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Template Role of Double-Stranded RNA in Tombusvirus Replication

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ABSTRACT

Replication of plus-strand RNA [(+)RNA] viruses of plants is a relatively simple process that involves complementary minus-strand RNA [(−)RNA] synthesis and subsequent (+)RNA synthesis. However, the actual replicative form of the (−)RNA template in the case of plant (+)RNA viruses is not yet established unambiguously. In this paper, using a cell-free replication assay supporting a full cycle of viral replication, we show that replication of *Tomato bushy stunt virus* (TBSV) leads to the formation of double-stranded RNA (dsRNA). Using RNase digestion, DNAzyme, and RNA mobility shift assays, we demonstrate the absence of naked (−)RNA templates during replication. Time course experiments showed the rapid appearance of dsRNA earlier than the bulk production of new (+)RNAs, suggesting an active role for dsRNA in replication. Radioactive nucleotide chase experiments showed that the mechanism of TBSV replication involves the use of dsRNA templates in strand displacement reactions, where the newly synthesized plus strand replaces the original (+)RNA in the dsRNA. We propose that the use of dsRNA as a template for (+)RNA synthesis by the viral replicase is facilitated by recruited host DEAD box helicases and the viral p33 RNA chaperone protein. Altogether, this replication strategy allows TBSV to separate minus- and plus-strand syntheses in time and regulate asymmetrical RNA replication that leads to abundant (+)RNA progeny.

IMPORTANCE

Positive-stranded RNA viruses of plants use their RNAs as the templates for replication. First, the minus strand is synthesized by the viral replicase complex (VRC), which then serves as a template for new plus-strand synthesis. To characterize the nature of the (−)RNA in the membrane-bound viral replicase, we performed complete RNA replication of *Tomato bushy stunt virus* (TBSV) in yeast cell-free extracts and in plant extracts. The experiments demonstrated that the TBSV (−)RNA is present as a double-stranded RNA that serves as the template for TBSV replication. During the production of new plus strands, the viral replicase displaces the old plus strand in the dsRNA template, leading to asymmetrical RNA synthesis. The presented data are in agreement with the model that the dsRNA is present in nuclease-resistant membranous VRCs. This strategy likely allows TBSV to protect the replicating viral RNA from degradation as well as to evade the early detection of viral dsRNAs by the host surveillance system.

Replication of plus-strand RNA [(+)RNA] viruses is a relatively simple process driven by the viral replicase complex (VRC). The original viral (+)RNAs are recruited from translation to replication, and the (+)RNAs become templates to produce the complementary minus-strand RNAs [(−)RNAs] in small amounts while sequestered in membrane-bound VRCs (1–6). The (−)RNAs are used as the templates to generate large amounts of new (+)RNA progeny, which are released from the VRCs for a new round of translation/replication, cell-to-cell movement, and packaging. However, the replicative structure of the (−)RNA within the VRC is currently not yet defined for plant (+)RNA viruses (7, 8). The existence of double-stranded RNA (dsRNA) or the replicative intermediate (RI) form with one partially annealed (−)RNA and several partially annealed (+)RNAs has been shown for several animal (+)RNA viruses after disruption of VRCs via phenol-chloroform extraction (9–15). However, these complete or partial dsRNAs might have formed during the removal of membranes and proteins from the RNA samples by phenol-chloroform extraction, thus not excluding the possibility that these structures formed artificially during RNA sample manipulation. Although dsRNA forms are detected via using monoclonal anti-dsRNA antibody in cell or tissue samples (9, 16–18), these experiments used denatured (fixed) samples that could promote the artifactual annealing between free (−)RNA and (+)RNA during sample preparation. In addition, dsRNAs could be the dead-end products of the replication process, accumulating mostly at the end of RNA replication and not being used as the templates for new RNA synthesis.

Indeed, the earliest-characterized (+)RNA virus replication system based on Qβ phage showed the generation of free (−)RNA that was used to generate abundant (+)RNA progeny by the viral replicase (19–21). Similarly, it has been shown that yeast single-stranded RNA (ssRNA) viruses utilize free (−)RNAs as the templates for (+)RNA synthesis during their replication (22). However, replication of plant and animal (+)RNA viruses is more complex than Qβ phage replication, and also, it takes place in membranous structures where the viral RNAs and proteins are sequestered (1, 3, 4, 6). What would make the naked viral (−)RNA prevent annealing with the (+)RNA in such a microenvironment, where the viral (+)RNAs and (−)RNAs are present in close proximity and at high concentrations? Indeed, the structure of the

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poliovirus (PV) RNA-dependent RNA polymerase (RdRp)-RNA complex does not support the separation of the minus and plus strands prior to the exit of these RNAs from the PV RdRp (23). Overall, after 50 years of research, we still do not know the replicative structure of the viral RNA for plant (+)RNA viruses.

In this paper, we have addressed the replicative structure of the viral RNA using *Tomato bushy stunt virus* (TBSV), a tombusvirus that infects plants and is capable of replication in a yeast (*Saccharomyces cerevisiae*) surrogate host (24, 25). Based on a cell extract prepared from yeast that supports complete TBSV replication *in vitro* (26, 27), we show the accumulation of dsRNA in the viral replicase complex. Importantly, we could not detect free (−)RNAs before phenol-chloroform extraction, thus reducing the possibility that dsRNA was formed artificially via annealing of naked viral (−)RNAs and (+)RNAs during RNA extraction. Time course radiolabeled nucleotide chase experiments revealed that the dsRNA accumulated at early time points before the bulk synthesis of plus strands. The data obtained support a strand displacement mechanism, where the plus-strand portion of the dsRNA is displaced by the newly synthesized plus strand. These results suggest that the replicative structure of a plant (+)RNA virus is different from that shown for Qβ phage, indicating that the membranous microenvironment used by eukaryotic (+)RNA viruses might provide different circumstances and mechanisms for viral RNA replication in comparison with the cytosolic environment exploited by (+)RNA phages.

MATERIALS AND METHODS

***In vitro* TBSV replication assay in cell-free yeast extract.** Three different kinds of yeast cell-free extracts (CFEs) capable of supporting TBSV replication *in vitro* were prepared as described previously (26, 27). The differences among yeast CFEs were based on the starting yeast materials used to prepare the CFEs, namely that (i) the yeast cells did not express any tombusvirus component, (ii) the yeast cells expressed p33 and p92 replication proteins (see the supplemental material), or (iii) the yeast cells expressed p33 and p92 replication proteins and DI-72 replicon RNA (repRNA). Briefly, the method for the CFE assay is as follows: *in vitro* TBSV replication assays were performed by using 2 μl of CFE, 0.25 μg DI-72 (+)repRNA T7-made transcripts, 200 ng affinity-purified maltose-binding protein (MBP)-p33, 200 ng MBP-p92^{pol}, buffer A (30 mM HEPES-KOH [pH 7.4], 150 mM potassium acetate, 5 mM magnesium acetate, 0.13 M sorbitol), 0.4 μl actinomycin D (5 mg/ml), 2 μl of 150 mM creatine phosphate, 0.2 μl of 10 mg/ml creatine kinase, 0.2 μl of RNase inhibitor, 0.2 μl of 1 M dithiothreitol (DTT), 2 μl of a ribonucleotide (rNTP) mixture (10 mM ATP, CTP, and GTP and 0.25 mM UTP), and 0.1 μl of [^{32}P]UTP in a 20-μl total volume. The CFE assay was performed at 25°C for 3 h and stopped by the addition of a 1/10 volume of 1% SDS and 50 mM EDTA. This was followed by phenol-chloroform extraction and RNA precipitation. The ^{32}P-labeled repRNA products from the CFE assays were separated by electrophoresis in 0.5× Tris-borate-EDTA (TBE) buffer in a 5% polyacrylamide gel (PAGE) containing 8 M urea. The CFEs obtained from yeast cells expressing viral components were prepared as described previously (27).

For detection of the dsRNA in the CFE assay, the obtained ^{32}P-labeled repRNA products from the CFE assays were divided into two halves: one was loaded onto the gel without heat treatment in the presence of 50% formamide, while the other one was heat denatured at 85°C for 5 min in the presence of 50% formamide (28).

To remove the excess amounts of (+)RNA and, thus, increase the sensitivity of the RNA probing assay, CFE was fractionated by centrifugation at 14,000 rpm at room temperature for 10 min to separate the "soluble" (supernatant) and "membrane" (pellet) fractions at the end of the CFE-based TBSV replication assay. The membrane fraction was resuspended in buffer A (30 mM HEPES-KOH [pH 7.4], 150 mM potassium acetate, 0.13 M sorbitol, and 5 mM magnesium acetate).

The tombusvirus replicase assembly assay was performed in a 20-μl volume by using 2 μl of a mixture of 10 mM ATP and 10 mM GTP followed by incubation for 1 h at room temperature (26). After centrifugation at 14,000 rpm at room temperature for 10 min, the pellet (i.e., the membranous fraction) was resuspended in buffer A. This was done to synchronize the start of RNA synthesis after the addition of rNTPs and [^{32}P]UTP to the CFE as described previously (26).

RNA-RNA hybridization with unlabeled RNA probes. The replication assay was performed by using [^{32}P]UTP, and the reaction products were centrifuged at room temperature at 14,000 rpm for 10 min. The membranous fraction (pellet) was then resuspended in buffer A containing different unlabeled T7^{pol}-made transcripts (0.3 μg) complementary to one of the four regions in DI-72 RNA in the presence of 0.1% Triton X-100. The mixture was incubated at room temperature for 10 min. A 5× volume of 1% SDS and 50 mM EDTA was then added to stop the reaction, followed by phenol-chloroform extraction and RNA precipitation.

In the second replication assay, the samples from the CFE-based replication assay with [^{32}P]UTP was centrifuged at room temperature at 14,000 rpm for 10 min. The membrane fraction (pellet) was then resuspended in buffer A containing different unlabeled T7^{pol}-made transcripts (0.3 g) complementary to one of the four regions in DI-72 RNA in the presence of 0.1% Triton X-100. After incubation at room temperature for 10 min, a 5× volume of 1% SDS and 50 mM EDTA was added, followed by phenol-chloroform extraction and RNA precipitation. The samples were dissolved in 1× RNA loading dye, incubated at 85°C for 10 min, and immediately cooled down at 4°C.

