Significance of the 3′-terminal region in minus-strand RNA synthesis of Hibiscus chlorotic ringspot virus

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RNA-dependent RNA polymerase (RdRp) was solubilized from crude extracts of Hibiscus cannabinus infected by Hibiscus chlorotic ringspot virus (HCRSV), a member of the Carmoviridae. After treatment of the extracts with micrococcal nuclease to remove the endogenous templates, the full-length genomic RNA and the two subgenomic RNAs were efficiently synthesized by the partially purified RdRp complex in vitro. When the full-length RNAs of Potato virus X, Tobacco mosaic virus, Odontoglossum ringspot virus and Cucumber mosaic virus were used as templates, no detectable RNA was synthesized. Synthesis of HCRSV minus-strand RNA was shown to initiate opposite the 3′-terminal two C residues at the 3′ end in vitro and in vivo. The CCC-3′ terminal nucleotide sequence was optimal and nucleotide variations from CCC-3′ diminished minus-strand synthesis. In addition, two putative stem–loops (SLs) located within the 3′-terminal 87 nt of HCRSV plus-strand RNA were also essential for minus-strand RNA synthesis. Deletion or disruption of the structure of these two SLs severely reduced or abolished RNA synthesis. HCRSV RNA in which the two SLs were replaced with the SLs of Turnip crinkle virus could replicate in kenaf protoplasts, indicating that functionally conserved structure, rather than nucleotide sequence, plays an important role in the minus-strand synthesis of HCRSV. Taken together, the specific sequence CCC at the 3′ terminus and the two SLs structures located in the 3′ UTR are essential for efficient minus-strand synthesis of HCRSV.

INTRODUCTION

The development of template-dependent, RNA-dependent RNA polymerase (RdRp) preparations from tissues or cells infected with plus-strand RNA viruses has facilitated analysis of the components and necessary factors required from the host for virus replication. Such systems have been developed for several viruses that infect a variety of hosts, including bacteriophage Qβ (Blumenthal & Carmichael, 1979), Black beetle virus (Saunders & Kaesberg, 1985), Flockhouse virus (Wu et al., 1992), Polio virus (Van Dyke & Flanagan, 1980; Baron & Baltimore, 1982), Sindbis virus (Lemm et al., 1998) and the plant viruses Alfalfa mosaic virus (AMV) (Houwing & Jaspers, 1986; Quadt et al., 1991), Brome mosaic virus (BMV) (Miller & Hall, 1983; Quadt & Jaspars, 1990), Cucumber mosaic virus (CMV) (Hayes & Buck, 1990; Quadt & Jaspars, 1991), Cucumber necrosis virus (CNV) and Tomato bushy stunt virus (TBSV) (Nagy & Pogany, 2000), Foxtail mosaic virus (Rouleau et al., 1993), Potato virus X (PVX) (Doronin & Hemenway, 1996; Plante et al., 2000), Red clover necrotic mosaic virus (Bates et al., 1995), Tobacco mosaic virus (TMV) (Osman & Buck, 1996; Watanabe et al., 1999), Turnip yellow mosaic virus (TYMV) (Mouches et al., 1974; Garbouri-Bouzid et al., 1991; Deiman et al., 1997; Singh & Dreher, 1997) and Turnip crinkle virus (TCV) (Song & Simon, 1994). Some of these systems have been used for analyses of host and viral protein components contained in the RdRp complexes. Several of the RdRp complexes have been useful for the determination of template requirements for initiation of RNA synthesis. The BMV and TCV RdRp complexes have been used additionally to study RNA recombination in vitro (Nagy & Simon, 1998a, b; Nagy et al., 1998). In general, initiation of RNA synthesis of positive-strand RNA viruses starts at the 3′ end of the genomic RNA. Therefore, various structures and cis sequences involved in the process serve as promoters or factors for minus-strand RNA synthesis (Buck, 1996). These include tRNA-like structures in the family Carmoviridae and genera Tobamovirus, Tymovirus and Hordeiviruses (Dreher, 1999). Some viruses do not have a tRNA-like structure, but contain various conserved elements that play a role in the initiation of RNA synthesis. For example, the satellite RNA C of TCV contains a stable stem–loop (SL) structure, which is indispensable for its synthesis (Song & Simon, 1995b). Sequences required for replication initiation are not confined to the 3′ untranslated region (UTR). Long-distance base pairing brings the RdRp to the 3′ end where initiation takes place (Klovins et al., 1998). In a very different mechanism, for the replication of Dengue virus, the
interactions between the 3′UTR and 5′UTR and the middle cyclization motifs are necessary for replication (You et al., 2001).

Hibiscus chlorotic ringspot virus (HCRSV) is a positive-strand RNA virus, a member of the Carmoviruses, and is composed of 3911 nt containing seven open reading frames (ORFs) with two subgenomes (Huang et al., 2000). The ORF encoding p23 is involved in the replication of RNA in kenaf (Liang et al., 2002).

The cis sequence elements involved in the in vitro and in vivo RNA synthesis of HCRSV have not been characterized. Preparation of a soluble, template-dependent RdRp complex of HCRSV would allow us to study which host or virus-encoded proteins, such as p23, p28 and p81, are involved in the replication of HCRSV. Here we have reported the successful purification of a template-specific RdRp complex from HCRSV-infected kenaf (Hibiscus cannabinus L.) and determination of the cis sequence requirements for initiation of minus-strand synthesis of HCRSV. In addition, the requirements for the two putative SLs for the accumulation of minus-strand RNA of HCRSV RNA in vitro and in vivo were investigated. The replication of chimeric constructs containing substitution of the two SLs of HCRSV with the three SLs in the 3′UTR of TCV were carried out in vivo.

