Role of an Internal and Two 3'-Terminal RNA Elements in Assembly of Tombusvirus Replicase†

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Received 9 March 2005/Accepted 8 May 2005

Plus-strand RNA virus replication requires the assembly of the viral replicase complexes on intracellular membranes in the host cells. The replicase of *Cucumber necrosis virus* (CNV), a tombusvirus, contains the viral p33 and p92 replication proteins and possible host factors. In addition, the assembly of CNV replicase is stimulated in the presence of plus-stranded viral RNA (Z. Panaviene et al., J. Virol. 78:8254–8263, 2004). To define *cis*-acting viral RNA sequences that stimulate replicase assembly, we performed a systematic deletion approach with a model tombusvirus replicon RNA in *Saccharomyces cerevisiae*, which also coexpressed p33 and p92 replication proteins. In vitro replicase assays performed with purified CNV replicase preparations from yeast revealed critical roles for three RNA elements in CNV replicase assembly: the internal p33 recognition element (p33RE), the replication silencer element (RSE), and the 3'-terminal minus-strand initiation promoter (gPR). Deletion or mutagenesis of these elements reduced the activity of the CNV replicase to a minimal level. In addition to the primary sequences of gPR, RSE, and p33RE, formation of two alternative structures among these elements may also play a role in replicase assembly. Altogether, the role of multiple RNA elements in tombusvirus replicase assembly could be an important factor to ensure fidelity of template selection during replication.

The genomes of plus-stranded RNA viruses are replicated by viral replicase complexes assembled on intracellular membranes (1, 4). The replicase complex consists of virus-encoded proteins, such as the RNA-dependent RNA polymerase (RdRp), auxiliary viral proteins, and possibly host-derived proteins and the viral RNA template (2, 10). The viral RNA serves a more complex role than just being used as a template for replication. For example, the assembly of viral replicases of *Brome mosaic virus* (BMV) and *Alfalfa mosaic virus* requires the plus-stranded viral RNA in order to be functional (28, 37). In the case of *Cucumber necrosis virus* (CNV), the viral (+)RNA, but not the (–)RNA, stimulated replicase assembly by 40-fold in yeast, a model host (22). All these works led to the model that the viral (+)RNA likely serves as a platform to bring the viral replication proteins and host protein factors together, leading to efficient assembly of the viral replicase.

Tombusviruses are nonsegmented plus-stranded viruses that code for five proteins. These include the p33 and p92 replication proteins (Fig. 1), a cell-to-cell movement protein (p22), a coat protein (p41), and a suppressor of gene silencing (p19). The overlapping p33 and p92 replicase proteins are essential for replication of the genomic RNA (gRNA) in plant cells (14, 20, 33, 40). The p92 replication protein has the RdRp signature motifs in its unique C terminus, whereas the auxiliary p33 plays a role in template selection and the recruitment of the viral RNA into replication (12, 26). In addition, mutagenesis of p33

within its RNA-binding site (an arginine-proline-rich motif, termed the RPR motif) (29) led to altered gRNA replication (20), subgenomic RNA synthesis (20), and RNA recombination (21), suggesting that p33 is a multifunctional protein. Another essential property of p33 is its interaction with other p33 proteins and with p92 that is supported by the p33–p33/p92 interaction domain (30).

A useful feature of tombusviruses for replication studies is the availability of small defective interfering (DI) RNAs, which are deletion derivatives of the viral gRNA (Fig. 1A) (40). These DI RNAs do not code for essential genes, allowing for convenient testing of the role of essential and/or regulatory *cis*-acting RNA elements. Replication of DI RNA has been tested in plants, in single plant cells (protoplasts), and in yeast, a model host (23, 40). In addition, in vitro replication assays based on (i) partially purified CNV replicase from CNV-infected plants (13) or (ii) purified recombinant CNV replicase from yeast (22) were used to define promoters (18, 19), replication enhancers (RE) (15, 17), a replication silencer element (RSE) (25), and an internal replication element (12, 26) also called the p33 recognition element (p33RE). The roles of these and other *cis*-acting elements were also studied in vivo during tombusvirus replication (6, 24, 25, 27, 31, 32). These studies have established that, for example, the minus-strand initiation promoter (genomic promoter, gPR) consists of a hairpin structure flanked by a short 3'-terminal tail (Fig. 2) (6, 19). On the contrary, the plus-strand initiation promoter (complementary promoter) consists of an 11-nucleotide (nt) single-stranded region at the 3' end of the minus-strand intermediate RNA. In addition to the above promoters, two types of regulatory elements were identified: RE and RSE, which either up- or down-regulate initiation, respectively, from the promoters in vitro (15, 25, 32). Interestingly, the RSE can down-regulate initiation of minus-strand synthesis by hybridizing to the very 3'

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† Supplemental material for this article may be found at <http://jvi.asm.org/>.

