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Suppression of Viral RNA Recombination by a Host Exoribonuclease

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RNA viruses of humans, animals, and plants evolve rapidly due to mutations and RNA recombination. A previous genome-wide screen in *Saccharomyces cerevisiae*, a model host, identified five host genes, including *XRN1*, encoding a 5′-3′ exoribonuclease, whose absence led to an ∼10- to 50-fold enhancement of RNA recombination in *Tomato bushy stunt virus* (E. Serviene, N. Shapka, C. P. Cheng, T. Panavas, B. Phuangrat, J. Baker, and P. D. Nagy, Proc. Natl. Acad. Sci. USA 102:10545–10550, 2005). In this study, we found abundant 5′-truncated viral RNAs in *xrn1*Δ mutant strains but not in the parental yeast strains, suggesting that these RNAs might serve as recombination substrates promoting RNA recombination in *xrn1*Δ mutant yeast. This model is supported by data showing that an enhanced level of viral recombinant accumulation occurred when two different 5′-truncated viral RNAs were expressed in the parental and *xrn1*Δ mutant yeast strains or electroporated into plant protoplasts. Moreover, we demonstrate that purified Xrn1p can degrade the 5′-truncated viral RNAs in vitro. Based on these findings, we propose that Xrn1p can suppress viral RNA recombination by rapidly removing the 5′-truncated RNAs, the substrates of recombination, and thus reducing the chance for recombination to occur in the parental yeast strain. In addition, we show that the 5′-truncated viral RNAs are generated by host endoribonucleases. Accordingly, overexpression of the Ngl2p endoribonuclease led to an increased accumulation of cleaved viral RNAs in vivo and in vitro. Altogether, this paper establishes that host ribonucleases and host-mediated viral RNA turnover play major roles in RNA virus recombination and evolution.

Human-, animal-, and plant-pathogenic RNA viruses are frequently subjected to RNA recombination (1, 3, 20, 51), a process that joins noncontiguous RNA segments together. The resulting novel combinations of genes, sequence motifs, and/or regulatory RNA sequences could cause dramatic changes in the infectious properties of RNA viruses that can potentially lead to the emergence of new viruses or strains. Therefore, RNA recombination can help viruses to "jump species," escape natural resistance mechanisms, and/or render antiviral methods ineffective. Indeed, there is a growing number of examples where RNA recombination likely contributed to viral outbreaks, including outbreaks of caliciviruses (15), astroviruses (48), poliovirus (12, 22), dengue virus (14, 52), enteroviruses (21, 29), influenza virus (17), bovine viral diarrhea virus (12), and the recombinant severe acute respiratory syndrome (SARS) coronavirus, a newly emerged viral pathogen of humans (4, 42, 46). RNA recombination can also increase virus fitness by facilitating virus genome repair, a process that leads to the correction of mistakes introduced during viral RNA replication by the error-prone viral RNA replicases or due to damage caused by host ribonucleases (1, 3, 20, 27, 51).

Viral RNA recombination is thought to occur when the viral replicase accidentally switches templates during complementary RNA synthesis (6, 20, 27, 28). Indeed, mutagenesis of viral replicase proteins affected the sites and frequency of recombination (10, 11, 25, 33). Moreover, purified viral replicases were used to demonstrate efficient generation of RNA recombinants in vitro in the presence of added RNA templates (5, 6, 18). Some recombination events might be due to a promiscuous RNA ligation process, as demonstrated for a small number of RNA viruses (8).

Similar to other processes that take place during virus propagation in infected cells, RNA recombination is possibly affected by host factors. Accordingly, a systematic, genome-wide analysis of individual host genes based on the *Saccharomyces cerevisiae* single-gene deletion library identified five host genes whose absence led to a 10- to 50-fold enhancement in viral recombinant RNA accumulation compared to the parental yeast strain (43). The above study was based on a model RNA virus, tomato bushy stunt virus (TBSV), which is a small, nonsegmented, plus-strand RNA virus of plants (50). Yeast has been shown to be a highly suitable host for studies of the replication and recombination of a small TBSV replicon RNA (30). The replicon RNA, which is a naturally generated defective interfering (DI) RNA, consists of four noncontiguous segments of the TBSV genomic RNA, named RI to RIV. Coexpression of the replicon RNA with the two essential tombusvirus replication proteins (i.e., p33 and p92 of cucumber necrosis virus [CNV], which is closely related to TBSV [50]) leads to robust RNA replication in yeast cells, which showed similar characteristics to plant cell infections (30, 35). In addition, small amounts of RNA recombinants, which were shown to be similar to those occurring in plants and plant protoplasts, were also detected in yeast, establishing that it is a suitable host for viral recombination studies. The identification of yeast genes that greatly affected the accumulation of TBSV recombinants opened up the possibility of dissecting the roles of the identified host genes in viral RNA recombination.