METHODS

Inoculation of plants. Two-week-old kenaf was inoculated with 10 µg in vitro RNA transcripts from a full-length HCRSV cDNA clone, pHCRSV223 (Huang et al., 2000), in 30 µl inoculation buffer (30 mM sodium acetate, pH 5.5). Systemically infected leaves were harvested 7 days after inoculation and stored at −80 °C.

Purification of RdRp. RdRp purification was carried out combining the methods of Nagy & Pogany (2000) and Song & Simon (1994). Frozen leaves (20 g) were ground with 10 g sterile white quartz sand in 60 ml buffer A [50 mM Tris/Cl, pH 8-2, 15 mM MgCl₂, 10 mM KCl, 2 mM EDTA, 20% (v/v) glycerol, 90 mM 2-mercaptoethanol] with 300 µl protease inhibitor cocktail (Sigma). After centrifugation at 30 000 g for 10 min, the supernatant was centrifuged at 43 000 g for 1 h. Subsequently, the supernatant was loaded on to a Sephacyl 500 HR column (1 × 75 cm) (Amersham Pharmacia Biotech), which was pre-equilibrated with buffer B containing 50 mM Tris/Cl, pH 8-0, 10 mM MgCl₂, 1 mM EDTA, 6% (v/v) glycerol, 1-2 M NaCl] containing 80 µl protease inhibitor cocktail and 50 µl 2-mercaptoethanol, followed by stirring for 20 min. The pellet was collected by centrifugation at 43 000 g for 20 min. The pellet in 8 ml pre-chilled buffer B [50 mM Tris/Cl, pH 8-0, 10 mM MgCl₂, 1 mM EDTA, 6% (v/v) glycerol, 1-2 M NaCl] containing 80 µl protease inhibitor cocktail and 50 µl 2-mercaptoethanol, followed by stirring for 20 min. The pellet was collected by centrifugation at 43 000 g for 20 min. The pellet in 8 ml pre-chilled buffer B containing 2% taurodeoxycholic acid (TDC) and 1-5 M LiCl and stirred for 1 h. The supernatant was centrifuged at 43 000 g for 20 min and the supernatant was centrifuged further at 100 000 g for 1 h. Subsequently, the supernatant was loaded on to a Sephacryl 500 HR column (1 × 75 cm) (Amersham Pharmacia Biotech), which was pre-equilibrated with buffer B containing 0-5% Triton X-100. Fractions (1 ml) were collected and stored at −80 °C for up to 6 months without loss of activity. To obtain highly template-dependent RdRp (Miller & Hall, 1983) and to remove endogenous RNA, micrococcal nuclease (nuclease S7; Roche) was added according to the method of Osman & Buck (1996) at 30 °C for 30 min. The micrococcal nuclease was inactivated by adjusting the EGTA concentration to a final value of 15 mM. All steps were carried out at 4 °C unless otherwise stated.

Preparation of RNA templates. For RdRp activity assay, the full-length in vitro transcripts were derived from pHCRSV223 (Huang et al., 2000), which was linearized by Smal to provide the exact length of the viral sequence and transcribed by T7 RNA polymerase. The RNA products transcribed separately from the full-length cDNA clones of PVX, TMV, Odonatum glossum ringspot virus (ORSV) and TCV were used to test template specificity of the extracted RdRp complex. CMV RNAs 1, 2, 3 and subgenomic RNA 4 were used as size markers and as templates for the RdRp assay. The Δ5′UTR plus-strand RNA template with nt 1–60 was derived from pHCRSV223 after digestion with BstUI and Smal, blunt-ending with T4 DNA polymerase, ligation into pBluescript SK(−) and transcription by T7 RNA polymerase. The Δ3′UTR was transcribed by T7 RNA polymerase from a cDNA construct Δ3′UTR 4′−(Koh et al., 2002). After digestion of the plasmid DNA templates with DNase I, the transcribed products were purified using the phenol/chloroform extraction method.

To investigate the effects of 3′-terminal nucleotides on minus-strand synthesis, a set of 3′UTR mutant constructs with different deletions or substitutions was generated by PCR using Pfu DNA polymerase (Promega). pHCRSV223 was used as the template and all resultant PCR products contained a T7 RNA polymerase promoter fused directly with the 5′ 21 nt of the 3′UTR of HCRSV. Appropriate PCR primers (Table 1) were designed. All mutated regions were confirmed by DNA sequencing. The mutant DNA constructs were transcribed into RNA templates for the RdRp activity assay. To compare the replication efficiency of various mutants in vitro and in vivo, full-length mutants containing corresponding mutations were similarly constructed by PCR. The primer designated S′Full + contained a T7 promoter fused immediately to the extreme 3′-end sequences of HCRSV.

To investigate the involvement of the predicted SLs of HCRSV in minus-strand synthesis and whether the SLs from TCV could substitute for the replication functions of HCRSV SLs, another set of full-length mutants containing various predicted SL deletions or alterations was generated from pHCRSV223 by PCR with appropriate primers (Table 1). All fragments were cloned into the pHCRSV223 Psfl and BamHI internal sites for in vivo studies. pTCV T1d1 (Carrington et al., 1989) and pHCRSV223 were used as templates to carry out overlapping PCR to construct the chimeric HCRSV mutants in which the 3′UTR contained individual TCV SL1, SL2 or SL3, or TCV (SL1 + SL2) or (SL1 + SL2 + SL3). The resulting PCR fragments containing the HCRSV 3′UTR with these different TCV SLs were digested with Psfl and BamHI and inserted into pHCRSV223. The full-length PCR products containing mutations at the 3′ end and the mutants with substituted SLs were used as templates for RNA transcription. Purified full-length transcripts of mutants were used for transfection of kenaf protoplasts.