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FIG. 1. Expression of RII(+) and RIV(+) of DI-72 RNA in yeast stimulates the activity of purified CNV replicase. (A) Plasmids used to express the CNV p33 and p92 replicase proteins and DI-72(+) RNA in yeast cells. pGBK-His33 and pGAD-His92 have a constitutive P_{ADH1} promoter, and DI-72(+) RNA is expressed from a galactose-inducible promoter (P_{GAL1}). Black boxes in pGBK-His33 and pGAD-His92 constructs represent the His₆ tag. The translation termination codon of p33 was replaced with a tyrosine (Y) codon, allowing p92 expression from the pGAD-His92 plasmid. There is a satTRSV(-) ribozyme (Rz sat) at the 3' end of DI-72. (B) Schematic representation of viral RNAs expressed in yeast. The first or last nucleotide positions present at the deletion junctions are indicated numerically based on the 621-nt DI-72 RNA (38). (C) In vitro activity of affinity-purified CNV replicase preparations. Each replicase preparation was tested in the presence of exogenous RI/III(-) template, which contains the minus-stranded regions I and III of DI-72 (panel A), in a standard CNV replicase assay. Radiolabeled RNA products (generated via de novo initiation on added RNA templates) from CNV replicase preparations were analyzed on denaturing 5% PAGE-8 M urea gels. For quantification, we measured the intensity of 32 P-labeled RNA products by using a phosphorimager. The relative activity of the CNV replicase is shown below the image as a percentage representing the mean of three separate experiments. The activity of the CNV replicase obtained from yeast expressing the full-length DI-72(+) corresponds to 100%. Lane numbers correspond to RNAs shown in panel B. Lane C0 represents the basal activity of CNV replicase obtained from yeast that expressed only p33 and p92, but not the replicon RNA. (D) Comparison of in vitro activity of affinity-purified CNV replicase preparations on plus- and minus-stranded templates. See further details in the legend for panel C.

terminus of gPR, which “masks” the promoter from recognition by the viral replicase (25). Another type of regulatory element is the p33RE, which is bound selectively by p33 replication protein, leading to selective recruitment of the viral RNA into replication (26). p33RE is present within an internal region (within the p92 gene) that forms a stem-loop structure named RII(+)-SL in the plus-stranded RNA (26). Identification of numerous *cis*-acting RNA elements in tombusvirus RNA suggests that various steps during replication might be controlled/regulated by coordinated action of several *cis*-acting elements.

In this paper, we tested the role of tombusviral RNA se-

quences in facilitating the assembly of the tombusvirus replicase in yeast (*Saccharomyces cerevisiae*) cells. Various regions of DI RNAs were coexpressed with CNV p33 and p92 replication proteins in yeast, followed by the purification of the recombinant replicase. An in vitro replicase assay revealed critical roles for p33RE, the RSE, and gPR in the viral RNA for stimulation of the assembly of the CNV replicase. Deletion or mutagenesis of any of these elements reduced the activity of the tombusvirus replicase to a basal (minimal) level. Altogether, the requirement for three separate *cis*-elements suggests that the assembly is a multistep process and likely requires multiple protein factors in infected cells.

FIG. 2. Deletions of SL3 and gPR sequences inhibit the activity of the purified CNV replicase. Construct R2-4 (Fig. 1B, lane 6) was used to generate a short deletion in RIV(+). The activity of the purified CNV replicase was tested as described in the legend to Fig. 1. Activity of the CNV replicase obtained from yeast expressing R2-4 (lane C6) corresponds to 100% (see also lane 6 in Fig. 1B). (Bottom) Western blot analyses of relative amounts of p33 and p92 present in purified CNV replicase preparations from yeast. Western blotting was performed using anti-His antibody.

MATERIALS AND METHODS

Expression of p33 and p92 replication proteins and the tombusvirus RNA in yeast. The CNV p33 and p92 proteins with N-terminal His₆ tags were expressed constitutively in *S. cerevisiae* strain INVSc1 (Invitrogen) from pGBK-His33 and pGAD-His92 plasmids carrying *ADHI* promoters (22). Various portions of the tombusvirus replicon RNA (based on DI-72 RNA [39]) were also expressed in yeast from the galactose-inducible *GAL1* promoter (16). The construction of the RNA expression plasmids [based on pYES-DI-72(+)-Rz, which expresses the full-length DI-72(+)-Rz RNA replicon (16, 22)] was based on PCR.

Yeast cells were cotransformed with all three plasmids [i.e., pGAD-His92, pGBK-His33, and various derivatives of pYES-DI-72(+)-Rz] using the LiAc/ssDNA/PEG method (8). In control experiments (Fig. 1, C0), we replaced the pYES-DI-72(+)-Rz plasmid with the original pYES plasmid lacking viral sequences. Transformed yeast cells were grown on selective SC medium without uracil, leucine, and tryptophan (SC-ULT) that contained 2% galactose at 23°C until reaching an optical density at 600 nm of 0.6 to 0.7 (approximately 24 h). Purification of the active CNV replicase complex from the above yeast cells was performed as described previously (22). Briefly, the enriched membrane fraction

of yeast was treated with the extraction buffer containing 1% Triton X-100, 5% SB3-10 (caprylyl sulfobetaine; Sigma), and 0.5 M KCl followed by His tag-based metal affinity purification using ProBond resin (Invitrogen). The recombinant proteins were recovered from the resin in the extraction buffer containing 150 mM imidazole, 1% SB3-10, and 0.1% Triton X-100. The purity of the obtained recombinant protein-containing preparations was tested with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22), whereas the amounts of the recombinant proteins in various samples were compared by Western blotting with monoclonal anti-His tag antibody (Amersham).

In vitro CNV replicase assay. To test the activity of various CNV replicase preparations, 0.5 μg RNA template [representing a minus-stranded template containing RI and RIII, named RI/RIII(-) (22)] was used with 25 μl of purified recombinant CNV replicase in a standard in vitro replicase assay as described previously (22). The RNA products were phenol-chloroform extracted and analyzed under denaturing conditions (i.e., 5% PAGE containing 8 M urea) (22).