For this study, we have tested the role of the *XRN1* gene, one of the identified host genes from the genome-wide genetic screen whose deletion increased TBSV recombination (43). Because *XRN1* encodes a 5′-3′ exoribonuclease (16, 36), we hypothesized that Xrn1p could be involved in viral RNA re-

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combination via affecting viral RNA degradation, which could potentially influence the amounts of viral RNA substrates available for recombination events. Accordingly, we demonstrate that Xrn1p is involved in rapid degradation of 5′-truncated viral replicon RNAs, which then leads to suppression of RNA recombination in the parental yeast strain. In contrast, in an *xrn1*Δ mutant strain, the partially degraded (i.e., 5′-truncated) replicon RNAs accumulate and serve as efficient templates for RNA recombination. In addition, we show that the partially degraded replicon RNAs are likely generated by Ngl2p and other endoribonucleases. Based on our results, we propose that Xrn1p RNase suppresses viral RNA recombination by quickly removing partially degraded replicon RNA templates used for RNA recombination events. Altogether, our results establish that host-mediated viral RNA turnover is a major factor in viral RNA recombination.

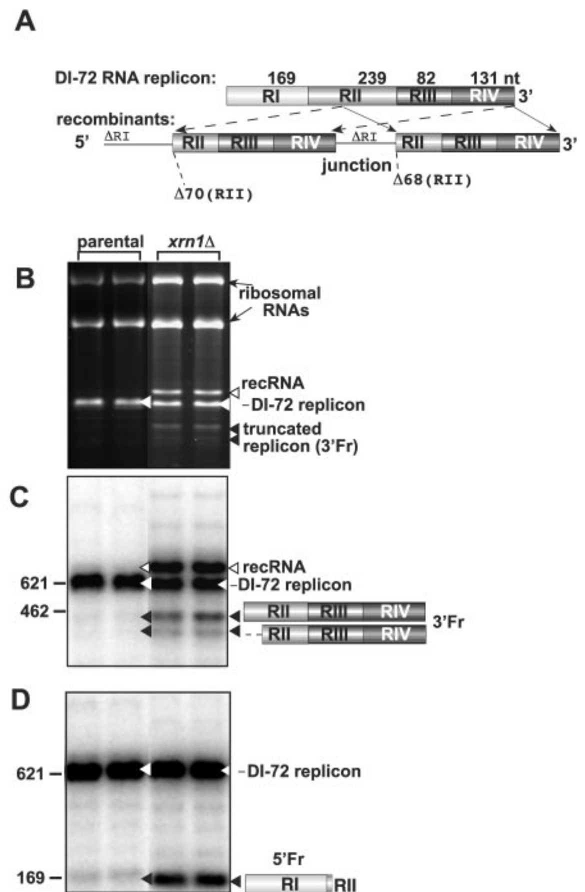

FIG. 1. Accumulation of partially degraded replicon RNAs in *xrn1*Δ mutant yeast. (A) Schematic representation of DI-72 replicon RNA and newly generated recombinant RNAs. The recombinant RNAs contain partially duplicated sequences from the 3′ half of the DI-72 replicon RNA joined in a head-to-tail fashion. (B) Ethidium bromide-stained agarose gel of total RNA extracts obtained from two yeast strains showing the accumulation of DI-72 replicon RNA (white arrowhead), recombinant RNA (open arrowhead), and putative partially degraded replicon RNAs (closed arrowheads). The yeast cells coexpressed DI-72 RNA and the p33 and p92 replication proteins. (C and D) Northern blot analysis of total RNA extracts with a ^{32}P-labeled RNA probe specific for RIII (C) or RI (D). The samples are the same as those used for panel B.