Time course experiments. To measure ssRNA versus dsRNA amounts in the CFE-based replication assay, the RNA products were allowed to become ^{32}P labeled to various time points at 25°C. At the end of the replication assay, the repRNAs in one half of the sample were hybridized with unlabeled R1(−) transcripts (0.3 μg) in the presence of 0.1% Triton X-100. Another half was untreated. After the addition of a 5× volume of 1% SDS and 50 mM EDTA, we performed phenol-chloroform extraction and RNA precipitation, and the samples were then dissolved in 1× RNA loading dye.

Cleavage of DI-72 (−)repRNA with a DNAzyme. A single-stranded DNAzyme, named 10-23 (with the sequence 5′-TCCTGTTTAGGCTAG CTACAACGAGAAAGTTAG-3′) was constructed to cleave DI-72 (−) repRNA at position 120 (counted from the 5′-end minus strand). The CFE-based replication assay was performed as described above for 90 min, followed by collection of the membrane fraction by centrifugation. The obtained membrane fractions were resuspended in 1× RdRp buffer (50 mM Tris [pH 8.0], 10 mM $MgCl_2$, 10 mM DTT) in the presence or absence of 0.1% Triton X-100. The DNAzyme 10-23 (250-pmol final concentration dissolved in water) was then added to the membrane fractions and incubated for 2 h at room temperature. The reaction was stopped by the addition of 50 mM EDTA and 1% SDS. The control assay mixture contained the full-length ^{32}P-labeled DI-72 (−)repRNA T7^{pol}-made transcript.

RNase H digestion of the CFE-based *in vitro* products. The CFE-based TBSV replication assay was conducted as described previously (26, 27). After 3 h of incubation, 0.1 μl RNase I was added to the reaction mixtures, followed by 15 min of incubation at room temperature. Treatment A was performed as follows: 100 pmol oligonucleotide and the stop solution (0.05 M EDTA [pH 8.0] and 1% SDS) were added to the CFE samples, followed by phenol-chloroform extraction and ethanol precipitation. The pellet was then dissolved in water, and RNase H digestion was carried out in a 100-μl final volume in the presence of 20 mM Tris (pH 8.0), 50 mM NaCl, and 10 mM $MgCl_2$ with 1 U of RNase H at 30°C for 15 min. The products were purified by phenol-chloroform extraction and isopropanol precipitated. Treatment B was performed as follows (29): after RNase I digestion, samples were extracted with phenol-chloroform and isopropanol precipitated. Following a 70% ethanol wash and drying,

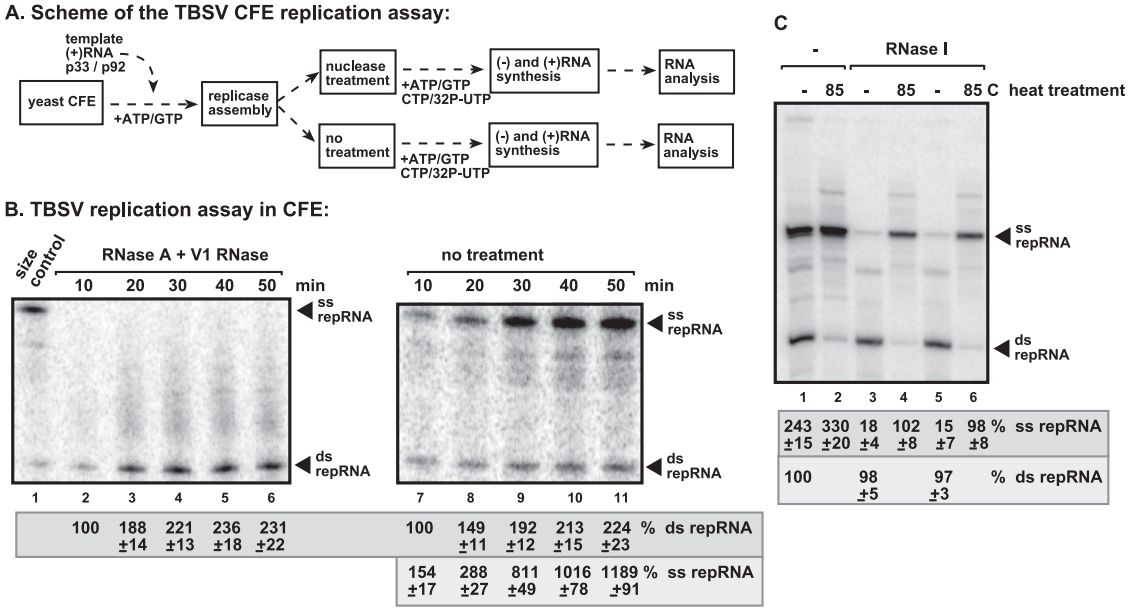


FIG 1 Cell-free TBSV replication assay showing the accumulation of RNase-protected viral dsRNA. (A) Scheme of the CFE-based TBSV replication assay. Purified recombinant p33 and p92^{pol} replication proteins of TBSV, *in vitro*-transcribed TBSV DI-72 (+)repRNA, GTP, and ATP were added to the whole-cell extract prepared from yeast strain BY4741. After replicase assembly on the membranes present in the CFE, the ssRNA-specific RNase RNase A and the dsRNA-specific V1 nuclease were added to the assay mixture to destroy unprotected RNAs. (B, left) PAGE analysis of the ^{32}P-labeled TBSV dsRNA products produced in the CFE-based TBSV replication assay in the presence of RNase A and RNase V1. (Right) Nondenaturing PAGE detection of ssRNA and dsRNA products produced in the CFE-based TBSV replication assay in the absence of RNases A and V1. The percentages of dsRNA in the samples are shown. (C) Characterization of dsRNA products at the end of the CFE assay. ssRNA-specific RNase I treatment was applied at the end of the replication assay, prior to phenol-chloroform extraction. The odd-numbered lanes represent replicase products that were not heat treated (thus, both ssRNA and dsRNA products are present), while the even-numbered lanes show the heat-treated replicase products (only ssRNA is present). Note that for the nondenatured samples, the dsRNA product represents the annealed (−)RNA and the (+)RNA, while the ssRNA products represent the newly made (+)RNA products. Each experiment was repeated at least three times, and the data were used to calculate standard deviations.

each sample was dissolved in 10 μl of sterile water. Ten microliters of 2× STE buffer (20 mM Tris [pH 8.0], 4 mM EDTA [pH 8.0], and 100 mM NaCl) and 100 pmol oligonucleotide was added to each sample. The samples were heated to 94°C in a PCR machine and gradually cooled to room temperature in 15 min. The RNase H digestion was carried out in a 100-μl final volume in the presence of 20 mM Tris (pH 8.0), 50 mM NaCl, and 10 mM $MgCl_2$ with 1 U of RNase H at 30°C for 15 min. Each sample was then phenol-chloroform extracted and ethanol precipitated.

Pulse-chase experiments. The CFE-based TBSV replication assay was performed for 1 h at 25°C in the presence of ATP, CTP, GTP, and [^{32}P]UTP. The samples were then centrifuged for 10 min at 14,000 rpm at room temperature, followed by resuspension of the pellet (membranous fraction) in buffer A. We then added 0.4 μl actinomycin D (5 mg/ml), 2 μl of 150 mM creatine phosphate, 0.2 μl of 10 mg/ml creatine kinase, 0.2 μl of RNase inhibitor, 0.2 μl of 1 M DTT, and 2 μl of rNTP mixture to continue the replication assay. For the cold assay, we used 10 mM ATP, 10 mM CTP, 10 mM GTP, and 10 mM UTP, while for the "labeling" assay, we used 10 mM ATP, 10 mM CTP, 10 mM GTP, 0.25 mM UTP, and 0.1 μl of [^{32}P]UTP. The assay mixture was incubated for 10 to 50 min at 25°C. After that, a 5× volume of 1% SDS and 50 mM EDTA was added, followed by phenol-chloroform extraction and RNA precipitation. The RNA samples were dissolved in 1× RNA loading dye, and one half of the sample volume was then loaded onto the gel directly, while the other half of the RNA samples was annealed with unlabeled R1(−) T7^{pol}-made RNA transcript as follows. After incubation at 85°C for 5 min, followed by immediate cooling to 4°C, the RNA samples were incubated at room temperature for 5 min with R1(−) (0.3 μg). Each sample was then phenol-chloroform extracted, ethanol precipitated, and analyzed.

TBSV replication assay using plant extracts. *Nicotiana benthamiana* plants were inoculated with sap obtained from TBSV-infected plants. The newly emerging systemically infected leaves were harvested at 5 days postinoculation. The leaf samples (~1 cm^2) were ground with a pestle in the presence of 0.2 ml buffer F (30 mM HEPES-KOH [pH 8.0], 50 mM potassium acetate, 250 mM sorbitol, 5 mM magnesium acetate, and 2 mM DTT), followed by centrifugation at 500 × *g* for 2 min at 4°C. The supernatant was centrifuged again at 1,000 × *g* for 5 min at 4°C. One microliter of supernatant from the last centrifugation step was used immediately for the *in vitro* replication reaction (20 μl). The reaction components were the same as those for the yeast CFE-based *in vitro* reaction, except that buffer F was used in the *in vitro* reaction for the plant extracts. No external RNA template or recombinant protein was added to the *in vitro* replication assay mixture.

RESULTS

RNase-resistant viral dsRNA is produced during TBSV replication in cell extracts. To test the nature of the viral RNAs produced during TBSV replication, we utilized a yeast cell-free extract (CFE) that is capable of supporting a single full cycle of authentic TBSV replication, starting with the *in vitro* assembly of the membrane-bound TBSV replicase and followed by the production of (−) RNA and abundant (+)RNA progeny in a membrane-dependent manner (26, 27, 30). As the final product of replication, the newly synthesized (+)RNAs are released from the VRC to the buffer, while the (−)RNA stays in the nuclease-resistant membrane-bound VRCs (26, 27).