RdRp assay. RdRp assays (40 µl) containing 1 mM ATP, 1 mM CTP, 1 mM GTP, 0-65 mM UTP and 0-35 mM DIG-11-UTP were carried out as described previously for TCV (Song & Simon, 1994; Nagy & Pogany, 2000) with the exception that each reaction contained 3 µg template RNA and was incubated at 25-7 °C for 1-5 h. To verify that the reaction products generated were double-stranded, half of the products were treated with nuclease S1 (Nagy & Pogany, 2000). The reaction was stopped by adding 40 µl TE buffer (10 mM Tris/Cl, pH 8-0, 1 mM EDTA) containing 0-2% SDS and 50 mM EGTA, followed by phenol/chloroform extraction twice and ammonium acetate/2-propanol precipitation. The input and nascent RNA were pelleted together and rinsed with 70% ethanol, dissolved in 6 µl nuclease-free water and subjected to formaldehyde-denaturing 1-5% agarose gel electrophoresis. RNA was transferred to a nylon membrane (Roche) and detection was carried out as described in the manual of the DIG-labelling detection kit with chemiluminescent detection (Roche). To quantify the relative amounts of RNA
**Table 1.** Primers used for construction of RdRp templates

<table>
<thead>
<tr>
<th>Name</th>
<th>HCRSV sequence†</th>
<th>HCRSV nucleotide positions</th>
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</thead>
<tbody>
<tr>
<td>5' Full(+)</td>
<td>CCAAATTAATACGACTCATATAAGGGGAAACCGTGAGCCATTTCCG</td>
<td>1–22</td>
</tr>
<tr>
<td>3' UTR(+)</td>
<td>CCAAATTAATACGACTCATATAAGGTGTATTGTATGAGGCCTCC</td>
<td>3628–3648</td>
</tr>
<tr>
<td>GCCC-3'</td>
<td>GGCTGCTCACAACATAGTCG</td>
<td>3911–3890</td>
</tr>
<tr>
<td>GCCCA-3'</td>
<td>TGAGGCTGCTCACAACATAGTCG</td>
<td>A + 3911–3890</td>
</tr>
<tr>
<td>GCCA-3'</td>
<td>TGAGGCTGCTCACAACATAGTCG</td>
<td>A + 3910–3890</td>
</tr>
<tr>
<td>GCC-3'</td>
<td>GGCTGCTCACAACATAGTCG</td>
<td>3910–3890</td>
</tr>
<tr>
<td>G-3'</td>
<td>GTCTGGGCTCACAACATAGTCG</td>
<td>3909–3890</td>
</tr>
<tr>
<td>GGGG-3'</td>
<td>CCCCCTGCTCACAACATAGTCG</td>
<td>3908–3890</td>
</tr>
<tr>
<td>ΔSL1</td>
<td>GGGCTGAGTATTTCAAACAAAGTACCC</td>
<td>3911–(Δ3905–3881)–3871</td>
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<tr>
<td>SL1Aloop</td>
<td>GGCTGGCTCACAACAGTTGTGAGGTATTTC</td>
<td>3911–(Δ3896–3890)–3874</td>
</tr>
<tr>
<td>SL1R</td>
<td>GGCTGGAGGTGGTGTATGAGGCTGAGGG</td>
<td>3911–3880</td>
</tr>
<tr>
<td>SL1L</td>
<td>GGCTGGCTCACAACATAGTCGCGGCAACACTCTGATTTCAAACAAAGTACCC</td>
<td>3911–3862</td>
</tr>
<tr>
<td>ΔSL2</td>
<td>GGCTGGCTCACAACATAGTCGCGGCAACACTCTGATTTCAAACAAAGTACCAAGGCTGTAGGAGGGG</td>
<td>3911–(Δ3862–3825)–3808</td>
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<tr>
<td>ΔSL1 + ΔSL2</td>
<td>GGGCTGAGTATTTCAAACAAAGTACCAAGGCTGTAGGAGGGG</td>
<td>3911–(Δ3905–3881)–3808</td>
</tr>
<tr>
<td>SL2L</td>
<td>GGCTGGCTCACAACATAGTCGCGGCAACACTCTGATTTCAAACAAAGTACCCGGATCCAAGCCCAAGGATTTATGG</td>
<td>3911–3831</td>
</tr>
<tr>
<td>SL2U:1</td>
<td>GGCTGGCTCACAACATAGTCGCGGCAACACTCTGATTTCAAACAAAGTACCCGGATCCAAGCCCAAGGATTTATGG</td>
<td>3911–3831</td>
</tr>
<tr>
<td>SL2AU</td>
<td>GGCTGGCTCACAACATAGTCGCGGCAACACTCTGATTTCAAACAAAGTACCCGGATCCAAGCCCAAGGATTTATGG</td>
<td>3911–(Δ3845–3842)–3824</td>
</tr>
<tr>
<td>SL2AUa1</td>
<td>GGCTGGCTCACAACATAGTCGCGGCAACACTCTGATTTCAAACAAAGTACCCGGATCCAAGCCCAAGGATTTATGG</td>
<td>3911–(Δ3855–3834)–3815</td>
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<td>HTSL1</td>
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<td>TCVSL1 + TCVSL2</td>
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<tr>
<td>HTS2L</td>
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<td>TCVSL2 + HCRSV</td>
</tr>
<tr>
<td>HTS3</td>
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<td>TCVSL3 + HCRSV</td>
</tr>
<tr>
<td>HTSL1 + 2</td>
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<td>TCVSL1 + HCRSV</td>
</tr>
<tr>
<td>HTSL1 + 2 + 3</td>
<td>GGGAAAAGGACGACTTTTTTTTCCGAGCTAATGTCCGAGGAGGAGGAGGAG</td>
<td>TCVSL3 + HCRSV</td>
</tr>
</tbody>
</table>

†The T7 RNA polymerase promoter sequence is indicated in italics. Mutations in the HCRSV sequence are shown in bold. The deleted nucleotides are preceded by Δ. The junction positions between TCV and HCRSV sequences are shown with an asterisk. The sequences of TCV are shown in bold and italic.

synthesis, standard curves were prepared by diluting the labelled positive- and minus-strand HCRSV RNA derived from in vitro transcription in RdRp reaction buffer or hybridization buffer (DIG-Easy Hyb; Roche). Band intensities were quantified by densitometry as described previously (Lewandowski & Dawson, 1998). All experiments were conducted independently at least three times.