MATERIALS AND METHODS

Yeast strains and expression plasmids. *Saccharomyces cerevisiae* strain BY4741 (*MAT***a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and a haploid deletion series (BY4741 strain background) were obtained from Open Biosystems (Huntville, AL). The expression plasmids pGBK-His33 (carrying the p33 gene of CNV, a closely related virus to TBSV, behind the *ADH1* promoter and *HIS3* marker), pGAD-His92 (containing the CNV p92 gene behind the *ADH1* promoter and *LEU2* marker), and pYC/DI-72 (expressing TBSV DI-72 RNA under the control of the *GAL1* promoter and containing the *URA3* marker gene) have been previously described (31, 35). Each yeast strain was cotransformed with all of the above three plasmids (except for the RNA stability experiments) using a lithium acetate/single-stranded DNA/polyethylene glycol method (13), and transformants were selected by complementation of auxotrophic markers. The transformed yeast strains were grown under inducing conditions as described earlier (31, 35). Xrn1p and Ngl2p were expressed from plasmids pAJ10 (16) and pESC/DI-NGL2HisFLAG, respectively.

Protoplast inoculation. Three different DI RNA constructs with different 5′-end deletions were generated by PCR and T7 transcription as described previously (35). The same amount of DI RNA transcripts (1 μg/sample), together with CNV genomic RNA transcripts (5 μg), was used for electroporation of 5×10^5 *Nicotiana benthamiana* protoplasts, prepared as described previously (32). Protoplasts were incubated in the dark, followed by RNA extraction as described previously (32, 44).

Viral RNA analysis. Total RNA isolation and Northern blot analysis were done as previously described (30). For detection of plus-strand DI RNAs, a ^{32}P-labeled RNA probe representing RIII (Fig. 1B) was prepared by T7 transcription from a PCR-generated cDNA, which was amplified with primers 1165 (AGCGAGTAAGACAGACTCTTCA) and 23 (GTAATACGACTCACTATA GGGACCCAACAAGAGTAACCTG). Total RNAs obtained from selected strains were also analyzed by Northern blotting using a probe representing RI of DI-72 (Fig. 1A) obtained from a PCR template (primers 15 [GTAATACGA CTCACTATAGGGCATGTCGCTTGTTTGTTGG] and 20 [GGAAATTCT CCAGGATTTCTC] were used for amplification).

RT-PCR analysis of junction sites. We used both yeast total RNA extracts and gel-isolated recombinants for reverse transcription-PCRs (RT-PCRs) to specifically amplify regions covering the junction sites (Fig. 1). First, the RT reaction included primer 14 (GTAATACGACTCACTATAGGGTTCTCTGCTTTTACG AAG) for cDNA synthesis, followed by PCR with primers 168 (TCGTCTTATTGG ACGAATTCCTGTTTACGAAAG) and 270 (TTGGAAATTCTCCTTCAGTCT GAGTTTGTGGA). The PCR products were cloned into the pGEM-T Easy vector (Promega) and sequenced with the M13 reverse primer (5).

5′ RACE and 3′ RACE of recombinants. The 5′ sequences of truncated RNAs were determined by using 5′ rapid amplification of cDNA ends (5′ RACE). We followed a GC-rich sequence protocol for 5′ RACE (Invitrogen). Briefly, total RNA samples obtained from yeast (43) were loaded in 4% denaturing polyacrylamide gel electrophoresis (PAGE) gels for gel isolation of the truncated RNAs. After recovery of the RNAs from gels, we performed 5′ RACE. For amplification of first-strand cDNAs, we used primer 116 (GTAATACGACTC ACTATAGGACACCTAACTTTCGT), which anneals to the 5′ end of RIV(+) of TBSV DI-72. Second-strand cDNAs were synthesized by using a 3′ RACE anchor primer (GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTT). A nested primer (291 [GTAATACGACTCACTATAGGAACCTGTATGCTA TGCC]), which anneals to RIII(+) of DI-72, and an abridged universal amplification primer (AUAP) (GGCCACGCGTCGACTAGTAC) were used for the first PCRs, and the second PCRs were done by using the AUAP primer and the second nested primer 14 (GTAATACGACTCACTATAGGGTTCTCTGCT TTTACGAAG), which anneals to RII(+). The resulting products were directly cloned into pGEM-T Easy (Promega) and sequenced with the M13 reverse primer.

