). Ion regulation of enzymic activities might depend on the reaction temperature: Mn2+ ions might be more effective for RdRp assays than Mg2+ ions when the reaction temperature is 23 or 25°C. However, when 37°C is used, Mg2+ ions are more effective than Mn2+ ions (Labonté et al., 2002). Reaction between the Mg2+ ion and NS5B protein might represent in vivo RdRp–ion interaction, because a magnesium ion has been found to bind to the active site of the crystal structure of HCV RdRp (Ago et al., 1999; Lesburg et al., 1999). In addition, we also found that the activity is low when calcium ions (Ca2+) serve as the divalent cation. Similarly, Hansen et al. (1997) have shown that the Ca2+ ion does not support poliovirus 3D polymerase activity. Therefore, 5 mM MgCl2 was used in the following RdRp assays.

Fig. 1. Expression of the CSFV wild-type and N-terminally truncated NS5B proteins. Expression was induced by addition of IPTG. The truncated NS5B proteins were purified by using Ni–NTA resin. The expression products were subjected to separation by SDS-PAGE followed by Coomassie blue staining. Lane 1, wild-type NS5B; lane 2, NS5BΔN45; lane 3, NS5BΔN62; lane 4, NS5BΔN73; lane 5, NS5BΔN100. Numbers on the left refer to molecular mass markers (in kDa).
Enzymic activity for CSFV NS5B protein with N-terminal deletion

Our previous report has shown that CSFV NS5B proteins with a C-terminal deletion of 65 aa still have RdRp activity, but cannot tolerate removal of 82 aa from the C terminus (Xiao et al., 2003). To analyse the effects of the N-terminal domain on CSFV RdRp activity, we produced several CSFV NS5B proteins with N-terminal deletions that were subjected to RdRp assays.

Firstly, elongative RNA synthesis was performed with the poly(C)–oligo(G)_{12} template–primer pair. The RdRp assays of NS5BΔN73 or NS5BΔN100 revealed that CSFV NS5B proteins with N-terminal deletion of 73 or 100 aa could not elongate the oligo(G)_{12} RNA strand on the poly(C) template. In contrast, NS5BΔN45 and NS5BΔN62 still had RdRp activities for elongative RNA synthesis (Fig. 2a). To examine de novo RNA synthesis on natural templates, the 3’-terminal sequence, including the 3’ UTR, was obtained from the CSFV genome as described previously (Xiao et al., 2004). The 3’-terminal hydroxyl groups of this sequence were oxidized. The resulting sequence served as the RNA template and was included in primer-independent RdRp assays. As shown in Fig. 2(b), all of the NS5B proteins with N-terminal deletions lost their activities except for NS5BΔN45. In our previous report, the CSFV NS5B protein lacking 65 C-terminal amino acids holds almost the same enzymic activity as the wild type (Xiao et al., 2003). It is suggested that the N terminus is more important than the C terminus for RdRp activity of the CSFV NS5B protein. This conclusion also holds true for HCV NS5B, but not for BVDV NS5B (Table 1).

RdRp activity for NS5B proteins with substitution mutations

To gain a deeper insight into the effect of the N-terminal domain of the CSFV NS5B, the N-terminal amino acid sequence (positions 1–300) of CSFV NS5B protein was compared with those of the BVDV and BDV NS5B proteins. The alignment revealed that two conserved amino acids, lysine (K) and arginine (R), occurred frequently in the fragment of positions 63–300, but did not exist in the fragment of positions 1–62 (Fig. 3). It is suggested that the N terminus, rich in arginines and lysines, might interact with template, primer or nucleotides in polymerization (Lai et al., 1999). To test this hypothesis, mutational analysis of the conserved lysines and arginines was performed individually. All of the conserved lysines and arginines in the fragment of positions 63–300 were substituted individually by alanine (A). The mutated proteins were expressed and purified by using Ni–NTA resin and analysed by SDS-PAGE. These mutated NS5B proteins were incubated with the poly(C)–oligo(G)_{12} template–primer pair for elongative RdRp reaction and with the oxidized CSFV 3’ UTR for de novo RNA synthesis.