First, we assembled the TBSV VRC in the CFE using purified recombinant TBSV p33 and p92^{pol} replication proteins plus DI-72 (+)repRNA (Fig. 1A). The VRC assembly assay mixture con-

tained only ATP and GTP that are needed for VRC assembly but lacked CTP and UTP to prevent the initiation of repRNA synthesis (26, 27). After the VRC assembly, we treated the samples with RNases (both ssRNA-specific RNase A and dsRNA-specific V1 nuclease) to destroy the RNAs not protected by the VRCs (Fig. 1A). After the addition of ATP/CTP/GTP and ^{32}P-labeled UTP, we measured the accumulation of new RNase-resistant viral RNA products in the CFE-based assay. Interestingly, we observed the accumulation of an RNase-resistant dsRNA product after 10 min of incubation (Fig. 1B, lane 2), and the amount of this RNA was doubled after 20 to 30 min but did not increase further after 30 min (Fig. 1B, lanes 5 and 6 versus lane 4). This dsRNA product likely represents annealed viral (−)RNAs and (+)RNAs, since it migrated as full-length ssRNA after heat denaturation (Fig. 1C, lanes 4 and 6). We also observed the accumulation of a small amount of RNase-resistant ssRNA (~20% of the dsRNA product) at the end of the assay (3-h time point) (Fig. 1C, lanes 3 and 5).

A similar CFE-based assay, but performed in the absence of RNases, showed a rapid accumulation of both ssRNA and dsRNA products (Fig. 1B, lanes 7 to 11). In contrast to the dsRNA, which reached a plateau after 30 min, the amount of newly synthesized ssRNA increased continuously. Comparison of the results of the CFE assays in Fig. 1B and C suggests that the dsRNA product is RNase resistant and part of the VRC, while most of the ssRNAs become RNase sensitive during replication *in vitro*, likely due to their release from the VRCs (see below) (26).

To confirm that the VRC-bound and RNase-protected viral RNA is indeed present as dsRNA species, we treated the CFE samples with RNases after disruption of the membranes in the CFE with small amounts of nonionic detergents (Fig. 2B, lanes 5, 6, 9, and 10). Treatment with dsRNA-specific V1 nuclease destroyed the dsRNA only in the presence of the detergent (Fig. 2B, lanes 5 and 6 versus lanes 3 and 4). On the other hand, treatment with ssRNA-specific RNase I did not affect the fast-migrating band in the presence of detergent (Fig. 2B, lanes 9 and 10 versus lanes 7 and 8), suggesting that this band represents dsRNA formed between (−)RNAs and (+)RNAs. The V1 RNase did not destroy the dsRNA in the absence of detergent (Fig. 2B, lanes 3 and 4), likely due to the protection provided by the membrane-bound VRCs. We also repeated these experiments with two other types of CFE-based assays, where the VRCs were preassembled in yeast (see Fig. S1C and S1D in the supplemental material) or where only the protein components of VRCs were preassembled in yeast and the repRNA was added only during the CFE assay (see Fig. S1A and S1B in the supplemental material). Altogether, these assays also confirmed the presence of dsRNAs in the VRCs. Moreover, we observed that only a tiny amount (0.05% Triton X-100 or 0.01% Nonidet P40) of detergent is needed to make the ssRNA in the membrane-bound VRC sensitive to RNase I (see Fig. S2A in the supplemental material). This amount of detergent is not enough to solubilize membrane-bound proteins (such as TBSV p33 and p92, which are integral membrane proteins) but is likely enough to disrupt the membrane-protected structure of VRCs and renders VRCs accessible to RNases. Also, the detergent did not interfere with binding of the p33 replication protein to the viral RNA *in vitro* (see Fig. S2B in the supplemental material), suggesting that the replicating RNA is still bound by the replication proteins within the VRCs after treatment with small amounts of detergents. The presence of a small amount of Triton X-100 did not promote annealing between (−)- and (+)repRNAs under the test

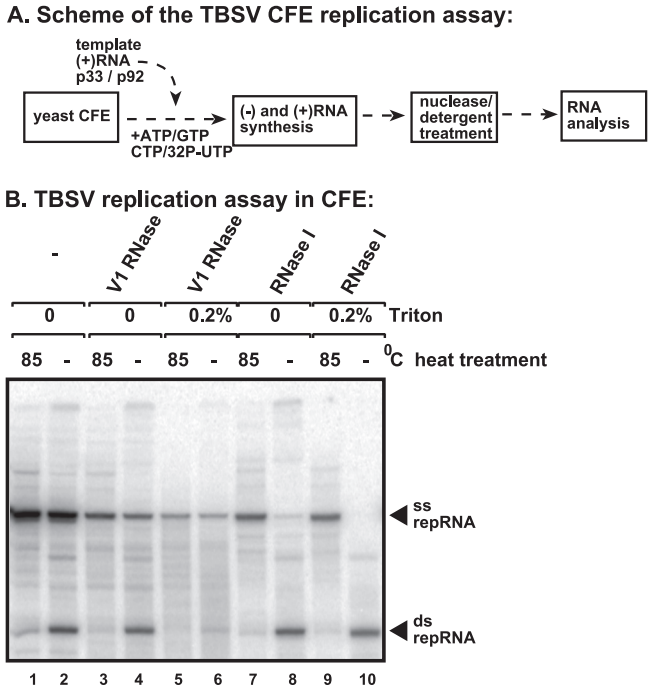
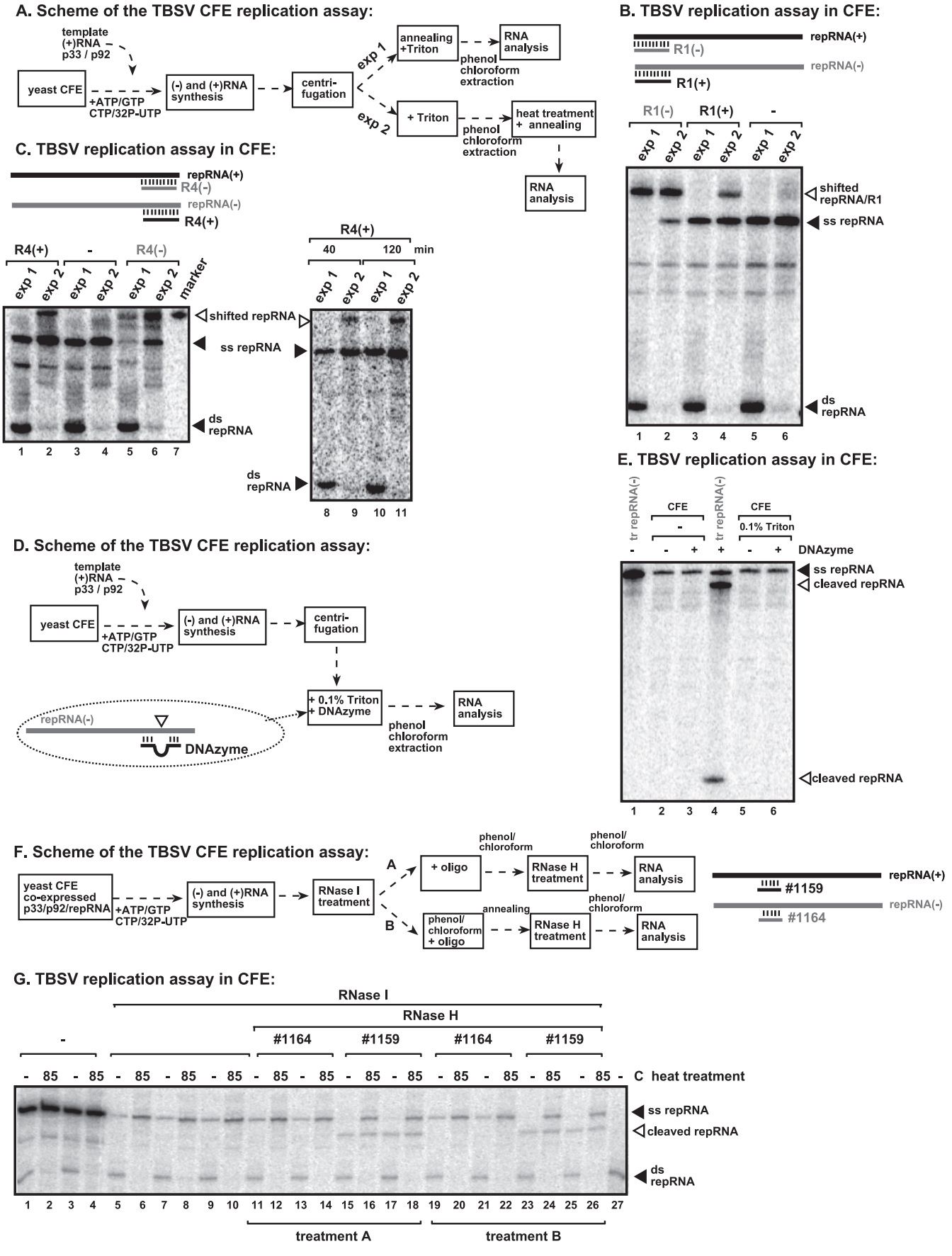


FIG 2 Detection of dsRNA-specific V1 nuclease-sensitive dsRNA products in the CFE-based TBSV replication assay. (A) Scheme of the CFE-based TBSV replication assay. Purified recombinant p33 and p92^{pol} replication proteins of TBSV and *in vitro*-transcribed TBSV DI-72 (+)repRNA were added to the whole-cell extract prepared from yeast strain BY4741. We added V1 or RNase I in the presence or absence of 0.2% Triton X-100 prior to phenol-chloroform extraction. (B) PAGE analysis of the ^{32}P-labeled TBSV repRNA products obtained in the CFE assays (for further details, see Fig. 1C). Note that RNase V1 can cleave highly structured hairpin regions in the ssRNAs, which likely causes some loss in the ssRNA bands in some samples. Each experiment was repeated at least three times.

conditions (see Fig. S2D and S2E in the supplemental material). The dsRNA was resistant to RNase I before and also after phenol-chloroform extraction (see Fig. S3 in the supplemental material).

Altogether, these CFE-based assays confirmed the accumulation of the viral dsRNA product during TBSV replication. The early accumulation of V1 nuclease-sensitive (in the presence of detergent) but RNase I-insensitive VRC-bound dsRNA product during TBSV replication suggests that the dsRNA might participate as a template for (+)RNA synthesis. Importantly, we detected viral dsRNA before phenol-chloroform extraction, thus reducing the possibility that dsRNA was formed artificially via annealing of naked viral (−)- and (+)RNAs during RNA extraction.