Western blotting. The amounts of p33 and p92 in the purified replicase preparations were analyzed with Western blotting as described elsewhere (22, 30). Briefly, aliquots of replicase preparations were mixed with SDS-PAGE sample loading buffer (30) in a 1:1 ratio, heated for 5 min at 85°C, electrophoresed in 8% SDS-PAGE gels, and electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad). Detection of p33 and p92 was based on monoclonal anti-His antibodies (Amersham) and secondary alkaline phosphatase-conjugated anti-mouse antibody (Sigma) as described previously (22).

Replication assay in yeast. First, we randomized three positions within RSE and gPR based on the pYES-DI-72(+)-Rz expression plasmid using a two-step PCR procedure. In the first step, two PCR fragments were generated with primer pair 542 (GCCCGAAGCTTGGAATTCCTCCAGGATTTC) and 1588 (CCCCCGAAGGGGTGAGATCAACCGTGTCT) and with primer pair 1589 (CAGACACGGTTGATCTCACCCCTTCGGGGGGNNNATAGAGATCGC) and 1590 (CCTTCTCTGTTTGGCAAGAAACAGGACTGGNNNGCATTTCGTCA) using pYES-DI-72(+)-Rz as a template. Then, the obtained PCR fragments were gel isolated and they were used together as templates for the second PCR with primers 542 and 1069 (CCGGTCGAGCTCTACCAGGTAATATACCACAACGTGTGT). This resulted in a PCR product representing the full-length DI-72 carrying the randomized nucleotides (underlined above) within the RSE and gPR. The PCR fragment was digested with HindIII and SacI and cloned into pYES-DI-72(+)-Rz (replacing the original DI-72 insert with the PCR fragment), resulting in pYC-DI-72randRSE/gPR. A similar approach was used to generate pYC-DI-72*randRSE/gPR, which carried a mutated RII(+)-SL (see construct 65 below) in addition to the randomized positions in RSE and gPR. Further information regarding the primers, templates, and randomized regions within the RSE and gPR of DI-72 RNA can be found in Tables S1, S2, and S3 in the supplemental material.

After transformation of competent *Escherichia coli* with either pYC-DI-72randRSE/gPR or pYC-DI-72*randRSE/gPR, we randomly selected 300 and 400 colonies, respectively, for separate isolation of pYC-DI-72randRSE/gPR and pYC-DI-72*randRSE/gPR plasmids. Then, three plasmids (pYC-DI-72randRSE/gPR [pYC-DI-72*randRSE/gPR] and pGBK-His33 and pGAD-His92) were used to transform *S. cerevisiae* strain INVSc1 (Invitrogen), and each transformed line was plated separately. Each of the 700 yeast lines was predicted to express DI-72 replicon with unique combinations of mutations in the RSE and gPR sequences. Each yeast transformant was separately tested for DI-72 RNA replication after growing for 2 days in SC-ULT medium with 2% galactose. Total RNA was extracted from each yeast line separately and tested with Northern blotting as described previously (16).

RESULTS

Both RII(+) and RIV(+) sequences are required for stimulation of assembly of the CNV replicase complex. Previous work demonstrated that a 621-nt replicon RNA based on DI-72(+)-Rz, a prototypical DI RNA, was capable of stimulation of CNV replicase assembly when coexpressed with p33 and p92 replication proteins in yeast (22). Because DI-72(-)-Rz RNA did not stimulate CNV replicase assembly (22), we assumed that DI-72(+)-Rz RNA must be needed to serve as a platform to facilitate the assembly of the functional CNV replicase in yeast cells (Fig. 1A). DI-72 RNA consists of four noncontiguous regions derived from the gRNA: RI represents the 5' noncoding region (NCR) and RII is derived from the

p92 gene, whereas RIII and RIV are from the 3' NCR (40). To delineate which regions are involved in CNV replicase assembly, we systematically deleted each region from DI-72(+) RNA, followed by coexpression of these deletion derivatives of the replicon RNA together with CNV p33 and p92 in yeast (Fig. 1B). Purified CNV replicase preparations obtained from the above yeast strains were then used in an in vitro assay with an added RNA template to measure replicase activity [based on de novo initiation of cRNA synthesis on the added RI/III(-) template] (schematically shown in Fig. 1A) (22). The purified CNV replicase preparation obtained from yeast expressing only CNV p33 and p92, but not the replicon RNA (Fig. 1C, lane C0), had only minimal (basal-level) replicase activity on the added RI/III(-) template in the standard in vitro replicase assay. On the other hand, similarly prepared CNV replicase from yeast coexpressing DI-72(+) RNA with p33 and p92 showed ~100-fold higher activity (Fig. 1C, lane 1) on the added RI/III(-) template than the C0 preparation.

In comparison with the full-length DI-72(+) RNA, deletion of RIII (construct R1-2-4) (Fig. 1B and C, lane 2) reduced the activity of the CNV replicase by 32%, whereas deletion of RI (construct R2-3-4, lane 3) increased the activity of the purified CNV replicase by 33%. These data suggested that neither region played a substantial role in assembly of the functional replicase. On the contrary, deletion of RIV (construct R1-2-3, lane 5) or the combined deletion of RI and RII (construct R3-4, lane 4) reduced the CNV replicase activity to a basal level, which was similar to that obtained with replicase preparations obtained from yeast expressing DI-72(-) RNA (lane 7). These data suggested that both RII(+) and RIV(+) were likely involved in the assembly of the functional CNV replicase. Accordingly, coexpression of R2-4 carrying only RII(+) and RIV(+) sequences with p33 and p92 in yeast led to a high level of CNV replicase activity [61% of that obtained with DI-72(+)] (Fig. 1B and C, lane 6).