**Fig. 2.** RdRp activity for the CSFV NS5B protein with N-terminal deletion. (a) RdRp activity for elongative RNA synthesis was investigated. The RdRp activity of wild-type or mutated NS5B protein (200 ng) was measured by using poly(C)–oligo(G)_{12} with 5 mM MgCl_{2}. Incorporation of radioactivity was performed in a 1 h standard reaction at 37°C. The labelled products were collected on a GF/C glass filter after being precipitated with 5% TCA. Incorporation of radioactivity was measured by scintillation counting. The assay was repeated three times with similar results and the data are expressed as means (10^3 c.p.m.) from three independent experiments. (b) RdRp activity for de novo RNA synthesis. The 3’-terminal hydroxyl group of the RNA fragment containing the CSFV 3’ UTR was oxidized. The resulting sequence was incubated with the mutant NS5B proteins in the presence of a single radiolabelled CTP, [^32]PCTP, at 37°C for 1 h. In comparison, the wild-type NS5B protein was included in parallel in the above assays. After precipitation with isopropl alcohol, the reaction products were loaded onto 1.5% agarose gels containing 2-2 M formaldehyde, 40 mM MOPS (pH 7.0), 10 mM sodium acetate and 1 mM EDTA for electrophoresis. Gels were dried and analysed by using X-ray films. Lanes 1–5 represent wild-type, NS5BΔN45, NS5BΔN62, NS5BΔN73 and NS5BΔN100, respectively.

**Table 1.** RdRp relative activity of the different truncated NS5B proteins (CSFV, BVDV and HCV)

<table>
<thead>
<tr>
<th>Mutant*</th>
<th>Reference</th>
<th>Relative activity (%)†</th>
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<tr>
<td>CSFV NS5BΔN62</td>
<td>37</td>
<td>Fig. 2</td>
</tr>
<tr>
<td>CSFV NS5BΔC65</td>
<td>100</td>
<td>Xiao et al. (2003)</td>
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<tr>
<td>BVDV NS5BΔN90</td>
<td>100</td>
<td>Lai et al. (1999)</td>
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<tr>
<td>BVDV NS5BΔC24</td>
<td>100</td>
<td>Lai et al. (1999)</td>
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<tr>
<td>HCV NS5BΔN19</td>
<td>0–46</td>
<td>Lohmann et al. (1997)</td>
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<tr>
<td>HCV NS5BΔC55</td>
<td>42</td>
<td>Lohmann et al. (1997)</td>
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*NS5BΔN62, the NS5B mutant lacking 62 N-terminal amino acids; NS5BΔC65, the NS5B mutant lacking 65 C-terminal amino acids, and so on.
†We expressed the RdRp activities of truncated NS5B proteins as a percentage (relative activity) of the activities of their wild-type NS5B proteins (100%).
Our data show that all of the substitutions of arginines were detrimental to RdRp activity, leading to reduced elongative RNA synthesis and de novo RNA synthesis (Fig. 4), and even the termination of both forms of RNA synthesis (Fig. 4, lanes 2, 8, 10 and 11). Substitutions of lysines produced two contrary results, i.e. some reduced or destroyed RdRp activity, but some enhanced RdRp activity. Substitution of lysine at positions 65, 74, 85, 113, 120, 152, 172 or 175 led to a complete inactivation of RdRp (Fig. 4a, b). However, substitutions of lysines at other positions did not decrease the enzymic activity; on the contrary, they increased the enzymic activity. The seven mutated NS5B proteins (K219A, K228A, K238A, K249A, K255A, K263A and K266A) possessed higher activities than did their parental proteins (Fig. 4a, b). Substitution of lysine at one of positions 282, 293 and 300 enhanced elongative RNA synthesis, but affected de novo RNA synthesis only a little (Fig. 4, lanes 24, 26 and 28). Our results are similar to those of the previous reports that deal with substitutions of lysine or arginine at the same positions in BVDV (Lai et al., 1999). Nevertheless, a slight difference exists between these studies. Substitution of lysine at position 282 of BVDV NS5B creates an enzyme with an activity of 200% of that of wild type when it is incubated with poly(C)–oligo(G) (Lai et al., 1999). In the present study, substitution at the same position of the CSFV NS5B protein enhanced enzymic activity by only 15% (Fig. 4a, lanes 1 and 24). In BVDV, substitution of arginine at position 285 or 295 destroys elongative synthesis and de novo synthesis almost completely (Lai et al., 1999). Substitution at the same position of the CSFV NS5B protein led to a complete inactivation of de novo synthesis, but the corresponding mutant still held 44 or 43% enzymic activity for elongative RNA synthesis (Fig. 4, lanes 25 and 27). A possible reason is that the BVDV NS5B protein used in the previous reports was truncated from the C terminus, whereas the NS5B protein used in this study was full length. The three-dimensional structure of the truncated NS5B might be slightly changed, compared with that of the full-length protein, so that the interactive site is altered to a certain degree.