Free (−)RNA is not produced during TBSV replication in cell extracts. To further test what form of (−)RNA is present in the VRCs, we probed the ^{32}P-labeled VRC products with short unlabeled RNAs prior to phenol-chloroform extraction (experiment 1) (Fig. 3A). Interestingly, annealing of the (+)repRNA-specific R1(−) RNA, but not the (−)repRNA-specific R1(+) RNA, to the ^{32}P-labeled VRC products resulted in a shift in migration of the ssRNA products (Fig. 3B, lane 1 versus lane 3). After heat denaturation of the repRNA samples (experiment 2) (Fig. 3A), both (+)RNA- and (−)RNA-specific R1(−) and R1(+) resulted in annealing and partial duplex formation with ssRNAs, confirming that R1(−) and R1(+) anneal efficiently to the com-


A. Scheme of the TBSV CFE replication assay:
template (+)RNA p33 / p92
yeast CFE
+ATP/GTP CTP/32P-UTP
(-) and (+)RNA synthesis
centri-fugation
exp 1
exp 2
annealing +Triton
RNA analysis
phenol chloroform extraction
+ Triton
heat treatment + annealing
phenol chloroform extraction
RNA analysis
B. TBSV replication assay in CFE:
repRNA(+)
R1(-)
repRNA(-)
R1(+)
R1(-)
R1(+)
-
exp 1
exp 2
exp 1
exp 2
exp 1
exp 2
shifted repRNA/R1
ss repRNA
ds repRNA
1 2 3 4 5 6
C. TBSV replication assay in CFE:
repRNA(+)
R4(-)
repRNA(-)
R4(+)
R4(+)
-
R4(-)
exp 1
exp 2
exp 1
exp 2
exp 1
exp 2
marker
shifted repRNA
ss repRNA
ds repRNA
1 2 3 4 5 6 7
R4(+)
40
120
min
exp 1
exp 2
exp 1
exp 2
ds repRNA
8 9 10 11
D. Scheme of the TBSV CFE replication assay:
template (+)RNA p33 / p92
yeast CFE
+ATP/GTP CTP/32P-UTP
(-) and (+)RNA synthesis
centri-fugation
repRNA(-)
DNAzyme
+ 0.1% Triton + DNAzyme
phenol chloroform extraction
RNA analysis
E. TBSV replication assay in CFE:
tr repRNA(-)
CFE
-
tr repRNA(-)
CFE
0.1% Triton
- - + + - + DNAzyme
ss repRNA
cleaved repRNA
cleaved repRNA
1 2 3 4 5 6
F. Scheme of the TBSV CFE replication assay:
yeast CFE co-expressed p33/p92/repRNA
+ATP/GTP CTP/32P-UTP
(-) and (+)RNA synthesis
RNase I treatment
A
+ oligo
phenol/ chloroform
RNase H treatment
phenol/ chloroform
RNA analysis
B
phenol/ chloroform + oligo
annealing
RNase H treatment
phenol/ chloroform
RNA analysis
repRNA(+)
#1159
repRNA(-)
#1164
G. TBSV replication assay in CFE:
RNase I
RNase H
-
#1164
#1159
#1164
#1159
- 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - 85 - C heat treatment
ss repRNA
cleaved repRNA
ds repRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
treatment A
treatment B

plementary sequences in the ^{32}P-labeled ssRNAs under the experimental conditions used (Fig. 3B, lanes 2 and 4). We also repeated this experiment with two other sets of short unlabeled RNAs annealing to the ^{32}P-labeled 3′ untranslated region (UTR) (Fig. 3C, lane 5 versus lane 1) or to a ^{32}P-labeled internal position of either plus or minus strands, with similar results (see Fig. S4, lanes 1 and 8 versus lanes 5 and 12, in the supplemental material). In addition, we have studied the presence of free minus strands of repRNAs with short unlabeled RNAs at various time points (40, 60, and 120 min) that showed that only free single-stranded and double-stranded (+)repRNAs were present in the CFE-based replication assay mixture (Fig. 3C, lanes 8 and 10; see also Fig. S4 in the supplemental material). Importantly, we could not detect free (−)repRNAs within the VRCs in these CFE-based replication assays.

To further test if free (−)repRNAs are present in the VRCs, we used a minus-strand-specific DNAzyme, which can cleave the (−)repRNA upon annealing (Fig. 3D). The DNAzyme was added at the end of the assay to the detergent-treated membrane fraction of the CFE, prior to phenol-chloroform extraction. We did not observe a DNAzyme-cleaved ^{32}P-labeled (−)repRNA product in the VRC assay (Fig. 3E, lanes 3 and 6), albeit the control (−)repRNA transcripts were cleaved by this DNAzyme (Fig. 3E, lane 4). This suggests that the (−)repRNA was present in the VRCs as part of double-stranded repRNA, which is not targeted by the DNAzyme due to the lack of efficient annealing to the duplexed repRNA.

Since the VRCs contained a small amount of free RNase I-resistant single-stranded repRNA (in the absence of detergent) (Fig. 1C, lanes 3 and 5), we analyzed the polarity of this repRNA species using strand-specific oligonucleotides and RNase H, which cleaves RNA/DNA hybrids (Fig. 3F). This assay revealed that the VRCs contained a small amount of free (not base-paired) single-stranded (+)repRNA but not single-stranded (−)repRNA (Fig. 3G, lanes 15 and 17 versus lanes 11 and 13).

All the above-described data suggested that the tombusvirus VRCs contain the (−)repRNA annealed to (+)repRNA forming dsRNA, which is used as the template by the VRC to produce (+)RNAs. On the other hand, the VRCs do not seem to contain detectable amounts of free/naked (−)repRNA, thus making it unlikely that free (−)repRNA could serve as a template for (+)RNA synthesis during tombusvirus replication.

Time course experiments reveal that dsRNA may be a template for production of new (+)RNA in cell-free extracts. To examine if dsRNA is used as a template for (+)RNA synthesis or if it is a dead-end product of RNA synthesis during TBSV replication, we performed time course experiments (Fig. 4A). We observed that ~70% of the double-stranded repRNA was produced within the first 30- to 60-min period of the CFE-based assay (Fig. 4B, lanes 10 to 12), while the bulk of the single-stranded repRNA was produced within the 90- to 120-min period of the assay (Fig. 4B, lanes 13 and14). The short unlabeled RNA probe-based mobility shift assay revealed that all the detectable free single-stranded repRNA was of positive polarity (Fig. 4B, lanes 1 to 4). Similar time course experiments characterizing the RNA content of the membrane-bound VRCs (after the removal of the released new repRNA progeny from the samples) also revealed that the dsRNA accumulated within the 30- to 60-min period of the assay and that most of the single-stranded repRNA content of the VRCs accumulated at later time points (90 to 120 min) and represented only (+)repRNA progeny (Fig. 4C and D). Based on these data, we suggest that the double-stranded repRNA in the membrane-bound VRCs serves as a template for (+)RNA synthesis during replication, and it is unlikely that the double-stranded repRNA is a dead-end product of replication.

TBSV RNA is replicated by a strand displacement mechanism on the dsRNA template in cell-free extracts. To test the mechanism of utilization of double-stranded repRNA as a template for (+)RNA synthesis during TBSV replication, we used radioactive chase experiments (Fig. 5A and B). First, we produced unlabeled double-stranded repRNA in the CFE by allowing replication for 2 h. During this period, double-stranded repRNA reaches a maximum level (data not shown). We then added ^{32}P-labeled UTP in three different sets of experiments, as shown schematically (Fig. 5B). In experiment 1, we treated the CFE at the 2-h time point with micrococcal nuclease to remove excess (+)repRNA, or in experiment 2, we collected the membrane fraction of the CFE (Fig. 5B). The micrococcal nuclease treatment or collection of the membrane fraction by centrifugation removes the released (+)repRNA from the VRCs, and thus, these treatments exclude the possibility that the (+)repRNA could participate in the assembly of new VRCs during the labeling phase of the assay. This design helps in obtaining synchronized VRC activities during the labeling phase of the assay. Interestingly, the double-

FIG 3 Lack of free TBSV (−)repRNA among the TBSV RNAs produced in the CFE assay. (A) Scheme of the CFE-based TBSV replication assay. In experiment 1, at the end of the replication assay, we added unlabeled short complementary RNAs to the membrane fraction of the CFE assay mixture in the presence of 0.1% Triton X-100 prior to phenol-chloroform extraction and RNA analysis. In experiment 2, at the end of the replication assay, we added 0.1% Triton X-100 to the membrane fraction of the CFE assay mixture, performed phenol-chloroform extraction, heat denatured the RNAs, and then added unlabeled short complementary RNAs. Note that experiment 2 tests the ability of the short complementary RNAs to specifically anneal to the target RNA in the assay mixture. (B, top) Scheme of the annealed unlabeled short complementary RNAs to the ^{32}P-labeled repRNA products. Note that the annealed RNA duplex changes the migration of the RNA in PAGE gels. (Bottom) Representative PAGE analysis of ^{32}P-labeled repRNA products synthesized by the tombusvirus replicase in the CFE assay. The positions of shifted repRNAs (due to annealing to short complementary RNAs), single-stranded repRNAs, and double-stranded repRNAs are shown. Each experiment was repeated three times. (C) Same assay as in panel B except with a different set of short complementary RNAs. (D) Scheme of the DNAzyme-based cleavage of the ^{32}P-labeled repRNA products. The DNAzyme and 0.1% Triton X-100 were added to the membrane fraction of the CFE assay mixture prior to phenol-chloroform extraction. Note that the DNAzyme must anneal to the free (−)repRNA to induce cleavage of the target (−)repRNA, as shown. (E) Representative denaturing PAGE analysis of the DNAzyme-treated ^{32}P-labeled repRNA products synthesized by the tombusvirus replicase in the CFE assay. Lane 4 shows the DNAzyme cleavage products of the *in vitro*-transcribed DI-72 (−)repRNA as a positive control that demonstrates the functionality of the DNAzyme. (F) Identification of the polarity of ssRNA present in the membrane-bound VRCs. Shown is a scheme of the CFE-based TBSV replication assay. The CFE was prepared from BY4741 yeast cells expressing tombusvirus p33 and p92^{pol} replication proteins and the TBSV DI-72 (+)repRNA. The RNase I treatment (in the absence of detergent) at the end of the CFE assay removed the released, unprotected, ^{32}P-labeled (+)repRNA, followed by phenol-chloroform extraction. The samples were then annealed to strand-specific DNA oligonucleotides (as shown schematically) and treated with RNase H. (G) PAGE analysis of the ^{32}P-labeled TBSV repRNA products obtained in the CFE assays after RNase H treatment. Note that the RNase I-protected ^{32}P-labeled ssRNA was cleaved by RNase H in the presence of oligonucleotide 1159, demonstrating that the ssRNA represents (+)repRNA in the membrane-bound VRC.