The isolated recombinant CNV replicase obtained from yeast expressing the full-length DI-72(+) RNA can actively transcribe both minus-stranded and plus-stranded templates in vitro (22). However, in the above experiments we only tested whether the CNV replicase preparations, such as that obtained from yeast expressing construct R1-2-3 (Fig. 1C, lane 5), showed deficiency in using minus-stranded template. To test if the same CNV preparation is also inefficient on a plus-stranded template, we performed in vitro replicase assays with template RII/IV(+). These experiments demonstrated that the CNV replicase preparation obtained from yeast expressing construct R1-2-3 was deficient in using both plus- and minus-stranded templates in vitro (Fig. 1D, lanes 2 and 4).

The replication silencer element and the gPR promoter in RIV(+) are involved in assembly of the CNV replicase. To delineate what sequence(s) within RIV(+) is the most critical for CNV replicase assembly, we deleted previously characterized RNA elements (40) within RIV(+) in construct R2-4 (Fig. 2). Deletion of the least-conserved single-stranded region (termed s4 [6, 25]) in R2-4 RNA slightly increased the activity of the CNV replicase (Fig. 2, lane 21). On the contrary, deletions of either the SL3 hairpin, which includes the RSE [construct R2-4(Δ SL3)], or gPR [construct R2-4(Δ gPR)] reduced the in vitro activity of the CNV replicase to basal levels (2 to 4%) (Fig. 2, lanes 22 and 24). Deletion of SL2, which is essen-

FIG. 3. Defining sequences in RII(+) that are critical for activity of the CNV replicase. Construct R2-4(Δ s4) (Fig. 2, lane 21) was used to generate a short deletion in RII(+). The stem-loop structure with an internal C · C mismatch, termed RII(+)-SL, which serves as a p33 recognition element, is shown schematically. The activity of the purified CNV replicase was tested as described in the legend to Fig. 1. Activity of the CNV replicase obtained from yeast expressing R2-4(Δ s4) (lane C21) corresponds to 100% (see also lane 21 in Fig. 2). Lane C0 represents the basal activity of CNV replicase obtained from yeast that expressed only p33 and p92, but not the replicon RNA.

tial for replication (6, 9) but the function of which is currently unknown, also resulted in a large drop (92%) in CNV replicase activity [construct R2-4(Δ SL2)] (Fig. 2, lane 23). These data suggest that RSE and gPR elements, and to a lesser extent SL2, are important for the assembly of the CNV replicase.

As we observed previously (22), the purified replicase preparations showing a basal level of activity contained similar amounts of p33 as those with high activity (Fig. 2). p92 levels, which were ~10-fold lower than p33 levels, were also comparable in these replicase preparations (Fig. 2). These results support the model that the assembly of p33 and p92 (and host factors) into an active replicase complex is stimulated by the viral RNA carrying RII(+) and RIV(+) sequences.

Role of the p33RE in RII(+) in assembly of the CNV replicase. To delineate what sequence(s) within RII(+) is critical for CNV replicase assembly, we used a similar deletion approach as described above. Deletions of 30 or 74 nt from the 5' end of RII(+) [constructs R2(5'Δ30)-4(Δs4) and R2(5'Δ74)-4(Δs4)] (Fig. 3, lanes 31 and 32), which did not affect p33RE located within the RII(+)-SL, reduced CNV replicase activity by 19 and 66%. In spite of the decrease, the remaining activity of the CNV replicase was still ~30- to 80-fold higher than the basal level (Fig. 3, lane C0), suggesting that these constructs could support the assembly of the CNV replicase. Deletion of 71 nt from the 3' end of RII(+) [construct R2(3'Δ71)-4(Δs4)] (Fig. 3, lane 34) reduced CNV replicase assembly moderately

FIG. 4. RII(+)-SL is involved in stimulation of CNV replicase activity. Construct R2-4(Δ s4) (Fig. 2, lane 21) was used to generate short deletions in RII(+)-SL. Replacement of various sequences at the top of RII(+)-SL with a 6-nt loop sequence (a BamHI site) is shown schematically. The activity of the purified CNV replicase was tested as described in the legend to Fig. 1. Activity of the CNV replicase obtained from yeast expressing R2-4(Δ s4) (lane C21) corresponds to 100% (see also lane 21 in Fig. 2).

(by 77%), suggesting that the 3' portion of RII(+) is not essential for CNV replicase assembly.

In contrast, a deletion which removed part of the RII(+)-SL stem-loop structure [construct R2(3' Δ 85)-4(Δ s4)] (Fig. 3, lane 35) reduced the purified CNV replicase activity to a basal level. Similarly, deletion of the entire RII(+)-SL [construct R2(Δ SL)-4(Δ s4)] (Fig. 4, lane 45) resulted in a basal level of replicase activity.

To further delineate the role of sequences within RII(+)-SL, we removed the 5' and 3' flanking sequences [R2(SL)-4(Δ s4)] and replaced the top of the stem-loop with a BamHI sequence [R2(SL-Bam)-4(Δ s4)] (Fig. 4). These minimal constructs still supported CNV replicase assembly (11 to 12%) (Fig. 4, lanes 41 and 42). Altogether, the highly variable CNV replicase activity obtained with the above RII(+) deletion constructs and the reduced activity obtained with the minimal constructs are likely due to suboptimal spacer sequences and/or the effect of the flanking sequences on the folding of central RII(+)-SL structure in these RNAs. Nevertheless, all the above deletion constructs supported an above-basal level of CNV replicase activity, suggesting that their role is indirect during the assembly process.