Conserved lysines relative to RdRp specificity

To understand why the substitution of lysine increased the enzymic activity, we further examined the RdRp activity of the mutated NS5B proteins by using three RNA templates: the first containing the 3′ UTR with a deletion at the 3′ terminus of the plus strand (designated +RNA5), the second containing the 3′ UTR with a deletion at the 3′ terminus of the minus strand (designated −RNA5) and the third containing an RNA random sequence corresponding to positions 1245–1700 of the plus-strand genome (designated RNAr2) (Xiao et al., 2004). These templates have been shown to lose the capacity for RNA synthesis (Xiao et al., 2004). When the hydroxyl groups of the 3′ termini of the RNA templates were oxidized and the resulting templates were respectively incubated with these mutated NS5B proteins, the seven mutants (K219A, K228A, K238A, K249A, K255A, K263A and K266A) possessed higher activities than did their parental proteins (Fig. 4a, b). Substitution of lysine at one of positions 282, 293 and 300 enhanced elongative RNA synthesis, but affected de novo RNA synthesis only a little (Fig. 4, lanes 24, 26 and 28). Our results are similar to those of the previous reports that deal with substitutions of lysine or arginine at the same positions in BVDV (Lai et al., 1999). Nevertheless, a slight difference exists between these studies. Substitution of lysine at position 282 of BVDV NS5B creates an enzyme with an activity of 200% of that of wild type when it is incubated with poly(C)–oligo(G) (Lai et al., 1999). In the present study, substitution at the same position of the CSFV NS5B protein enhanced enzymic activity by only 15% (Fig. 4a, lanes 1 and 24). In BVDV, substitution of arginine at position 285 or 295 destroys elongative synthesis and de novo synthesis almost completely (Lai et al., 1999). Substitution at the same position of the CSFV NS5B protein led to a complete inactivation of de novo synthesis, but the corresponding mutant still held 44 or 43% enzymic activity for elongative RNA synthesis (Fig. 4, lanes 25 and 27). A possible reason is that the BVDV NS5B protein used in the previous reports was truncated from the C terminus, whereas the NS5B protein used in this study was full length. The three-dimensional structure of the truncated NS5B might be slightly changed, compared with that of the full-length protein, so that the interactive site is altered to a certain degree.
conserved lysines at positions 219, 228, 238, 249, 255, 263 and 266 might be related to the CSFV RdRp specificity for template. Substitutions of the conserved lysines destroyed the specificity, so these mutants might synthesize RNA on any template. Investigation of the RdRp activity of K282A, K293A and K300A was performed with the above RNA templates. These mutants were unable to initiate RNA synthesis on the RNA templates (Fig. 4a, lanes 25–33). To be convincing, other NS5B mutants resulting from substitution of conserved arginines or from deletion of the N terminus were included in the RdRp assays with the above three types of RNA template. The three proteins, R218A, R252A and R267A, could not synthesise RNA on the template variants (Fig. 5b, lanes 1–9). The same results were obtained in R285A and R295A only with elongative activity (Fig. 5b, lanes 10–15). The 12 NS5B mutants losing RdRp activity entirely (Fig. 4a, b) were still unable to produce detectable RNA on the template variants. The same results were also observed in the four truncated NS5B proteins (Fig. 5b, lanes 16–27). Therefore, these amino acids at positions 1–100, the conserved arginines in the N-terminal domain and the lysines at positions 65, 74, 85, 113, 120, 152, 172, 175, 282, 293 and 300 are dispensable for the enzymic specificity of CSFV RdRp.