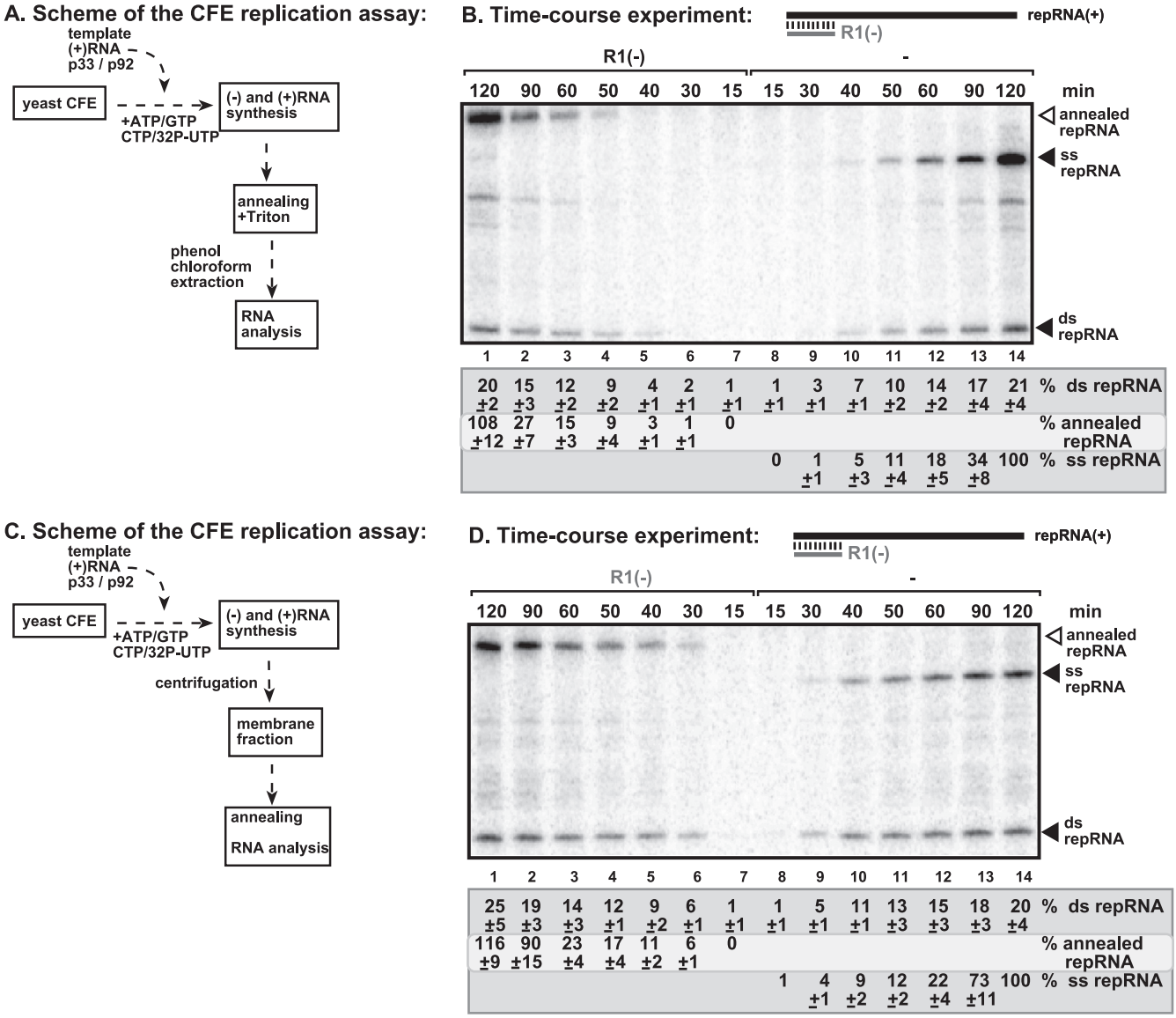


FIG 4 Time course to detect the appearance of TBSV repRNA products generated in the CFE assay. (A) Scheme of the CFE-based TBSV replication assay. At the end of the replication assay, we added unlabeled R1(−) RNA to the CFE assay mixture to test the polarity of the ssRNA products. (B) Representative nondenaturing PAGE analysis of ^{32}P-labeled repRNA products synthesized by the tombusvirus replicase in the CFE assay. The positions of shifted repRNAs [due to the annealing to R1(−) RNA], single-stranded repRNAs, and double-stranded repRNAs are shown. Note that the production of dsRNA and ssRNA products requires a longer time in this assay than in the assay depicted in Fig. 1 because here we did not preassemble the replicase complex prior to the addition of ribonucleotides. Each experiment was repeated three times. (C) Scheme of the CFE-based TBSV replication assay. At the end of the time course of the replication assay, we removed the supernatant by centrifugation and added unlabeled R1(−) RNA (in the presence of 0.1% Triton X-100) to the membrane fraction of the CFE to test the polarity of the ^{32}P-labeled ssRNA products. Note that only the membrane-bound ^{32}P-labeled repRNA products were analyzed in order to get rid of the large amount of original (+)repRNA transcripts added at the start of the CFE assay. (D) Representative PAGE analysis of ^{32}P-labeled repRNA products synthesized by the tombusvirus replicase in the CFE assay. The positions of shifted repRNAs [due to the annealing to R1(−) RNA], single-stranded repRNAs, and double-stranded repRNAs are shown. Note that the bulk amount of dsRNA appeared earlier (between 30 and 50 min) than that of the (+)ssRNA product (between 60 and 120 min), suggesting that the dsRNA might serve as a template for new (+)RNA synthesis and less likely that the dsRNA is a dead-end product of replication. Each experiment was repeated three times.

stranded repRNA became ^{32}P labeled during all three CFE-based assays (Fig. 5C). This observation is consistent with the plus-strand displacement model (model 1) (Fig. 5A) while excluding the conservative model as the mechanism of replication (model 2, in which the double-stranded repRNA should not be labeled due to a lack of new minus-strand synthesis) (Fig. 5A).

To further analyze this model, we performed time course experiments in combination with the above-described radioactive chase approach, as shown in Fig. 5B and D (experiment 3). After the production of unlabeled double-stranded repRNA in the CFE for 2 h, we added ^{32}P-labeled UTP and continued with the CFE-based assay. This time course study revealed that double-stranded repRNA became rapidly labeled (after 10 min of incubation in the presence of ^{32}P-labeled UTP), and the amount of double-stranded