**Prediction of RNA structure.** The RNA secondary structure of the 3' UTR of TCV (Carrington et al., 1989) and HCRSV (Huang et al., 2000) was predicted using MFOLD, version 3.0 (www.bioinform. rpi.edu/applications/mfold) (Mathews et al., 1999; Zuker & Jacobson, 1998). Since the hairpin structure of TCV satellite RNA C has been determined by enzymic digestion and chemical modification and the first SL nearest to the 3' terminus of the TCV 3' UTR has already been predicted (Song & Simon, 1995b), we compared the 3' UTR sequence of HCRSV with that of TCV and predicted two SL structures, which was in agreement with the MFOLD prediction.

**Isolation and transfection of kenaf protoplasts.** Isolation and transfection of kenaf protoplasts were carried out as described previously (Liang et al., 2002). In each of the three experiments, 10 µg of in vitro transcripts of the full-length mutants was transfected into a suspension of 1 x 10^6 kenaf protoplasts using a PEG-mediated method. All experiments were carried out independently at least three times.

**Northern blot analysis of mutants replicated in kenaf protoplasts.** At 24 h post-inoculation (p.i.), protoplasts were pelleted by centrifugation and 100 µl sterile water and 100 µl RNA extraction...
confirmed the ability of crude RdRp preparation to utilize double-stranded RNA in the S1 nuclease-treated extracts out independently at least three times.

3911–3206 or nt 3206–3911) were used to detect plus- or minus-strand RNA synthesis by the mutants. All experiments were carried out independently at least three times.

RESULTS

RdRp activity is associated with HCRSV infection

To purify an extract containing HCRSV RdRp, we first isolated a membrane-containing fraction from kenaf plants infected with HCRSV. Similar extraction procedures were carried out using mock-inoculated plants to obtain protein extract for use as a negative control. Following the procedures published for extraction of TCV RdRp (Song & Simon, 1994) and CNV RdRp (Nagy & Pogany, 2000), several detergents were tested and 2 % TDC was chosen as it solubilized the RdRp complex from membranes the most efficiently (data not shown). The solubilized complex was fractionated by Sephacryl S500HR column chromatography and three peaks containing RdRp activity were separated successfully. The RdRp extract from each peak was treated with micrococcal nuclease to remove endogenous RNA. The peak II fraction was used to test RdRp activity due to its higher specificity for template-dependent activity (data not shown). Similar results have been obtained previously with TCV (Song & Simon, 1994), CNV and TBSV (Nagy & Pogany, 2000).

The ability of RdRp to utilize an endogenous RNA template to produce both single- and double-stranded RNA progeny has been documented previously (Plante et al., 2000; Singh & Dreher, 1997). To investigate the activity of the crude RdRp preparation, the RdRp complex was assayed with endogenous RNA template purified from HCRSV-infected kenaf leaves and protein extracts from mock-inoculated kenaf leaves using the same RdRp purification procedures. RdRp activity was observed in the preparations from HCRSV-infected kenaf leaves, and both genomic and subgenomic RNAs were generated when endogenous RNA was present. Protein extracts prepared from the mock-inoculated kenaf leaves did not show any RdRp activity (Fig. 1).

To test whether the HCRSV RdRp complex could synthesize the complementary RNA strand using the endogenous RNA template, half of the reaction products were treated with S1 nuclease to digest away single-stranded RNA templates and their progeny. The S1 nuclease assay mixture was run on a 1-5 % agarose/formaldehyde gel. The presence of double-stranded RNA in the S1 nuclease-treated extracts confirmed the ability of crude RdRp preparation to utilize endogenous viral RNA templates to synthesize complementary strand RNA (Fig. 1). In addition, RdRp activity was not detected when endogenous templates were digested or when one of the four NTPs was absent from the RdRp assay mixture (data not shown), demonstrating that RdRp activity was detected only in the presence of the RdRp complex associated with HCRSV infection.

RdRp preparation is template dependent

To investigate the specificity of the RdRp extract, RNAs from TMV, PVX, ORSV and CMV were extracted and used as templates to test the activity of the solubilized RdRp after pre-treatment with micrococcal nuclease to remove endogenous template. Results showed that no progeny RNA was produced from these templates (Fig. 2A) compared with that of HCRSV RNA [Fig. 2, lane HCRSV (wt)]. Thus, the extracted HCRSV RdRp complex was highly template dependent in vitro. In addition, the 5′UTR was not required for the RNA synthesis of HCRCV in vitro, but the presence of the 3′UTR was necessary for its minus-strand synthesis (Fig. 2A). The HCRSV RdRp complex also successfully supported TCV RNA synthesis in vitro (Fig. 2B).
3’-terminal C with an A (GCCA-3’) resulted in a drop in activity to 41 %. Replacement of the three terminal Cs with Gs (GGGG-3’) resulted in only 5 % of activity compared with that of the wt (Fig. 3C). Taken together, these results showed that the three 3’-terminal C nucleotides are important for minus-strand synthesis and that the two 3’-terminal Cs in particular are crucial.