**DISCUSSION**

The N-terminal domain of RdRp, rich in arginine/lysine, is assumed to interact with template, primer or nucleotides. To test this hypothesis, we first examined the optimal conditions for RdRp activity. It was found that the CSFV RdRp increased elongative RNA synthesis when...
poly(C)–oligo(G)$_{12}$ served as the template–primer pair, 37 °C–1 h as the temperature–time combination and Mg$^{2+}$ as the divalent cation. The conditions identified here are similar to those used in other studies (Lohmann et al., 1997; Lai et al., 1999; Labonte et al., 2002). We also used oligonucleotide site-directed mutagenesis to perform deletion analysis and single substitution of the conserved arginines and lysines in the N-terminal domain of the CSFV NS5B protein. The RdRp activities of these mutated NS5B proteins were compared with those of their wild-type NS5B proteins.

Our results show that the N-terminal domain (positions 1–300) of the CSFV NS5B protein might be divided artificially into four different functional regions, designated N1–N4 (Fig. 3), which have different effects on RdRp activity. The N1 region, from positions 1 to 62, contains neither conserved lysine nor arginine. The N1 region-deleted NS5B protein has the capacity for elongative RNA synthesis, but not for de novo RNA synthesis on natural template (Fig. 2, lane 3). Although up to 90 N-terminal amino acids can be truncated from BVDV RdRp without loss of enzymic activity (Lai et al., 1999), this region in other polymerases is required for binding proteins in the replication complex (Shirako et al., 2000).

The N2 region (positions 63–216) is rich in conserved lysines and arginines. All substitutions of the conserved lysines and arginines in this region destroyed RdRp activity completely (Fig. 4a, b, lanes 2–13). Therefore, the N2 region of the CSFV NS5B protein is necessary for polymerase activity, which is consistent with the conclusion drawn from the recently published crystal structure of the BVDV NS5B protein (Choi et al., 2004). The crystal structure of this protein shows that the entrance to the template-binding channel of the RdRp is created by the β-hairpin motif of the N-terminal domain, which is rich in positively charged amino acids and may be used to open up secondary structure before the single-stranded RNA template entering the active centre (Choi et al., 2004). The β-hairpin motif corresponds with the fragment (positions 123–133) of the CSFV NS5B N2 region (Figs 3 and 6). Studies of the HCV NS5B crystal structure indicate that the conserved K51 of the N-terminal domain is probably involved in binding nucleotide and RNA during polymerization (Bressanelli

**Fig. 5.** Conserved lysines relative to RdRp specificity. The mutated NS5B proteins were included in the RdRp assays for investigating RdRp specificity. The results were for the mutated NS5B proteins in which the conserved lysines in the N3 and N4 regions were substituted individually (a) and for the mutated NS5B proteins in which the conserved arginines at N3 and N4 region were substituted individually and for four truncated NS5B proteins (b). Each mutated NS5B protein was incubated with three RNA templates with oxidized 3′-terminal hydroxyl groups, respectively. Lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28 and 31 are for assays with the first RNA template containing the plus 3′ UTR with deletion at the 3′ terminus (designated +RNA5); lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29 and 32 are for assays with the second RNA template containing the minus 3′ UTR with deletion at the 3′ terminus (designated −RNA5); lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30 and 33 are for the third RNA template containing the RNA random sequence corresponding to positions 1245–1700 of the plus-strand genome (designated +RNAr2).
Fig. 6. Stereo view of the structure of BVDV polymerase (Choi et al., 2004; PDB ID code 1S48). The structural elements tagged by $\tilde{N}x1$, $\tilde{N}/\tilde{N}$, $\tilde{N}/\tilde{N}/\tilde{N}$, $\tilde{N}/\tilde{N}/\tilde{N}$, $\tilde{N}/\tilde{N}/\tilde{N}$, $\tilde{N}/\tilde{N}/\tilde{N}/\tilde{N}/\tilde{N}$, and $\tilde{x}5$ constitute the N2 region (Fig. 3). The structural elements tagged by $\tilde{N}/\tilde{N}$, $\tilde{N}/\tilde{N}/\tilde{N}$, $\tilde{N}/\tilde{N}/\tilde{N}$, $\tilde{N}/\tilde{N}/\tilde{N}$, $\tilde{N}/\tilde{N}/\tilde{N}$, and $\tilde{x}8$ constitute the N4 region (Fig. 3). The N1 region is not included in the stereo view of the structure.