Deletions of either the top [R2(SL- Δ top)-4(Δ s4)] (Fig. 4, lane 43) or the bottom [R2(SL- Δ bottom)-4(Δ s4), lane 44] parts of RII(+)-SL reduced the activity of the CNV replicase to a

basal level. Overall, these experiments strongly established a role for the RII(+)-SL in the assembly of the CNV replicase complex.

Identification of core sequences involved in CNV replicase assembly. To identify the core sequences critical for CNV replicase assembly, we chose one of the minimal constructs [R2(SL)-4(Δ s4)] (Fig. 4, construct 42) to minimize the effect of flanking sequences on RNA folding. First, we introduced targeted mutations into the p33RE element, which includes the C · C mismatch and the flanking G-C base pairs in RII(+)-SL (26). Mutants C₉₉-G (Fig. 5, construct 57) and G₁₄₄-C (construct 58) in RII(+)-SL, which are known to debilitate binding to p33 (26), also resulted in a basal level of CNV replicase activity. On the contrary, GA_{96,97}-UU mutations (Fig. 5, construct 56), which had only a moderate effect on p33-RNA interaction (26), reduced replicase activity only moderately (by 75%) (Fig. 5, lane 56). Altogether, these data established a close correlation between p33 binding to the viral RNA (26) and the assembly of functional CNV replicase (see Discussion). The only exception is the stem-strengthening mutation (Fig. 5, construct 54), which reduced CNV replicase activity by 91%, but it had only moderate effects on p33-RNA interaction (26) (see Discussion).

To define the role of RSE, which consists of the 5'-GGGC U-3' sequence within the asymmetrical loop in SL3 (25), in the assembly of the CNV replicase, we deleted the three Gs (Fig. 5, construct 51) or mutated C to G (construct 59) in the RSE. In these mutants, RSE could not interact with gPR (25) and the CNV replicase showed basal-level activity (Fig. 5). Mutations within the gPR, such as replacement of CCC with AAA (Fig. 5, construct 53) and G₄-C (construct 55), which debilitated the ability of gPR to interact with RSE, also decreased the CNV replicase activity by 97 to 99%. These data suggest that the interacting sequences in RSE and gPR are critical for replicase assembly (see Discussion).

To test if additional sequences within these regions could also affect CNV replicase assembly, we mutagenized selected sequences in our minimal R2(SL)-4(Δ s4) construct (Fig. 4, lane 42). For example, mutagenesis of RII(+)-SL around the critical C · C mismatch had a remarkable inhibitory effect on CNV replicase activity (Fig. 6, constructs 61 to 65). Also, mutations in the tetraloop sequence in SL3 (Fig. 5, construct 60) affected CNV replicase activity by 97%, suggesting that this sequence could also play a role in replicase assembly. Replacement of SL2 with a similar hairpin (Fig. 5, construct 52) reduced CNV replicase activity by 92%. Thus, SL2 also plays a role in replicase assembly, but its effect is not as pronounced as that of gPR, RSE, or p33RE.

Mutations within gPR, which weakened the stem region (Fig. 6, construct 66), inhibited CNV replicase activity only moderately (51%) (Fig. 6), whereas mutations that replaced the GAAA tetraloop with ACAA (construct 68) had a more inhibitory effect (87%) (Fig. 6). However, introduction of a more stable G-C base pair next to the altered tetraloop (Fig. 6, construct 69) restored CNV replicase activity to 64% of the wild-type (wt) level, suggesting that the sequence of the tetraloop in gPR is not critical for the replicase assembly. Increasing the stability of the stem (construct 67) decreased the efficiency of CNV replicase assembly to the highest extent (by 90%) among the gPR mutants carrying the wt AGCCC sequence

FIG. 5. Defining the core sequences critical for stimulation of CNV replicase activity. Minimal construct R2(SL)-4(Δs4) (Fig. 4, lane 42) was used to generate the shown mutations. The critical C · C mismatch in RII(+)-SL involved in selective binding to p33 is shown in boldface, and the non-Watson-Crick base pairs are shown based on references 12 and 26. The activity of the purified CNV replicase was tested as described in the legend to Fig. 1. Activity of the CNV replicase obtained from yeast expressing R2(SL)-4(Δs4) (lane C42) corresponds to 100% (see also lane 42 in Fig. 4).

(Fig. 6). Altogether, these data suggest that the sequences in the stem and within the tetraloop of gPR are unlikely to play specific roles in replicase assembly. The structure of gPR seems to be more important, however, because both less- and more-stable structures inhibited the assembly of the CNV replicase.

The core interacting sequences in RSE and gPR are important for assembly of the CNV replicase. Previous work defined the importance of base pairing between 5-nt-long sequences (5'-GGGCU-3') of RSE and gPR (5'-AGCCC-3') (Fig. 7, top) for tombusvirus replication (25). These sequences are also important for the assembly of the replicase, as shown in Fig. 5.