To investigate further the accumulation of the virus mutants in vivo, the corresponding full-length mutants were tested in kenaf protoplasts. At 24 h p.i., the RNA synthesis efficiency of each mutant was compared (Fig. 3D). The results showed that when the 3’-terminal C nucleotide was deleted, virus accumulation clearly decreased compared with that of the wt. When the 3’-terminal C was changed to an A (GCCA-3’), virus accumulation was detectable at only a very low level. When the two 3’-terminal Cs were deleted, virus accumulation was undetectable. Surprisingly, when one extra A was added to the 3’ terminus (GCCA-3’), there was no detectable virus accumulation, indicating that the CCC-3’ sequence is essential for RNA synthesis. All other mutants, including deletion of the three 3’-terminal Cs and replacement of the three Cs with three Gs resulted in no RNA synthesis (Fig. 3D). These results were consistent with those of the in vitro assays (Fig. 3C). Thus, mutants with nucleotide deletions or substitutions that could not be tolerated by the RdRp were not replicated efficiently in the in vivo protoplast system (Fig. 3D).

Prediction of the secondary structure of the HCRSV 3’UTR

To investigate further the secondary structure of the HCRSV 3’UTR sequences, HCRSV (accession no. NC003608) and TCV (accession no. NC003821) were aligned. Approximately 50 % of the sequences were identical. The last 122 nt showed 56 % sequence alignment. In addition, comparison of the 3’UTR secondary structures of HCRSV and TCV using the MFOLD software (Mathews et al., 1999) showed that two SLs were predicted in the 87 nt at the 3’-proximal end of HCRSV (ΔG = -33.2) and three SLs in the 122 nt at the 3’ end of TCV (ΔG = -52.7) (Fig. 4). The predicted SL1 structures of HCRSV and TCV matched those of the satellite RNA C of TCV determined using a chemical method (Song & Simon, 1995b).

Two SLs in the 3’UTR are essential for RNA synthesis

To test the importance of the SLs in the 3’UTR, constructs containing deletions or alterations thatspanned each SL were made (Table 2, Fig. 5A). Following deletion of the first (ASL1), second (ASL2) or both (ASL1+ASL2) SLs, there was no detectable RNA synthesis (Fig. 5B). Disruption of the first SL by replacing the nucleotides on the right-hand side of the stem with those on the left-hand side on stem 1 (denoted as SL1LL) resulted in only 2 % of products being synthesized in vitro, compared with the wt. In contrast, replacing the left-hand side nucleotides of SL1 with those
from the right-hand side (SL1RR) resulted in a synthesis efficiency of 10% compared with the wt. When the single loop of the SL1 was deleted (SL1Dloop), the template activity was maintained at 24% of the wt level (Fig. 5B), indicating that the stem plays a more important role than the loop in RNA synthesis.

For SL2, a U loop and an internal (I) loop were predicted
When the U loop was joined directly to the I loop to form SL2U:1, RNA synthesis was greatly reduced to only 13% (Fig. 5B). In contrast, when only the U loop was deleted (SL2ΔU), the level of RNA synthesis was 91%.

When both the U and I loops were deleted (SL2ΔUΔI), RNA synthesis was a mere 3%. When the stem of SL2 was disrupted by replacing the right-hand side nucleotides with the left-hand side nucleotides (SL2LL), RNA synthesis
Table 2. SL mutants of HCRSV used in this study

<table>
<thead>
<tr>
<th>Mutants</th>
<th>HCRSV nucleotide positions</th>
<th>Purpose</th>
</tr>
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<tbody>
<tr>
<td>HCRSV mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL1 + SL2 (wt)</td>
<td>(3881–3905) + (3825–3862)</td>
<td>Two individual SLs of wt HCRSV</td>
</tr>
<tr>
<td>ΔSL1 + ΔSL2</td>
<td>Δ(3881–3905) + Δ(3825–3862)</td>
<td>Deletion of two individual SLs</td>
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<tr>
<td>ΔSL1</td>
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<td>Deletion of SL1 only</td>
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<tr>
<td>SL1 loop</td>
<td>Δ(3890–3896)</td>
<td>Deletion of the loop on SL1</td>
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<tr>
<td>SL1LL</td>
<td>3897–3905</td>
<td>To destroy the left-hand side stem of SL1</td>
</tr>
<tr>
<td>SL1RR</td>
<td>3881–3889</td>
<td>To destroy the right-hand side stem of SL1</td>
</tr>
<tr>
<td>ΔSL2</td>
<td>Δ(3825–3862)</td>
<td>Deletion of SL2 only</td>
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<tr>
<td>SL2 ΔU</td>
<td>Δ(3842–3845)</td>
<td>Deletion of the upper loop of SL2</td>
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<tr>
<td>SL2LL</td>
<td>3854–3862</td>
<td>Disruption of left-hand side stem of SL2</td>
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<td>SL2 U : I</td>
<td>3846–3847</td>
<td>Fusion of upper and internal loops of SL2</td>
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<td>SL2 ΔUΔI</td>
<td>Δ(3834–3853)</td>
<td>Deletion of upper and internal loops of SL2</td>
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<td>HCRSV : TCV fusion mutants</td>
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<td>HCRSV : TCV SL1</td>
<td>(1–3880) : TCV SL1 : (3906–3911)</td>
<td>HCRSV SL1 substituted with TCV SL1</td>
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<td>HCRSV SL2 substituted with TCV SL2</td>
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<td>HCRSV : TCV SL3</td>
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<td>HCRSV SL2 substituted with TCV SL3</td>
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<td>HCRSV : TCV (SL1 + SL2 + SL3)</td>
<td>(1–3824) : TCV (SL1 + SL2) : (3863–3880) : TCV SL1 : (3906–3911)</td>
<td>HCRSV SL1 and SL2 substituted with TCV SL3</td>
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</table>

reached 41%. These results indicated that initiation of minus-strand synthesis of HCRSV requires the presence of both SLs in the 3′UTR.