Three conserved arginines and seven conserved lysines are harboured in the N3 region, from positions 217 to 280. Substitutions of these conserved arginines in this region decreased the RdRp activity (Fig. 4a, b, lanes 14, 19 and 23). However, all of the substitutions of the conserved lysines in this region enhanced RNA synthesis (Fig. 4a, b, lanes 15–18 and 20–22) and made the mutated proteins lose specificity for templates (Fig. 5a, lanes 4–24), which was not found in other regions. Similar results were not observed for the substitutions of the conserved arginines. Therefore, it is believed that the N3 region is related to enzymic specificity for template. The conserved lysines are necessary for this specificity. Substitution of any conserved lysines in the N3 region might have led to initiating RNA genomic replication on any template. Structural analysis suggests that BVDV RdRp N-terminal motif 1 (positions 261–266) is close to the NTP-binding site, indicating that its function is to bind the incoming NTP (Choi et al., 2004). The sequence of positions 261–266 in the N3 region is consistent with that of motif 1 and may be involved in binding of the incoming NTP (Figs 3 and 6). Analysis of HCV crystal structure has established the viral RdRp N-terminal motif F as an NTP-binding site (Lesburg et al., 1999). Several basic residues of motif F have been found by co-crystallization with NTPs to participate in nucleotide binding (Ago et al., 1999; Bruenn, 2003; Lesburg et al., 1999). Alignment analysis reveals that the N-terminal motif F of HCV RdRp and the N3 region of the CSFV RdRp overlap (results not shown), which supports our supposition that the CSFV N3 region may participate in binding of the NTP. Furthermore, our data also reveal that the N3 region is related to the enzymic specificity for template, indicating that the N3 region might be also involved in binding of the template. The analysis of BVDV NS5B crystal structure shows that aa 136–164 and 266–280 form a fingertip region that is involved in NTP binding (Choi et al., 2004). Our CSFV RdRp N2 and N3 regions overlap the fingertip region (Figs 3 and 6). Therefore, the N2 and N3 regions are very important for the CSFV RdRp activity.

The N4 region (positions 281–300) is the shortest fragment and contains fewer conserved lysines and arginines. Substitution of arginine in this region reduced elongative synthesis and destroyed de novo RNA synthesis (Fig. 4a, b, lanes 25 and 27). The mutated proteins with substitution of lysine in the N4 region still possessed the same activity for de novo RNA synthesis as their parental NS5B proteins and slightly increased the ability for elongative synthesis (Fig. 4a, b, lanes 24, 26 and 28). They could not, however, initiate RNA synthesis on +RNA5, −RNA5 or +RNA2, indicating that substitution of conserved lysines in the N4 region is not detrimental to the RdRp specificity for template (Fig. 5a, lanes 25–29). In the BVDV NS5B crystal structure, the fragment (positions 281–308) is also important and constitutes motif 2 of the fingers domain. Motif 2 might be in contact with the phosphate backbone and bases, indicating that this motif plays a role in template binding (Choi et al., 2004). Our N4 region (positions 281–300) corresponds with motif 2 (Figs 3 and 6). In motif 2, some important amino acids, such as R285 and R295, are believed to be critical (Choi et al., 2004; Lai et al., 1999). In the N4 region of our CSFV NS5B protein, R285 and R295 are so important that substitution of either destroys de novo RNA synthesis. Therefore, the N4 region might be in contact with the phosphate backbone and bases in template binding. The results obtained here suggest that the conserved lysines in the N4 region were unimportant, which is consistent with the conclusion drawn from the previous report on the BVDV NS5B protein, in which the conserved lysines were believed to be less critical for RNA synthesis (Lai et al., 1999). The reason(s) for this remains unclear.

Taken together, our N2, N3 and N4 regions correspond with some important structural elements of the BVDV NS5B crystal structure, such as the $\beta$-hairpin motif, motif 1, motif 2 and the fingertip regions. Our results are consistent with the analyses of the crystal structures of other RdRps of members of the family Flaviviridae, supporting the speculation that the N terminus, rich in arginines and lysines, may...
interact with template, primer or nucleotides. The observation that the N3 region is related to the enzymatic specificity for template is interesting; thus, more experimental studies may be conducted to explore the specificity for template in the N3 region.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (30170214), by the Science and Technology Foundation of Shanghai Higher Education and by Shanghai Municipal Education Commission (CL200502).

REFERENCES