However, there is a possibility for alternative base pairing between RSE and gPR versus RSE and p33RE, as shown in Fig. 7. This model predicts that the 5-nt RSE sequence (5'-GGGCU-3') and the tetraloop sequence (5'-UUCG-3') of SL3 form base pairs with complementary sequences present within the internal loop region of RII(+)-SL, harboring the critical p33RE (Fig. 7A, bottom panel). To test the importance of the putative alternative base pairing, we tested single and combined mutations, which either interrupted or restored base pairing between either RSE and gPR or RSE and p33RE. For example, single mutations were introduced to RII(+)-SL (AcCCC), SL3 (GGGgU) and gPR (AcCCC), which main-

FIG. 6. Defining the role of sequences flanking the core sequences in p33RE and gPR in stimulation of CNV replicase activity. The minimal construct R2(SL)-4(Δs4) (Fig. 4, lane 42) was used to generate the shown mutations. The mutated sequences are shown in boldface. The activity of the purified CNV replicase was tested as described in the legend to Fig. 1. Activity of the CNV replicase obtained from yeast expressing R2(SL)-4(Δs4) (lane C42) corresponds to 100% (see also lane 42 in Fig. 4).

tained the possibility of alternative base pairing between these elements. The resulting construct (Fig. 7B, Comp-1), however, did not support the assembly of the CNV replicase. Testing the CNV replicase activity of control constructs that carried single mutations in only (i) one of the three elements (such as RII-SL-1, SL3-1, and gPR-1) (Fig. 7B) or (ii) in two of the three elements (such as RII-SL-1/SL3-1, RII-SL-1/gPR-1, and SL3-1/gPR-1) (Fig. 7B) demonstrated that the RSE and gPR sequences cannot be modified without the loss of replicase assembly. Similar results were obtained when two or three mutations were introduced into each of the three elements (Fig. 7B, Comp-2 and Comp-3 series of constructs). Based on these experiments, we suggest that the primary sequences of RSE and gPR are important for the assembly process and that compensatory mutations which, albeit, maintain the base-pairing potential, have detrimental effects.

Mutations within the core sequences of RSE and gPR inhibit replication of the DI-72 replicon RNA in yeast. To test if the above-identified core elements, which are critical for the assembly of the CNV replicase, are important during replication of the full-length replicon RNA in yeast, we randomly mutagenized three of the five positions in RSE (5'-GGnnn)

and gPR (5'-nnnCC). We did not change other sequences in the replicon RNA, such as RII(+)-SL or the 3'-terminal CC sequence in gPR, because that is required for efficient initiation of cRNA synthesis *in vitro* (25) and it can also be repaired *in vivo* (5). In these experiments, we randomly picked 300 yeast colonies, each carrying one of the replicon RNA variants with presumably unique combination of mutations in RSE and gPR, and separately tested DI RNA accumulation in total RNA extracts obtained from yeast 48 h after induction with galactose using Northern blotting. Only 1 of the 300 yeast strains accumulated DI RNA at a detectable level (not shown). The sequence of the replicating DI RNA contained only one mutation (cGCCC) within gPR, whereas the RSE sequence was wt (GGGCU). To check the sequence diversity within the 300 colonies, we randomly chose and sequenced 32 separate pYC/DI-72 expression plasmids derived from separate colonies and found that (i) none contained wt DI-72 sequence and (ii) all had different sequences within the randomized stretches (not shown). Therefore, it is likely that each of the 300 yeast strains expressed DI RNA carrying unique combination of mutations in RSE and gPR. Altogether, the observation that the vast majority of RSE and gPR mutants of DI-72 RNA (299 out of

FIG. 7. (A) Schematic representation of alternative base pairing between SL3 (which includes RSE) and gPR versus SL3 and RII(+)-SL sequences. The putative interacting sequences are marked with solid lines. Note that the predicted interaction between SL3 and RII(+)-SL could be facilitated by two sequence stretches (RSE-B and RSE'-B'). Also, the bottom portion of gPR is predicted to have two alternative structures. (B) Testing the role of alternative base pairing between SL3 and gPR versus SL3 and RII(+)-SL in replication of the DI-72(+) replicon RNA in yeast coexpressing p33 and p92. Single, double, and triple mutations within RII(+)-SL, SL3, and gPR, which either disrupted or restored base pairing, are shown in boldface. The replication of DI-72(+) replicon RNA and its derivatives in yeast was measured in total RNA extracts by Northern blotting. The replication levels were compared to that of DI-72(+) replicon RNA (100%). Note that mutations within SL3 and gPR abolished replication of the replicon RNA.

300) did not replicate in yeast supports the role for the primary sequence of the core regions of RSE and gPR in replication.

Because sequences within the p33RE were not changed in the above experiments, we could not exclude that the absence

of viable mutations within the RSE was due to the inability of RSE mutants to form alternative base pairing with both gPR and p33RE (Fig. 7A). Therefore, we modified three nucleotides in p33RE (Fig. 7B, construct RII-SL-3), which are known to allow the formation of active replicase, to test if viable DI RNAs could emerge from the pool of DI RNAs carrying randomized RSE and gPR sequences (see the randomized sequences above). Testing DI RNA accumulation in 400 separate yeast strains (derived from separate colonies, each of which carried one of the DI RNAs from the randomized pool [see Materials and Methods]) by Northern blotting revealed that none accumulated DI RNAs at a detectable level 48 h after induction with galactose (not shown). Based on the lack of replicating DI RNA among the 400 variants tested in yeast, we conclude that mutation(s) within the core sequences of RSE and gPR made the replicon RNA incompatible for replication. This finding supports the role for the primary sequences within the interacting RSE and gPR in tombusvirus replication.