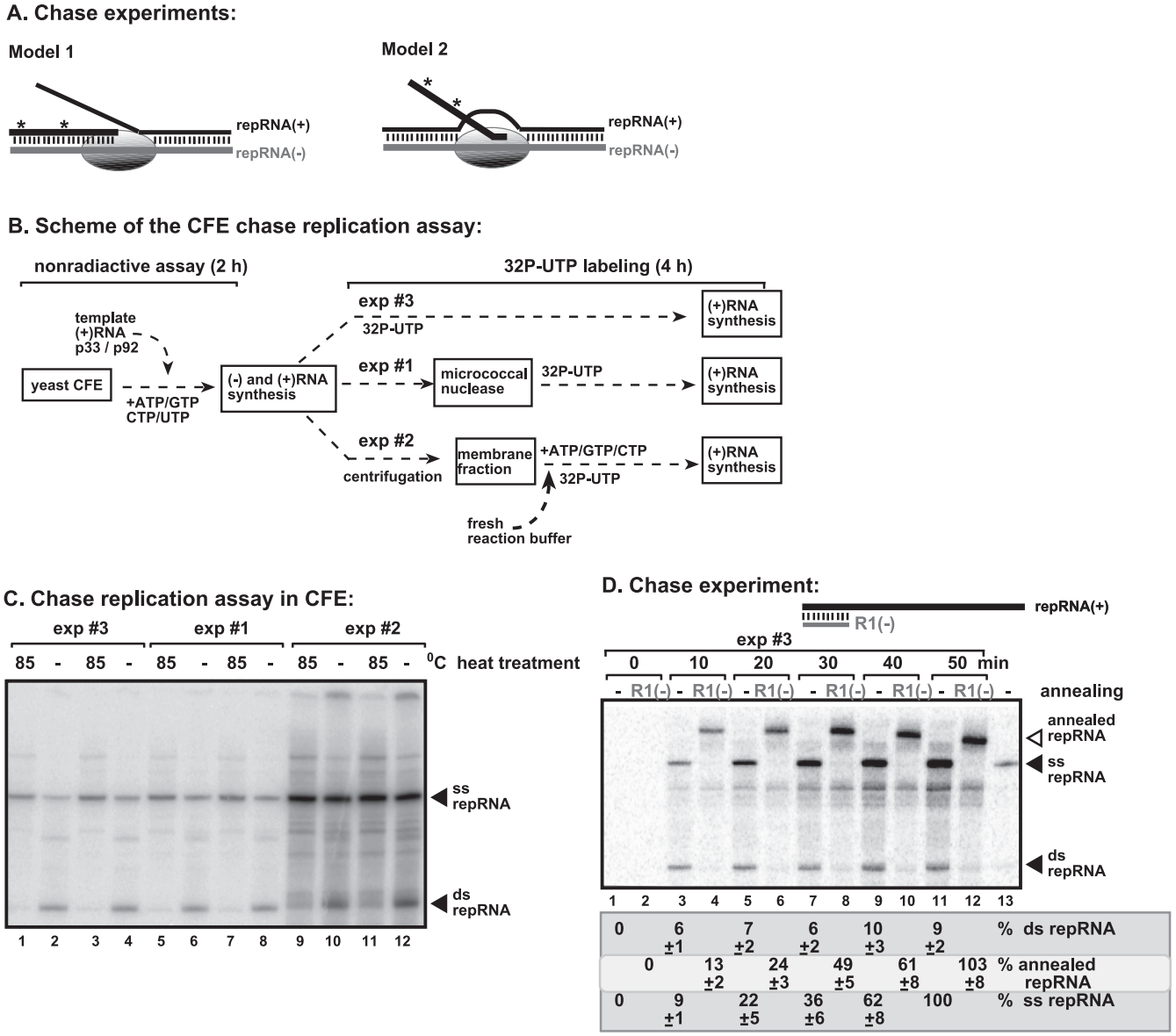


FIG 5 TBSV replicates via a strand displacement mechanism during plus-strand synthesis. (A) Semiconservative (strand displacement) (model 1) and conservative (model 2) models of (+)RNA synthesis based on dsRNA templates. Note the different fate of the newly synthesized ^{32}P-labeled (+)RNA depending on the mechanism of replication. (B) Schemes of the ^{32}P-labeled UTP chase experiments. In experiment 1, micrococcal nuclease treatment (lasting for 15 min followed by inactivation) was applied prior to the addition of ^{32}P-labeled UTP. In experiment 2, the membrane fraction from the prior CFE assay was collected to remove unincorporated nonlabeled ribonucleotides, followed by the addition of new buffer and a new batch of ribonucleotides, including ^{32}P-labeled UTP. The reaction assay was then continued for another 4 h. In experiment 3, ^{32}P-labeled UTP was added to the CFE-based replication assay mixture at the 2-h time point, followed by an additional 4-h reaction. Note that nuclease treatment or centrifugation in experiments 1 and 2 was done to prevent the synthesis of new minus-stranded RNAs in the CFE after the addition of ^{32}P-labeled UTP. (C) PAGE analysis of the ^{32}P-labeled TBSV repRNA products obtained in the CFE assays described above for panel B. The even-numbered lanes represent replicase products without heat treatment (thus, both ssRNA and dsRNA products are visible), while the odd-numbered lanes show the heat-treated replicase products (only ssRNA is present) (for further details, see Fig. 1). (D) RNA shift assay based on annealing of short unlabeled cRNA in chase time course studies. The assay was performed as described above for the experiment 3 scheme in panel B, except that the reaction was stopped after 10 to 50 min, as shown, and after heat denaturation, short R1(−) RNA complementary to the 5′ end of (+)repRNA was added to every second sample (even-numbered rows) to form a partial duplex. The PAGE gel shows the complete shift of the newly synthesized repRNAs (derived from both ssRNA and dsRNA), demonstrating that the new ^{32}P-labeled RNAs represented (+)repRNAs, while the level of ^{32}P-labeled (−)RNA was below the detection limit.

repRNA did not change after the first 10 to 20 min during the experiment (Fig. 5D, odd-numbered lanes). In contrast, the synthesis of new single-stranded repRNAs continued for at least another 40 min. Moreover, as predicted by model 1, all the newly labeled repRNAs, present in either the dsRNA or ssRNA form, are of (+)RNA polarity based on the complete shift in repRNA migration after annealing with the short plus-strand-specific unlabeled R1(−) RNA probes (Fig. 5D, even-numbered lanes). Therefore, our data demonstrate the lack of new (−)repRNA production after the addition of ^{32}P-labeled UTP to the assay mixture. Thus, the rapid labeling of double-stranded repRNA must be due to a replacement of the unlabeled (+)repRNA portion of

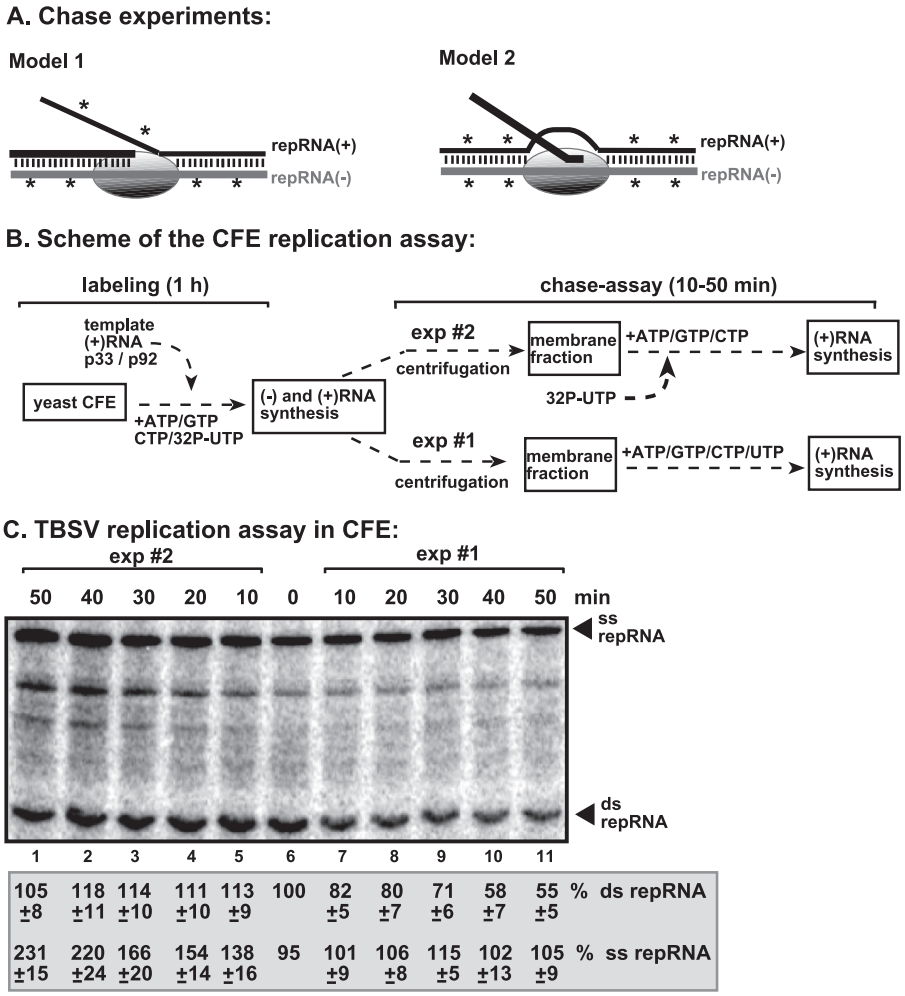


FIG 6 Additional evidence supporting that TBSV replicates via a strand displacement mechanism. (A) Prediction of the different fates of the newly synthesized unlabeled (+)repRNA depending on the mechanism of replication. The ^{32}P-labeled strands are shown with asterisks. (B) Schemes of the unlabeled UTP chase experiments. In experiment 1, the CFE-based TBSV replication assay mixture contained ^{32}P-labeled UTP and additional nonlabeled ribonucleotides to obtain ^{32}P-labeled ssRNAs and dsRNAs. We then removed the soluble ^{32}P-labeled (+)repRNAs (which were released from the VRCs during replication) and the unincorporated unlabeled and ^{32}P-labeled ribonucleotides by collecting the membrane fraction of the CFE. This was followed by the addition of nonlabeled UTP in combination with unlabeled ATP/CTP/GTP to the CFE-based replication assay mixture at the 1-h time point, followed by an additional 10- to 50-min reaction. In experiment 2, we used the same procedure as that used for experiment 1 except with the addition of ^{32}P-labeled UTP during the chase period (10- to 50-min reactions). Thus, this experiment is a positive control that could show if additional RNA synthesis took place during the chase period. (C) PAGE analysis of the ^{32}P-labeled TBSV repRNA products obtained in the CFE assays described above for panel B. For further details, see Fig. 1. Time point "0" (lane 6) shows the amount and single-stranded or double-stranded nature of repRNAs present in the CFE assay mixture prior to the chase period.

double-stranded repRNA with new ^{32}P-labeled (+)repRNA, as predicted by the strand displacement model (model 1) (Fig. 5A).

Another important observation is that the double-stranded repRNA becomes labeled at the beginning of the assay, while the bulk of the new labeled (+)repRNA is being produced at a later time point (Fig. 5D). This could be interpreted as evidence that the double-stranded repRNA serves as a template for plus-strand repRNA synthesis during replication, while the single-stranded (+)repRNA is the final product of replication. In other words, it is unlikely that the double-stranded repRNA would be a "dead-end" product in TBSV replication, since by then, the double-stranded repRNA should accumulate mostly at the end of the assay, which was not the case here.

We also performed similar chase experiments after isolation of the membrane fraction of CFE (see Fig. S5 in the supplemental material), which should completely prevent the formation of new VRCs during the labeling step. This time course study also revealed that double-stranded repRNA became labeled after 10 min of incubation in the presence of ^{32}P-labeled UTP, and the amount of double-stranded repRNA did not change during further incubation. Also, all the newly labeled repRNAs were of (+)RNA polarity (see Fig. S5, even-numbered lanes, in the supplemental material).