For the in vivo tests using kenaf protoplasts, replication of the corresponding mutants was analysed by Northern blotting. When deletion mutants of SL1 (ΔSL1), SL2 (ΔSL2) or SL1 + SL2 (ΔSL1 + ΔSL2) were tested, no replication was detected (Fig. 5C). When the stem structure of SL1 (SL1LL or SL1RR) was disrupted, the two mutants were unable to replicate. However, when the SL1 loop was deleted (SL1Δloop), replication was still detectable (Fig. 5C).

For SL2, when the U and I loops were merged (SL2U : I) or deleted (SL2ΔUΔI), the mutants could not replicate. When the stem of SL2 was disrupted (SL2LL), the mutant could still replicate. On the other hand, when the U loop of SL2 was deleted (SL2ΔU), the mutant was unable to replicate. From these results, it was concluded that the U loop of SL2 is more important in replication than the stem, since minus-strand synthesis was not detected among these mutants (Fig. 5C). The in vitro and in vivo assays thus indicated that both SL1 and SL2 are required for replication.

Replication of HCRSV containing TCV SLs

To investigate further the template recognition of HCRSV RdRp in protoplasts, constructs were generated in which HCRSV SLs were substituted with TCV SLs in the 3′UTR. Mutants of HCRSV : TCV SL1, HCRSV : TCV SL2, HCRSV : TCV SL3, HCRSV : TCV (SL1 + SL2) and HCRSV : TCV (SL1 + SL2 + SL3) were constructed (Fig. 6A). At 24 h p.i., RNA blots showed that there was no detectable RNA synthesis generated from the HCRSV mutants substituted with single a TCV SL (Fig. 6B, lanes 2, 3 and 5). In contrast, the HCRSV mutant substituted with two TCV SLs (Fig. 6B, lane 4) replicated in protoplasts, but without generating subgenomic RNAs. This could be a result of the corresponding sequences of SL1 and SL2 of TCV in the HCRSV : TCV chimeric minus-strand RNA affecting the accumulation of its plus-strand RNA in protoplasts. While HCRSV : TCV (SL1 + SL2 + SL3) was able to replicate successfully with both genomic and subgenomic RNAs, the replication efficiency was reduced (Fig. 6B). This result clearly indicated that the two SLs in the 3′UTR of HCRSV can be replaced by the three TCV SLs located in its 3′UTR. In addition, these data suggested that TCV SL3 might be involved in the initiation of subgenomic RNA synthesis.

DISCUSSION

In this study, we established a template-dependent HCRSV RdRp in vitro transcription system. This HCRSV RdRp complex is template specific, similar to the reported RdRp of TCV and CNV (Song & Simon, 1994; Nagy & Pogany, 2000). From our experiments, the in vitro system was able to determine the essential elements required for RNA synthesis of HCRSV. Results from the in vitro system were also consistent with those obtained from kenaf protoplasts in vivo. This is believed to be the first report of the direct use of genomic RNA as a template to investigate RNA synthesis in Carmoviridae. In TCV, only satellite RNA has been used as a template for studies (Song & Simon, 1995a, b; Nagy et al., 2001) and defective-interfering RNAs have been used in CNV and TBSV (Nagy & Pogany, 2000). Thus, no
information is available for the minus-strand synthesis of TCV genomic RNA. In this study, the minus-strand RNA synthesis of HCRSV, which may share some common features with RNA synthesis in carmoviruses, was studied in vitro. Since a partial 3′UTR sequence may not reflect the actual secondary structure of a virus, we used the entire 3′UTR as the template and full-length mutants to carry out the RdRp assay both in vitro and in vivo.

**HCRSV RNA synthesis in vitro versus in vivo**

Generally, an in vitro system of RNA synthesis can be used to dissect the mechanism and roles of proteins involved in RNA replication (Ahluquist et al., 2003). However, an in vitro system may lack important properties that can only be found in vivo, since some host proteins are involved in the replication process (Miller & Koev, 2000). Therefore, in this study, we used an in vivo system to confirm the results obtained from the in vitro system. The requirement of the stem–loop C (SLC) of BMV in vitro in minus-strand synthesis has been confirmed in barley protoplasts (Sivakumaran et al., 2003). While the in vivo results could be predicted from those in vitro, the correlation of the amount of RNA synthesized in vitro was not proportional to that in vivo (Fig. 3C and D; Fig. 5B and C). For example, determination of the initiation site as the two 3′-terminal Cs in the 3′UTR of HCRSV was consistent in both in vitro and in vivo systems, but the amount of RNA synthesized in vitro varied compared with that obtained in vivo (Fig. 3C and D). Also, the addition of one extra A at the 3′ terminus reduced the in vitro replication to 13% of the wt, while this mutant was unable to replicate in vivo (Fig. 3C and D).