DISCUSSION

P33RE, RSE, and gPR are critical RNA elements in CNV replicase assembly. The viral plus-stranded RNA template plays a remarkable role during the assembly of the CNV replicase by enhancing the activity of the purified replicase on added RNA templates by ~40-fold (22) to 100-fold (this study). Systematic deletion analysis of sequences in the 621-nt DI-72(+) RNA, which carries all the sequences required for CNV replicase assembly in yeast (22), led to the identification of three distinct *cis*-active elements that were required for CNV replicase assembly. These elements include RII(+)-SL harboring p33RE, SL3 containing RSE, and gPR sequences. Interestingly, only short sequence stretches (core sequences) within these elements are critical, whereas the additional sequences within these elements might only have indirect functions. The nature and possible roles of these core sequences will be discussed below.

The first major RNA element is the internally located RII(+)-SL, which contains a 4-nt-long symmetrical internal loop harboring the p33RE (Fig. 5). The most-conserved feature within the p33RE is a C · C mismatch, which is flanked by stable G-C base pairs from each side, among tombusviruses (12, 26). In addition to the G-C base pairs, non-Watson-Crick-type base pairing likely contributes to the stability of the unusual structure formed by the internal loop (12, 26). The CNV p33 replication protein has been shown to bind (as a dimer and/or multimer) selectively to p33RE within RII(+)-SL via recognition of the C · C mismatch (26). The same C · C mismatch is also important for the assembly of the CNV replicase in yeast (Fig. 5), suggesting that p33 binding to the viral RNA is likely a critical step in the replicase assembly process. Because p33 is also known to bind to p92 RdRp protein (30), we propose that the assembly of the CNV replicase might start with binding of p33/p92 complex to p33RE within RII(+)-SL. While this step could be sufficient for template selection and recruitment into replication (12, 26), it is unlikely that this step alone would be enough for replicase assembly. This is because the replicase assembly also requires additional RNA elements and possibly protein factors (see below).

The second major element for CNV replicase assembly is the 3'-terminal 5 nt of gPR (5'-AGCCC-3'). Mutations within this core sequence interfered with replicase assembly in yeast (although mutation in one position was tolerated [cGCCC]). Complementary mutagenesis of gPR, which changed the primary sequence but maintained the base pairing potential with the RSE (a 5-nt sequence within the internal loop in SL3) (Fig. 2) (25) revealed that the primary sequence in the 3' terminus of gPR is critical for the assembly process (Fig. 5 and 7B). This is seemingly contradictory with *in vivo* replication studies in plant protoplasts, where compensatory mutations in gPR and RSE did not completely abolish DI-72 RNA replication (25). We should point out, however, that in the plant protoplast experiments, DI-72 RNA replication was studied in the presence of wt helper virus, which could have supplied the necessary wt gPR and RSE sequences for the replicase assembly. Thus, the mutated DI-72 RNA only had to "borrow" preassembled replicase from the helper virus in order to replicate in plant protoplasts. In contrast, the replicon RNA itself has to function during the replicase assembly in yeast, where the helper virus is absent (Fig. 7B). Overall, these data support the role for the AGCCC sequence within the 3' terminus of gPR in replicase assembly.

Interestingly, 2 nt of the core gPR sequence (AG) are also predicted to base pair with 5' gPR sequences, forming the bottom of SL1 stem-loop structure (Fig. 6) (25). This alternative base pairing of AG (i.e., with RSE or within SL1) could be important for the function of gPR, because selective strengthening of SL1 led to a decreased level of replicase assembly (Fig. 6, construct 67). Additional sequences in gPR, including the tetraloop, will likely play indirect roles, possibly by forming a stem-loop structure, whose stability could be important (Fig. 6, construct 69).

The third RNA element required for the assembly of the CNV replicase is the RSE, which is present within the asymmetrical internal loop in SL3 (Fig. 5 and 7). Single and multiple mutations introduced within the 5-nt-long (5'-GGGCU-3') RSE completely inhibited the assembly of functional CNV replicase complexes (Fig. 7B). However, these mutations not only changed the primary sequence of the RSE but also inhibited its interaction with the core sequence in gPR (Fig. 7B). Complementary mutations, which restored base pairing between RSE and gPR, did not reverse the detrimental effect of mutations (Fig. 7B), suggesting that the primary sequence of the RSE is important in replicase assembly. Therefore, the sequences of RSE and gPR and their abilities to base pair are likely important, possibly by binding to host and/or viral protein factors (see below).

Whereas deletion and mutagenesis of DI-72(+) RNA firmly established the essential role of p33RE, RSE, and gPR during the assembly of the CNV replicase, these experiments suggest a lesser contribution of the remaining sequences in DI-72(+). Deletion of RI(+) and RIII(+) changed the efficiency of replicase assembly by less than 50% (Fig. 1B and C). In addition, partial deletions of RII(+) and RIV(+) sequences flanking p33RE and RSE, respectively (Fig. 3) affected replicase assembly to a lesser extent. Therefore, we propose that these sequences serve mostly as spacers, which are separating the critical elements, and/or stabilize the essential structures of RII(+)-SL, SL3, and gPR during CNV replicase assembly. The

exception is the SL2 hairpin in RIV(+) (located between SL3 and gPR) (Fig. 5), which might also play a direct role in replicase assembly, because its replacement with a different sequence forming a stem-loop structure had an inhibitory effect on replicase assembly (Fig. 5). Altogether, our results confirmed the roles of short core sequences/structures present in internal and 3'-terminal locations in the assembly of the functional CNV replicase complex.