To obtain further evidence on the mechanism of TBSV replication, we performed additional radioactive chase experiments (Fig. 6A). Here, we first produced ^{32}P-labeled double-stranded repRNA in the CFE by allowing replication to occur for 1 h, followed by a nonradioactive chase assay, as shown in Fig. 6B (experiment 1). As predicted by model 1, the amount of ^{32}P label in the double-stranded repRNA was reduced continuously, reaching

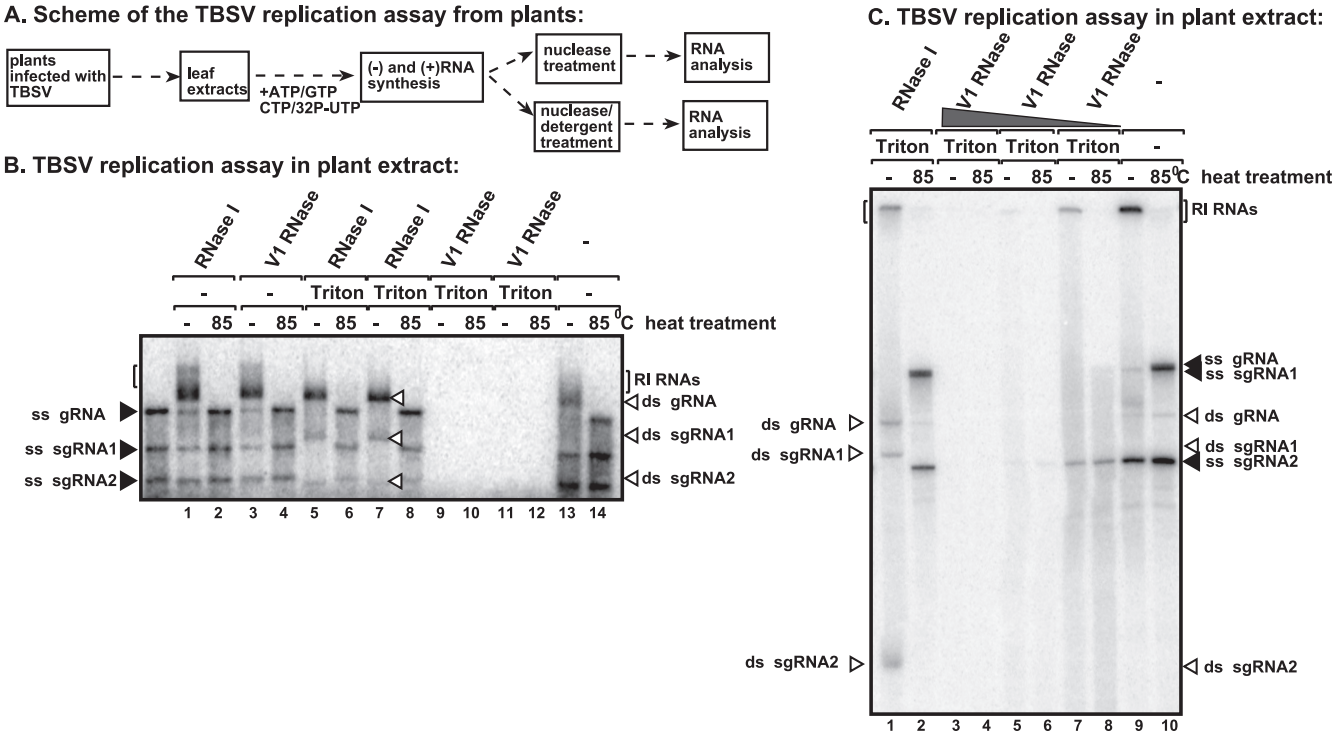


FIG 7 *In vitro* TBSV replication assay showing the accumulation of RNase-protected viral dsRNA in plants. (A) Scheme of the plant extract-based TBSV replication assay. *N. benthamiana* leaves replicating TBSV genomic RNA were used to prepare plant extracts, which were used in *in vitro* assays in the presence of rNTPs and ^{32}P-labeled UTP. (B) Nondenaturing agarose gel analysis of the ^{32}P-labeled TBSV ssRNA and dsRNA products produced in the plant extract-based TBSV replication assays in the presence of RNases I and V1 and 0.1% Triton X-100. The odd-numbered lanes represent replicase products, which were not heat treated (thus, both ssRNA and dsRNA products are present), while the even-numbered lanes show the heat-treated replicase products (only ssRNA is present). Note that the dsRNA product represents the annealed (−)RNA and the (+)RNA, while the ssRNA products represent the newly made (+)RNA products. The replication intermediates (RI) are likely present as a "smeary" band in the gel, as indicated. Each experiment was repeated three times. (C) PAGE analysis of *in vitro* TBSV replication products in plant extracts. Shown is nondenaturing PAGE analysis of the ^{32}P-labeled TBSV ssRNA and dsRNA products produced in the plant extract-based TBSV replication assay in the presence of RNase I or RNase V1 and 0.1% Triton X-100. The odd-numbered lanes represent replicase products, which were not heat treated (thus, both ssRNA and dsRNA products are present), while the even-numbered lanes show the heat-treated replicase products (only ssRNA is present). Note that the dsRNA product represents the annealed (−)RNA and the (+)RNA, while the ssRNA products represent the newly made (+)RNA products. The replication intermediates are present at the top of the gel, as indicated. Note that single-stranded gRNA and single-stranded sgRNA1 comigrate in this gel. Each experiment was repeated three times.

~50% by the end of the experiment (Fig. 6C, lanes 10 and 11). The easiest interpretation of this observation is that during ongoing replication, the ^{32}P-labeled (+)repRNA component of the double-stranded repRNA template was replaced by an unlabeled newly synthesized (+)repRNA (model 1) (Fig. 6A). In a parallel set of experiments, after the production of ^{32}P-labeled double-stranded repRNA in the CFE for 1 h, the replication assay was performed in the presence of ^{32}P-labeled UTP (experiment 2) (Fig. 6B). Increasing levels of (+)repRNA during the time course was observed, suggesting ongoing new (+)repRNA synthesis (Fig. 6C, lanes 1 to 5). Also, the level of ^{32}P-labeled double-stranded repRNA did not change during experiment 2 [due to the new ^{32}P-labeled (+)repRNA replacing the "old" ^{32}P-labeled (+) repRNA] (Fig. 6C, lanes 1 to 5), showing that there was no new generation of double-stranded repRNAs under these conditions.

Overall, the data obtained in both types of chase replication experiments support the strand displacement model (model 1) (Fig. 5A) and suggest that the dsRNA is used as a template for (+)RNA synthesis during TBSV replication in the CFE-based assay.

RNase-resistant tombusvirus dsRNA is also present in a plant extract. Since the above-described CFE-based studies involved yeast CFE and a short repRNA, we wanted to test if similar RNA forms are also present in plant extracts containing the infectious TBSV genomic RNA (gRNA). To this end, we prepared a plant extract from *Nicotiana benthamiana* leaves infected with TBSV gRNA (Fig. 7A). The plant extract contained the membrane-bound VRCs, including the viral RNAs. We then added ATP/CTP/GTP and ^{32}P-labeled UTP, followed by measuring the accumulation of new TBSV RNAs synthesized *in vitro* in the plant extract (Fig. 7B). As expected, the plant extracts accumulated RNase I- and RNase V1-resistant TBSV RNAs, which were present mostly as slow-migrating partial dsRNAs (replication intermediate [RI] product) (Fig. 7B, lanes 1 and 3), as suggested by changes in their migration after heat denaturation (lanes 2 and 4). Interestingly, treatment with a small amount of Triton X-100 made the ^{32}P-labeled TBSV RNAs RNase V1 sensitive (Fig. 7B, lanes 9 to 12), but the RNA products were still, at least partially, RNase I resistant (lanes 5 to 8). However, RNase I treatment did change the migration pattern of the TBSV RNAs, which moved as completely base-paired dsRNAs (gRNA, subgenomic RNA1 [sgRNA1], and sgRNA2), instead of the slowly migrating "smeary" band in the untreated sample (Fig. 7B, compare lane 5 with lane 13). These data suggest that TBSV dsRNAs are present as

RIs (forming a duplex between one minus strand and two or more plus strands) (model 1) (Fig. 5A). Indeed, completely base-paired dsRNAs of gRNA, sgRNA1, and sgRNA2 were detected in larger amounts only after RNase I/detergent treatment (Fig. 7C, lane 1), while these RNAs were removed by the RNase V1/detergent treatment (Fig. 7C, lanes 3, 5, and 7).

Since the detergent/nuclease treatment was performed before the phenol-chloroform extraction (Fig. 7B and C), the data suggest that the replicating TBSV RNAs were present mostly as dsRNAs (likely in the form of replication intermediates) in plant extracts.

DISCUSSION

The (−)RNA is present only in a dsRNA form during CFE-based TBSV replication. The (−)RNAs are produced in small amounts and sequestered in membrane-bound VRCs during TBSV (+)RNA virus replication (26, 31). This makes it difficult to study if free (−)RNA strands are present or the (−)RNA is part of dsRNA formed by annealing of plus and minus strands within the VRC during replication. By using abundant free (−)RNA-sensing short complementary (+)RNA probes and a (−)RNA-specific DNAzyme during the disruption of VRCs via a low concentration of detergent, we show that free (−)RNA is not present in a detectable amount in the VRCs. Therefore, free/naked TBSV (−)RNA is unlikely to serve as a template or as a replicative intermediate for (+)RNA synthesis (Fig. 3; see also Fig. S4 in the supplemental material). In addition, we find that V1 dsRNA-specific nuclease (in the presence of a small amount of detergent) could destroy, while the ssRNA-specific RNase I could not eliminate, the (−)RNA present in the membrane-bound VRCs (Fig. 2; see also Fig. S1 in the supplemental material). Based on these and other findings (Fig. 3), we propose that the TBSV (−)RNA is present in a dsRNA form during replication *in vitro*.

The dsRNA is unlikely to form artifactually during the above-described *in vitro* studies, because we performed our assays prior to phenol-chloroform extraction, which could facilitate the annealing of naked minus and plus strands due to the removal of lipids and proteins and provide a nonpolar environment. Also, we used only a small amount of nonionic detergents (as low as 0.01%) (see Fig. S2 in the supplemental material), which was enough to distort membranes and disrupt the structure of the VRC but not enough to solubilize the viral replication proteins or disrupt p33-repRNA interactions (see Fig. S2C in the supplemental material). Based on these data, we suggest that the TBSV (−)RNA is sequestered into dsRNA during replication in membrane-bound VRCs.

Another interesting observation in this work is the presence of a small amount of free (+)RNAs in the membrane-bound VRCs (Fig. 3G). We propose that these (+)RNAs represent the recently synthesized ssRNAs that are waiting to be released into the solution from the VRCs. Accordingly, a small amount of (+)RNAs can be copurified with the affinity-purified tombusvirus replicase (Z. Panaviene and P. D. Nagy, unpublished data).