The 3′ end sequences of the truncated DI RNAs were identified using 3′ RACE as described previously (5, 43), based on a 3′ RACE kit (Invitrogen). Purified RNAs were first polyadenylated [poly(A) polymerase; USB], followed by phenol-chloroform extraction prior to cDNA synthesis. First-strand cDNAs were synthesized with the 3′ RACE anchor primer, followed by PCRs using the AUAP primer and primer 20 (TBSV/CNV/1F+G [GGAAATTCTCCAGGA TTTCTC]), which anneals to the 5′ end of RI(−) of DI-72. The PCR products were cloned and sequenced as mentioned for 5′ RACE.

Purification of recombinant Xrn1p from yeast. We used a yeast strain carrying tandem affinity purification (TAP)-tagged Xrn1p from a TAP fusion library (Open Biosystems) to obtain recombinant Xrn1p in a single-step purification with calmodulin affinity resin (Stratagene) (39). Briefly, the yeast lysate contain-

ing TAP-tagged Xrn1p was loaded on 300 μl calmodulin resin that was equilibrated with 5 ml IPP150 calmodulin-binding buffer (10 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% NP-40). After washing of the resin three times with 10 ml of IPP150 calmodulin-binding buffer in a cold room, recombinant Xrn1p was eluted with 1 ml IPP150 calmodulin elution buffer (10 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, 0.1% NP-40). The presence and quality of Xrn1p (200 kDa) were checked by 10% sodium dodecyl sulfate-PAGE (SDS-PAGE) and silver staining. In some of the in vitro experiments, we also used purified recombinant Xrn1p-His6 provided by A. W. Johnson (16), with comparable results to those obtained with TAP-based purification (not shown).

RNA preparation for in vitro assays. Full-length DI-72(+) and DI-ΔRI(+) RNAs with 5′ triphosphate or 5′ monophosphate were synthesized by T7 polymerase and labeled with [^{32}P]UTP. RNAs with 5′ monophosphate were generated by T7 polymerase with 10 mM ATP, 10 mM CTP, 10 mM GMP, and 0.5 mM GTP. All transcripts were purified on MicroBio-Spin P-30 columns (Bio-Rad) prior to the in vitro assay with Xrn1p.

In vitro Xrn1p assay. The standard assay was based on the procedure of Stevens, using 1 to 10 μl of purified Xrn1p (47). Briefly, we used 2 pmol of ^{32}P-labeled RNA transcripts in 70 μl RNA-dependent RNA polymerase buffer (50 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$, 10 mM dithiothreitol, 100 mM potassium glutamate) (26) in the presence of 10 U of RNase inhibitor (Amersham) and bovine serum albumin at 37°C for various lengths of time (see Fig. 7), followed by phenol-chloroform extraction. The control preparation for Xrn1p was obtained using the same calmodulin-based procedure (see above), except that the yeast extract was derived from the parental yeast strain (lacking TAP-tagged Xrn1p).

Purification of recombinant Ngl2p. Recombinant FLAG-tagged Ngl2p was affinity purified from yeast strain BY4741 transformed with pESC/DI-NGL2HisFLAG. Briefly, the yeast extract was loaded on a column containing the FLAG M2 resin (Sigma) equilibrated with Tris-glycine (TG) buffer containing 0.01% NP-40 (Nonidet P-40). After careful washing of the resin two times with 600 μl TG buffer (50 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 10 mM KCl, and 10% glycerol), Ngl2p was eluted with TG buffer containing 100 mM β-mercaptoethanol. The quality of the purified Ngl2p (~70 kDa) preparation was checked by 8% SDS-PAGE, staining with Coomassie brilliant blue, and Western blotting using anti-His antibody (35).