Possible roles for alternative base pairing between RSE and gPR or RSE and p33RE. The RSE is currently the most intriguing *cis*-acting RNA element, because it might be involved in alternative RNA-RNA interactions. For example, in addition to the previously documented base pairing between the GGGCU sequence in RSE and the AGCCC sequence in gPR (25), we also predict an alternative base pairing of the GGGCU sequence of RSE with the internal loop region in RII(+)-SL (AGCCC) (Fig. 7A). This alternative base pairing could be further strengthened by base pairing between flanking sequences and the tetraloop in SL3 and the right-side sequence in the internal loop of RII(+)-SL, as shown in Fig. 7A. Importantly, the alternative base pairing between RSE and RII(+)-SL would allow the "exposure" of the 3'-terminal CCC tail of gPR for interaction with the viral replicase. This, in turn, could facilitate both the assembly of the replicase and initiation of minus-strand synthesis (see model below).

The evidence supporting the formation of alternative base pairing between RII(+)-SL and SL3 is not conclusive, however, because several disruptive mutations within sequences forming the alternative base pairing inhibited only moderately viral RNA accumulation (Fig. 7B, mutant RII-SL3-3). The supportive evidence for the role of alternative base pairing between RII(+)-SL and SL3 includes constructs 62 and 64 (Fig. 6), carrying mutations which reduced the strength of the putative alternative base pairing between RII(+)-SL and SL3 sequences and also decreased replicase activity by 97%. These mutations in RII(+)-SL did not inhibit binding to p33 *in vitro* (26). In addition, mutations in the tetraloop in SL3 (Fig. 5, construct 60), which are predicted to decrease the base pairing potential between SL3 and RII(+)-SL, also inhibited replicase activity by 97%. More extensive mutagenesis of the internal loop of RII(+)-SL (26) and of SL3 (25) is known to interfere with the primary functions of these elements (i.e., binding to p33 and base pairing with gPR, respectively), therefore making more rigorous testing of the alternative base pairing via complementary mutagenesis impractical (Fig. 7B). Alternative base pairing between *cis*-acting elements, termed repressor and promoter versus the repressor and derepressor, has also been proposed for *Turnip crinkle virus*, a related carmovirus (41), suggesting that analogous replicase-RNA interactions might also take place during replication of other plus-stranded RNA viruses.

The assembly of the BMV replicase complex on the viral RNA3(+) template also requires an internal element (termed RE [34]) and the 3' untranslated region (28). Another similarity between the BMV and tombusvirus replicase assembly is that the internal *cis*-element serves an additional role in template selection for replication (26, 34). Thus, plus-stranded RNA viruses belonging to different supergroups might use similar mechanisms to assemble functional replicases on intracellular membranes.

The requirement for specific RNA sequences in replicase activation is also known for hepadnaviruses, which use an internal sequence to activate the reverse transcriptase (RT) (36). The RNA-based activation of hepadnavirus RT leads to structural changes in the RT, which has been proposed to stimulate polymerase activity (35, 36). The activity of the influenza virus RNA polymerase is also stimulated by binding to the 5' and 3' ends of the template RNA (11). The stimulation is the result of activation of the high-affinity binding site of the polymerase, which prefers binding to primer-length RNAs (11).

Model for CNV replicase assembly. Assembly of the CNV replicase is likely initiated by selective binding of p33 dimers and/or multimers to p33RE present within RII of DI-72 and the p92 gene in gRNA (26). p33 has also been proposed to recruit p92 RdRp protein (T. Panavas et al., unpublished data). These events likely lead to the formation of DI RNA: p33:p92 complex (which likely includes host factors). However, the gPR promoter could be inaccessible for initiation because the 5-nt-long GGCGU sequence in SL3 functions as an RSE, base pairing with the 3' terminus of gPR (thus masking the initiation site in gPR). However, an as-yet-unidentified event, possibly (i) binding of host factor(s), (ii) binding of p33 to the structure formed between RSE and gPR, or (iii) membrane association of the template/p33/p92/host factor complex, might lead to structural changes in the template RNA and/or in the bound proteins. This could then (i) result in disruption of the RSE-gPR interaction, (ii) possibly followed by formation of putative alternative base pairing between RSE and RII(+)-SL (see above), (iii) assembly of the functional replicase, and (iv) initiation of minus-strand synthesis. The proposed mechanism would reduce the possibility that the replicase complex could start initiation of minus-strand synthesis prematurely, for example, during translation of viral RNA (in case of genomic RNA) or in the cytoplasm before the association of the replicase complex with peroxisomal membranes, which represent the site of CNV replication (T. Panavas et al., submitted for publication). Premature initiation of minus-strand synthesis could result in collision between the viral replicase and the ribosome, which travel in opposite directions in the template RNA (3, 7). In addition, premature initiation would produce double-stranded replicating RNAs (putative replication intermediates [4]) in the cytoplasm, which could trigger rapid antiviral responses, such as gene silencing (1).

Altogether, the proposed model would ensure the formation of robust CNV replicase complexes only in the right place and only at the right time. Moreover, the proposed mechanism would ensure high template fidelity for the CNV replicase. This is because only those RNAs would be replicated by the CNV replicase that contain the suitable *cis*-acting elements (i.e., p33RE, RSE, and gPR) to promote the assembly of functional replicase complexes in cells.

ACKNOWLEDGMENTS

We thank Judit Pogany and Saulius Serva for their critical comments and helpful suggestions.

This work was supported by the NIH and by the Kentucky Tobacco Research and Development Center at the University of Kentucky.

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