Experiments with plant extracts also indicated that the replicating TBSV RNAs are present as V1 nuclease-sensitive dsRNAs (in the presence of detergent) (Fig. 7B and C). The difference between the yeast CFE and plant extracts was the abundant presence of the complete dsRNA form in the yeast CFE, while the plant extract contained mostly the replication intermediate (indeed, RNase I/detergent treatment was needed to convert the RI into completely base-paired dsRNAs) (Fig. 7B and C), which was less abundant in the yeast CFE. This is likely due to the difference in the replicating RNAs: the CFE contained the 621-nucleotide (nt) DI-72 repRNA, which likely replicates rapidly, making it more difficult to visualize the RI form. In contrast, the plant extract contained the 8-times-longer gRNA and subgenomic RNAs, for which the replicase likely needs a much longer time to complete plus-strand synthesis. This favors the isolation of RI-like dsRNAs in the plant extract. Nevertheless, TBSV replication likely follows the same mechanism which we established for TBSV repRNA replication in yeast CFE.

dsRNA likely serves as the template for (+)RNA synthesis during CFE-based TBSV replication. Although the above-described experiments supported the existence of dsRNA during TBSV replication, it is possible that dsRNAs accumulate only as dead-end products of replication. However, time course experiments (Fig. 1B and 4) indicate that dsRNA is produced prior to the synthesis of the bulk (+)RNAs. This finding is more in line with the model that dsRNAs are not final, dead-end products but instead are used as the templates for (+)RNA synthesis during TBSV replication. This model is further supported by results from chase experiments (Fig. 4 to 6; see also Fig. S5 in the supplemental material), which showed that ^{32}P-labeled UTP was incorporated into the dsRNA fraction during the first 10 min of incubation, while the bulk of ^{32}P-labeled UTP was incorporated to the newly made (+)RNA at latter time points. In addition, the chase experiments with prelabeled dsRNA present in the VRC showed a ~50% decrease of ^{32}P-labeled dsRNA at the end of the assay, suggesting that one strand (the plus strand) was replaced by an unlabeled new (+)RNA during replication (Fig. 6). Altogether, our data are more consistent with an active template role for dsRNAs for (+)RNA synthesis and in disagreement with the model that dsRNAs are only dead-end products during TBSV replication.

Mechanism of dsRNA-based replication. Time course chase experiments revealed that ^{32}P-labeled UTP was incorporated only into the newly made plus strands (Fig. 4 and 5; see also Fig. S5 in the supplemental material), while the unlabeled UTP replaced only ~50% of the original ^{32}P label during the course of replication (Fig. 6). The strand displacement model, in which the minus strand in the dsRNA is used to make new plus strands that replace the original plus strand in the duplex, could explain the obtained data (Fig. 5 and 6). This model also explains asymmetrical replication if sequential initiation events always take place on the (−)RNA component of the dsRNA template to produce (+)RNA products (Fig. 8). Accordingly, we have shown previously that dsRNA templates could be used by the tombusvirus replicase to generate plus strands *in vitro* (32, 33).

The initiation of (+)RNA synthesis at the 3′ end of the (−)RNA component within the dsRNA template is likely facilitated by the AU-rich nature of this part of the dsRNA template. The weak base pairs within the AU-rich stretch might promote the limited opening of the dsRNA structure prior to initiation (32). Moreover, the role of the RNA chaperone function of the tombusvirus p33 replication protein and coopted host DEAD box RNA helicases, such as yeast Ded1p and Dbp2p and *Arabidopsis* RH20, in opening the AU-rich stretch-containing portion of the dsRNA structure to render the 3′ terminus of the (−)RNA component of the dsRNA accessible to the viral replicase was shown previously (33–35). The p33 chaperone and the recruited host DEAD box

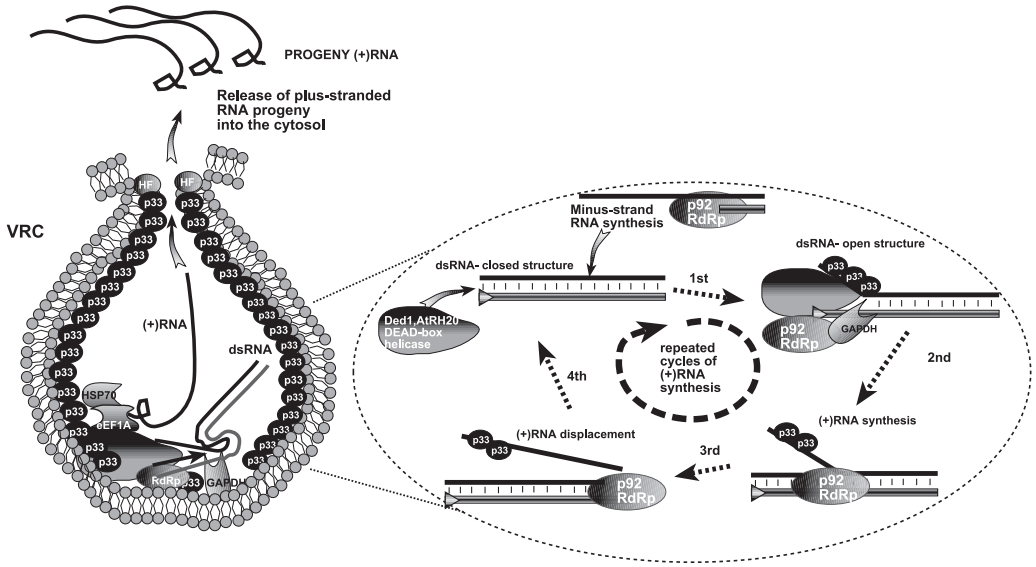


FIG 8 Model for the use of dsRNA as a template for (+)RNA synthesis by the tombusvirus replicase. The membrane-bound VRC containing viral and host factors synthesizes (−)RNA (gray line) that becomes part of the dsRNA. The formation of dsRNA disfavors additional rounds of (−)RNA synthesis by sequestering the original (+)RNA (black line) into the dsRNA structure as shown. The AU-rich side of the dsRNA (within and in the vicinity of the plus-strand promoter, represented by an arrowhead) is then opened up with the help of recruited host DEAD box helicases (Ded1p and Dbp2 in yeast and RH20 in plants) and the viral p33 RNA chaperone (first step). The exposed plus-strand promoter is then used by the tombusvirus $p92^{Pol}$ RdRp to synthesize a new plus-strand RNA (second step) by displacing the original plus-strand RNA, which is then released from the VRC (third step). Finally, the re-formed dsRNA within the VRC is reused for additional rounds of (+)RNA synthesis, leading to asymmetrical RNA replication. It is likely that one or more RdRps could simultaneously work on the same template (RI form) in the case of the long genomic RNA.

helicases are part of the tombusvirus replicase, and they could greatly facilitate the utilization of the viral dsRNA as the template for plus-strand synthesis (Fig. 8) (33–35). Therefore, the viral dsRNA seems to behave as a dynamic structure (alternating between closed/base-paired and partially open forms) due to the presence of coopted cellular helicases and the p33 RNA chaperone in the viral replicase.

What is the advantage for the virus to use dsRNA as the template for (+)RNA synthesis instead of the naked (−)RNA? We speculate that the formation of dsRNA templates allows more effective regulation of minus- than plus-strand synthesis since the dsRNAs can be used to generate only plus strands (32–35). This would effectively separate the timing of (−)RNA and (+)RNA syntheses, which could be useful for the regulation of asymmetrical replication. The use of the dsRNA template for (+)RNA synthesis would also favor the generation of a limited number of minus-strand RNA [possibly one dsRNA per one (+)RNA template], since the original plus-strand RNA would be sequestered into the dsRNA right after the (−)RNA synthesis step.

The formation of dsRNAs within the VRCs could also limit the number of *cis*-acting RNA elements that are accessible to the viral replicase after (−)RNA synthesis. For example, *cis*-acting RNA elements located on the plus strand, such as the internal p33 recognition element (p33RE), the 3′-proximal silencer element, and the 3′-terminal minus-strand initiation promoter (gPR), consist of secondary structures needed for function, but these hairpin structures are unlikely to exist and are not functional when they are part of dsRNA structures (32, 33, 36–38). Thus, masking of *cis*-acting elements located on the plus strand through the formation of dsRNA structures prevents competition between (+)- and (−)RNAs for the replicase, and it allows the tombusvirus replicase become committed to plus-strand synthesis at later time points. Also, the formation of dsRNAs would limit the possibility of internal initiation and possibly 3′-terminal extensions by the viral replicase, which are rather common processes on naked (−)RNA, resulting in the generation of nonfunctional viral RNAs *in vitro* with purified replicase preparations (39–41).

The dsRNA nature of the template could also prevent the template from being accidentally lost/released from the membrane-bound VRCs or exposed to the cytosol due to the large size of the dsRNA. In contrast, the "thinner" (+)RNA product could exit VRCs, ending up in the cytosol (or buffer in the case of the CFE assay) (Fig. 8). This is plausible since the tombusvirus VRCs, similar to many other (+)RNA virus VRCs, form spherule-like structures/vesicles that are connected with the cytosol only via a narrow opening, called the "neck" (42, 43). We propose that the bulky dsRNA might not be able to exit through this narrow neck structure of the spherule, essentially trapping the dsRNA and thus the (−)RNA component in the VRCs during the entire replication cycle. This strategy could serve a dual purpose: avoidance of recognition by the host foreign RNA surveillance system and protection against degradation by host ribonucleases.

The possible disadvantage for the formation of dsRNAs during (+)RNA virus replication is the prompt recognition of dsRNAs by the host anti-dsRNA surveillance system, based on Dicer RNase III enzymes for gene silencing in plants and animals, dsRNA protein kinase PKR, or RIG-I and MDA5 RNA sensors in mammals (44–51). The viral dsRNAs could be destroyed by cellular RNase III-like nucleases or other induced host responses, such as PKR or interferon responses (44–48, 50, 51). However, sequestration of dsRNA templates into virus-induced spherule-like structures/vesicles containing the VRCs could greatly reduce the ability of the

host cells to sense the presence of viral dsRNAs. Thus, the viral dsRNA templates hidden within the membrane-bound VRCs might be well protected against effective antiviral responses for several hours after the start of viral infection.

Similarities between TBSV and dsRNA virus replication. The formation of dsRNAs within the VRCs of tombusviruses adds another piece of evidence for the similarity between (+)RNA viruses and other viruses such as dsRNA viruses and retroviruses, as noted previously by Ahlquist (52). The presence of a small amount of recently synthesized (+)RNAs within the tombusvirus VRCs is another feature shared with dsRNA viruses. These viruses, TBSV, dsRNA viruses, and retroviruses, use RNA-binding proteins, such as RNA chaperones and helicases, within the replicase complex to facilitate RNA or cDNA synthesis (33–35, 53–56).

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