In vitro Ngl2p assay. One microgram of RNA transcripts (see above) was used with 1 μl of purified Ngl2p in buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml bovine serum albumin, and yeast tRNA [0.1 μg]). Reactions were carried out at 25°C for 10 min, followed by phenol-chloroform extraction. Some of the samples were further treated with 1 μl of Xrn1p-His6 (a gift from Arlen W. Johnson) and 10 units of RNase inhibitor at 37°C for 20 min, followed by phenol-chloroform extraction. The obtained RNAs were used in RT reactions with a ^{32}P-labeled primer (1163 [CCCGAAGCTTCCCAACAAGAGTAACCTGTATGCT]), using SuperScript II reverse transcriptase (Invitrogen). After 60 min at 50°C, the RT reactions were stopped by adding 2× loading dye, and the RT samples were loaded into 5% denaturing PAGE gels.

RESULTS

Rationale. A previous systematic, genome-wide screen of individual yeast genes led to the identification of five host genes whose separate deletion enhanced the accumulation of novel viral RNA recombinants >10-fold compared to that in the parental yeast strain (43). One of the identified genes was *XRN1*, which codes for a cytoplasmic 5′-3′ exoribonuclease (16, 36). Interestingly, the isolated recombinant RNAs had 5′ deletions and 3′ duplications of replicon RNA sequences, which resulted in incomplete dimeric recombinant RNAs (43) (shown schematically in Fig. 1A). Based on the lack of Xrn1p 5′-3′ exoribonuclease and the nature of junction sequences, we hypothesized that viral RNA degradation might be slow in *xrn1*Δ mutant cells, which could then lead to an accumulation of 5′-truncated viral RNA degradation products. This in turn might promote viral recombination if the incompletely degraded replicon RNAs could serve as efficient templates for recombination events, resulting in the generation of abundant

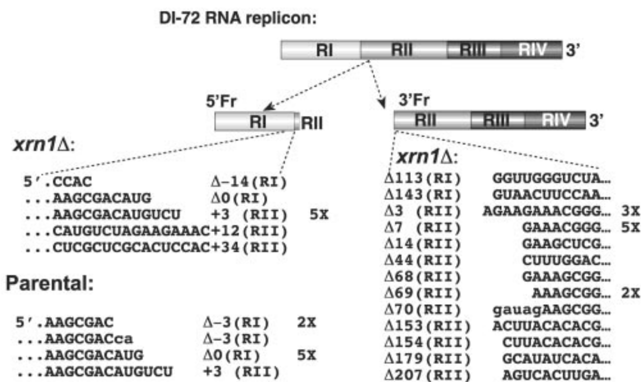

FIG. 2. Terminal sequences of partially degraded replicon RNAs. (Top) The original replicating DI-72 replicon RNA and the partially degraded RNA products are shown schematically. (Bottom left) 3′-Terminal sequences in the 5′Fr RNA products were obtained by 3′ RACE from the *xrn1*Δ mutant and parental yeast strains. The terminal sequences represent either RI or RII sequences, as indicated. (Bottom right) 5′-Terminal sequences in the 3′Fr RNA products were obtained by 5′ RACE from *xrn1*Δ mutant strains. The lengths of the missing 5′ sequences are indicated with numbers behind the "Δ" symbols, whereas the 5′ sequences in the 3′Fr RNAs are shown. Lowercase letters indicate nonviral sequences.

dimeric recombinant viral RNAs. This model was tested by using in vivo and in vitro approaches.

Characterization of partially degraded viral RNAs in *xrn1*Δ mutant cells. To dissect the role of *XRN1* in recombination of the TBSV replicon, we first characterized partially degraded viral replicon RNAs, which are suggested to serve as recombination intermediates in *xrn1*Δ mutant yeast. We performed Northern blotting analysis with probes specific for 5′ and 3′ replicon sequences, using total RNA samples obtained from cells actively replicating DI-72 replicon RNA. As predicted, we observed the accumulation of abundant amounts of partially degraded 3′ fragments of viral RNAs (termed 3′Fr), which represented two different sizes (the abundant ~Δ170 and the minor Δ240 RNAs, which were 170 to 240 nucleotides [nt] shorter than DI-72 replicon RNA), in *xrn1*Δ mutant cells (Fig. 1B to D). In contrast, the accumulation of 3′Fr RNA was barely detectable in the parental yeast cells (Fig. 1C). Interestingly, we also detected 5′ fragments of DI-72 replicon RNAs (5′Fr RNA, ~170 nt in length), which were detectable in the parental cells and abundant in *xrn1*Δ mutant cells (Fig. 1D).