Some differences also existed in the influence of SLs on replication between the in vitro and in vivo systems. Deletion of the U loop of SL2 (SL2AU) slightly affected the replication efficiency in vitro but the mutant could not replicate in kenaf protoplasts (Fig. 5). For mutants SL1Δloop and SL2LL, replication was apparently more reduced in protoplasts than in the in vitro assays (Fig. 5B and C). The requirements of SLs for minus-strand synthesis of TMV carried out in vitro (Osman et al., 2000) were the same as in tobacco protoplasts. However, differences were noted in the replication efficiency of some TMV mutants in vivo (Chandrika et al., 2000). Similarly, insertion of 3 nt in the SLC of BMV RNA 3 showed reduced RNA synthesis in vitro and an even greater reduction in vivo. However, a nucleotide change in the SLC led to less RNA synthesis in vitro but not in vivo (Sivakumaran et al., 2003). It is likely that other RNA elements may modulate RNA replication in vivo resulting in the differences observed in vitro. Other possible reasons for the differences may be that some host factors participate in the interaction between the RdRp replicase and different RNA templates (Osman et al., 2000) or other host factors, which may contribute to the initiation of minus-strand RNA synthesis by recognizing and utilizing the 3′ end of positive-strand RNA (Ahluquist et al., 2003). Therefore, cis sequence elements and structural requirements for minus-strand RNA synthesis need to be analysed using a combination of in vitro and in vivo approaches.

**Essential role of the 3′-terminal CCC nucleotides in minus-strand RNA synthesis**

The HCRSV RdRp complex was shown to be template dependent and able to initiate RNA synthesis in vitro. The in vitro assay showed that the 5′UTR was not involved in minus-strand RNA synthesis, but that the 3′UTR was (Fig. 1A). Both in vitro and in vivo experiments showed that the CCC-3′ sequence is crucial for minus-strand RNA synthesis, since its initiation began at the 3′-terminal two Cs. When the single 3′-terminal C was removed in vitro or in vivo, the template could still be replicated. However, when the two Cs at the 3′ terminus were deleted, no RNA synthesis was detected either in vitro or in vivo. When the 3′-terminal C was substituted with an A, template activity was reduced but detectable, with the RNA synthesis efficiency being reduced to 41% and 7% of the wt in vitro and in vivo, respectively (Fig. 3C and D). Our results are similar to those obtained from BMV (Chapman et al., 1998), TMV (Osman & Buck, 1996) and TYMV (Singh & Dreher, 1997), where initiation of minus-strand RNA synthesis started opposite the two Cs and removal or substitution of the terminal A in the CCA box reduced minus-strand synthesis.

The wt TMV can tolerate up to seven extra nucleotides at the 3′ terminus without significant loss of infectivity (Dawson et al., 1986) and an additional C at the 3′ terminus of Cistrus tristeza virus (CTV) also did not affect infectivity (Satyanarayana et al., 2002). For HCRSV, an additional A added to the 3′ terminus resulted in a severe reduction in RNA synthesis (Fig. 3C and D), indicating that CCC-3′ is a key element for minus-strand synthesis. The presence of an extra A at the terminus of the 3′UTR might affect the accessibility of HCRSV RdRp to the template.

For TCV, the initiation site of minus-strand synthesis has not yet been identified (Song & Simon, 1995b). In TYMV RNA, the 3′ RNA-like structure presents the CCA-3′ in a conformation that is easily accessible to the replicase (Dreher, 1999). The importance of CCA-3′ was demonstrated in the end-to-end replication by Qβ replicase (Treheway et al., 2001). From our experiments, we speculate that CCC-3′ may also be involved in forming a conformation for replicase access both in vitro and in vivo in HCRSV. However, the presence of a CCC-3′ terminal sequence alone is not sufficient for RNA synthesis. The two SLs in the 3′UTR must also be present and they cannot be disrupted or deleted for RNA synthesis to occur (Fig. 3C and D).

**Crucial role of the predicted SLs in minus-strand RNA synthesis**

In general, RNA viruses have a specific structure at the 3′ end of the genome that is required for initiation of minus-strand RNA synthesis (Ahluquist et al., 2003). In this study, the 3′UTR of HCRSV was predicted to constitute two SLs,
with six unpaired nucleotides located at the 3’ terminus. The first SL was very similar in structure to the single SL previously described as a minimum promoter for minus-strand synthesis of TCV satellite RNA C (Song & Simon, 1995b). This suggested that the two predicted SLs in HCRSV might also play a similar role(s) to the TCV satellite RNA C. However, when SL1 or SL2 was deleted, no RNA synthesis was detected in vitro or in vivo, demonstrating that both SLs were indispensable for RNA synthesis of HCRSV (Fig. 5B and C). This is different from the TCV RdRp interaction with the single SL of satellite RNA C in minus-strand synthesis. Also, disruption of certain SLs in BMV (Dreher & Hall, 1988; Chapman & Kao, 1999), TYMV (Deiman et al., 1997), AMV (Olsthoorn et al., 1999) and CTV (Satyanarayana et al., 2002) has resulted in a reduction in minus-strand synthesis, although not inhibiting RNA