To find out the possible relationship between these partially degraded replicon RNAs and the recombinant RNAs, we determined the sequences of the truncated RNAs, using gel-isolated RNAs after cloning of RT-PCR, 3′ RACE, and 5′ RACE products (43). The 3′Fr RNAs had 5′ deletions ranging from 172 to 376 nt from the 5′ end of the replicon RNA (Fig. 2). Interestingly, the deletions removed the entire RI sequence and part of the RII sequence of DI-72 replicon RNA. Altogether, the 5′-terminal sequences in many 3′Fr RNAs (i.e., 5′ deletions of 68 to 70 nt in RII; Fig. 1A) resembled the sequences present at the 5′ ends and at the recombination junctions in the most abundant recombinant RNAs, supporting the model that some of the partially degraded 3′Fr RNAs are likely involved in RNA recombination events as intermediates. This is based on the observation that recombination-driven

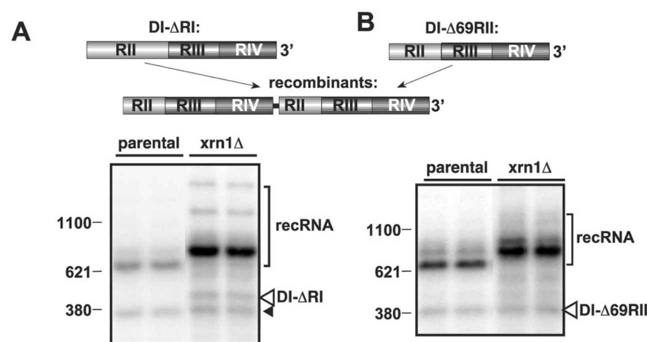

FIG. 3. Efficient generation of viral recombinants from 3′Fr RNAs in yeast. Two representative 3′Fr RNAs, DI-ΔRI RNA (A) and DI-Δ69RII RNA (B), were separately expressed in yeast (together with p33 and p92) from the *GAL1* promoter. Northern blot analysis of the total RNAs obtained 24 h after induction with galactose revealed the expressed 3′Fr RNAs (open arrowheads). The newly generated recombinants are bracketed. The black arrowhead indicates that DI-ΔRI RNA also generated a partially degraded RNA (possibly 5′-truncated, DI-Δ69RII-like RNA) in the parental and *xrn1*Δ mutant strains.

joining of two partially degraded 3′Fr RNAs in a head-to-tail fashion could give rise to the recombinants identified in *xrn1*Δ mutant cells (Fig. 1A).

Sequence analysis of the 5′Fr RNAs from both wild-type (wt) and *xrn1*Δ mutant cells by 3′ RACE revealed the presence of RI sequences and short stretches of RII (Fig. 2). The corresponding 5′ sequences were missing in the recombinant RNAs (Fig. 1D), suggesting that the observed 5′ fragments of DI-72 replicon RNA did not participate in viral recombination events (see Discussion). Overall, sequences present in 3′Fr and 5′Fr RNAs did not overlap significantly (Fig. 2). The presence of nonoverlapping 5′Fr and 3′Fr RNAs in *xrn1*Δ mutant cells suggests that they were generated via cleavage of the replicon RNA within RII sequences (or in the vicinity of the junction between RI and RII) by a putative endoribonuclease instead of by incomplete degradation from either the 5′ or 3′ end by exoribonucleases in parental and *xrn1*Δ mutant cells.