**Fig. 5.** Effects of SL structures in the 3’ UTR on minus-strand synthesis of HCRSV in vitro and in vivo. (A) Predicted SL structures located in the 3’ UTR of HCRSV. Nucleotide variations that disrupt (SL2U↓) or delete (SL2ΔU, SL2ΔU↓, SL1↓loop) secondary structures are indicated. The left- and right-hand side nucleotides of SL1 and SL2 are indicated as SL1L and SL1R, and SL2L and SL2R, respectively. (B) In vitro RdRp assays of different mutants. Templates are shown below the products after ethidium bromide staining to indicate the relative amounts of template. The percentage of the mean amount of each product is indicated. SL1LL and SL2LL indicate that the right-hand side stem nucleotides were changed to left-hand side stem nucleotides to disrupt the stem structures. Similarly, SL1RR and SL2RR indicate that the left-hand side stem nucleotides were changed to the right-hand side stem nucleotides. (C) Northern blot analysis of plus- and minus-strand RNA synthesis generated from different constructs in kenaf protoplasts at 24 h p.i. DIG-UTP-labelled HCRSV cRNA probes (nt 3911–3206 or nt 3206–3911) were used to detect the plus- or minus-strand RNA synthesis of the mutants. The amounts of 28S rRNA are shown to indicate the relative amounts of sample loaded.
synthesis completely. In another case, elements located hundreds of nucleotides upstream of the TCV 3' UTR were found to be needed for efficient replication (Carpenter et al., 1995). However, not all of the 10 SLs of CTV are critical for RNA synthesis (Satyanarayana et al., 2002). In this study, the two SLs of HCRSV were demonstrated to be absolutely essential for RNA synthesis. In addition, when the U loop located on SL2 was deleted (SL2D), no RNA synthesis could be detected either in vitro or in vivo. When both the U and I loops were merged (SL2U:1), there was also no RNA synthesis. In contrast, when the left-hand side stem of SL2 was disrupted by replacing the right-hand side stem with the left-hand side stem nucleotides (SL2LL), marginal synthesis was detected (Fig. 5B and C). Therefore, the U loop is an essential structure for RNA synthesis. The secondary structures of SLs in the 3' UTR of RNA viruses have been shown to be required for protein binding (Lai, 1998). For example, the hpE loop of AMV is not essential for RNA synthesis, whereas the stem and base pairing of the lower tri-loop are essential. Reducing the size of the bulge loop of hpE triggered transcription from an internal site, similar to the process of subgenomic transcription (Olsthoorn & Bol, 2002). It may be that failure to bind replication factors by HCRSV mutants with disruption or deletion of SL1 or SL2 renders them unable to initiate minus-strand synthesis.

### Substitution of the two HCRSV SLs with three TCV SLs generates both genomic and subgenomic RNAs

In this study, we demonstrated that HCRSV RdRp could support synthesis of TCV genomic RNA in vitro. We then examined whether the replacement of the two HCRSV SLs with the three from the TCV 3' UTR was functional with the HCRSV RdRp complex in vivo. Sequence alignment showed that the 3' UTR of TCV shares 50% nucleotide identity with the HCRSV 3' UTR. The TCV 3' UTR folds into three predicted SLs. Our results showed that substitution of SL1 or SL2 of HCRSV individually with one of the SLs of TCV resulted in no RNA replication. However, substitution of both SL1 and SL2 with those of TCV resulted in replication of the genomic RNA of HCRSV. Therefore, it seems that the SL1 and SL2 cis-acting sequences are not organized as modules for RNA synthesis. In addition, substitution of both SL1 and SL2 with all three SLs from TCV generated both genomic and subgenomic RNAs of HCRSV (Fig. 6B). This indicated that recognition of heterologous 3' replication elements may be extended to other carmoviruses as a result of their structural conservation. This supports the view that higher-order structure, rather than the primary sequence of the 3' UTR, is an important replication element (Koev et al., 2002).

When the 3' UTR of BMV RNA3 was replaced by the 3' UTR of CMV-Fny, this resulted in BMV replication in vivo (Rao & Grantham, 1994). Although there was only 56% sequence identity between the two 3' UTRs, CMV-Fny replicase could synthesize RNA from the BMV tRNA-like structure in vitro (Sivakumaran et al., 2000). However, substitution of the BMV RNA 3 SLC with that of CMV did not result in replication in vivo, indicating that the requirement of the BMV SLC is highly specific to BMV replicase (Sivakumaran et al., 2003). Reciprocal experiments of TCV SLs replaced with those of HCRSV could be carried out using TCV RdRp or in protoplasts of a common host.

It was interesting to note that in the absence of TCV SL3, no subgenomic RNA was synthesized (Fig. 6B). Perhaps the subgenomic RNAs of HCRSV were generated but degraded due to lack of encapsidation by the HCRSV capsid protein. However, the presence of genomic RNA indicated that it was not degraded and therefore it is unlikely that encapsidation and RNA degradation of subgenomic RNAs were involved. When the two SLs of HCRSV were replaced by the SL1 and SL2 of TCV to form HCRSV:TCV(SL1+SL2), no subgenomic RNA was detected, compared with HCRSV:TCV (SL1 + SL2 + SL3), indicating that TCV SL3 may be involved in the synthesis of both subgenomic RNAs. The predicted secondary structures of SL1 and SL2 of TCV and HCRSV were very similar (Fig. 4A and B). In SL1, both TCV and HCRSV possessed a long stem (10 versus 9 bp) and a small loop (5 versus 7 nt), respectively. The similarities of SL2
Fig. 6. Effects of replacing the two SLs of HCRSV with the three SLs of TCV on RNA synthesis in transfected kenaf protoplasts. (A) The sequence of HCRSV is indicated by a dotted line and the nucleotide positions for substitution of the two SLs with those of TCV are shown, based on the nucleotide sequence of HCRSV. (B) Northern blot analysis showing that TCV SL1 and SL2 can replace those of HCRSV for genomic RNA replication, and that TCV SL1, SL2 and SL3 can replace HCRSV SL1 and SL2 to generate both genomic and subgenomic RNAs. A DIG-UTP-labelled HCRSV cRNA probe (nt 3911–3206) was used. The relative amount of total RNA of each sample used in the transfection is indicated by the 28S rRNA levels.
included the same number of base pairs (9 nt) in the stem, the exact sequence of the I loop (12 nt), identical nucleotides in the upper stem (3 versus 2 bp) and same size (4 nt) of the U loop (Fig. 4A and B). Since both SL1 and SL2 of TCV and HCRSV are similar in sequence and/or in structure and HCRSV SL1 and SL2 could support subgenomic RNA synthesis, it is puzzling that HCRSV : TCV (SL1 + SL2) could not support replication of subgenomic RNAs of HCRSV in vivo. Additional functions of HCRSV SL1 and SL2 and replication of subgenomic RNA will be delineated in comparative studies with TCV.

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