3′Fr RNAs serve as substrates for RNA recombination in yeast cells and plant protoplasts. To test if the 3′Fr RNAs could participate in recombination, we constructed two yeast plasmids for the expression of TBSV RNAs (i.e., DI-ΔRI [Fig. 3A] and DI-Δ69RII [Fig. 3B]) that represent 3′Fr RNAs (Fig. 2). Then we expressed DI-ΔRI and DI-Δ69RII, together with the p33 and p92 replicase proteins, in wt and *xrn1*Δ mutant yeast to see if these RNAs can replicate and recombine. As predicted above, total RNA extracts obtained from *xrn1*Δ yeast cells expressing DI-Δ69RII RNA contained abundant amounts of recombinant RNAs (Fig. 3B). Sequencing the junctions in these recombinants confirmed that the expressed DI-Δ69RII RNA (a representative 3′Fr RNA species; Fig. 2) did indeed participate in RNA recombination (not shown) by generating head-to-tail dimers similar to that shown schematically in Fig. 1A. Interestingly, expression of a longer 3′Fr RNA (i.e., DI-ΔRI RNA; Fig. 3A) led to the occurrence of shorter 3′Fr RNAs with additional 60- to 150-nt deletions from the 5′ end of RII (Fig. 3A [sequences are not shown]). Sequence analysis of the generated recombinants obtained with yeast expressing DI-ΔRI revealed that the shorter 3′Fr RNAs (which quickly evolved to sizes similar to that of DI-Δ69RII) participated in the generation of recombinants with higher frequencies than the originally expressed DI-ΔRI RNA (not shown), suggesting that the short version (similar to DI-Δ69RI) is the more active substrate in recombination.

Unlike the case with full-length DI-72 replicon RNA, the control parental yeast strain also generated abundant recombinants when it expressed either of the two 3′Fr RNAs (Fig. 3B). The sizes of the recombinants, however, were shorter in the parental yeast than in *xrn1*Δ mutant yeast due to ~100-nt 5′ truncations in the recombinants (Fig. 3B [sequencing data are not shown]). Nevertheless, the occurrence of abundant recombinant RNAs in the parental strain suggests that the presence of plentiful 3′Fr RNAs promotes RNA recombination events. Thus, the parental strain is also recombination competent if a sufficient amount of recombination substrates is available. Overall, these data support the model that some of the 3′Fr RNAs can serve as recombination intermediates (substrates) during RNA recombination events, leading to the formation of partially dimeric recombinant RNA.

To test if 3′Fr RNAs can also serve as recombination intermediates in plant cells, we separately electroporated three different 3′Fr RNAs into *Nicotiana benthamiana* protoplasts. The wt CNV (a closely related virus to TBSV) genomic RNA was also coelectroporated into these cells to serve as a helper virus (which produces the replicase proteins) for the replication/recombination of the 3′Fr RNAs (44). Total RNA extracts obtained from protoplasts 24 h after electroporation contained not only the replicating 3′Fr RNAs but also novel dimeric recombinant RNAs in the case of two of the three 3′Fr RNAs tested (Fig. 4). The amounts of 3′Fr RNAs and recombinant RNAs increased over time (Fig. 4). Sequencing of the junctions in these recombinants revealed that they consisted of head-to-tail dimers, similar to the recombinants obtained in yeast cells (not shown). Similar to its replication in parental yeast, DI-ΔRI also generated a shorter, DI-Δ69RII-sized replicating RNA in *N. benthamiana* protoplasts (indicated by a black asterisk in Fig. 4), suggesting that the evolution and replication of DI-ΔRI RNA are similar in yeast and plant hosts. Construct DI-Δ179RII did not replicate and generate recombinants in *N. benthamiana* protoplasts (Fig. 4), consistent with previous findings that the central portion of RII includes an essential *cis*-acting element which is involved in template selection by the tombusvirus replication protein (23, 38). The stabilities of all three 3′Fr RNAs were comparable in *N. benthamiana* protoplasts (Fig. 4), supporting the model that the differences among these constructs in the presence of the helper virus are due to their different abilities to participate in replication/recombination. Overall, these experiments demonstrated that some 3′Fr RNAs can participate in similar recombination events in both yeast and plant cells.

The above data support the model that 3′Fr RNAs are generated via partial degradation of the full-length DI-72 replicon RNA in cells and that some of the 3′Fr RNAs can efficiently participate in RNA recombination. Due to the central role of 3′Fr RNAs in viral recombination, below we examine the proposed roles of host endoribonucleases and Xrn1p in the generation and degradation of 3′Fr RNAs and their effects on tombusvirus recombination.

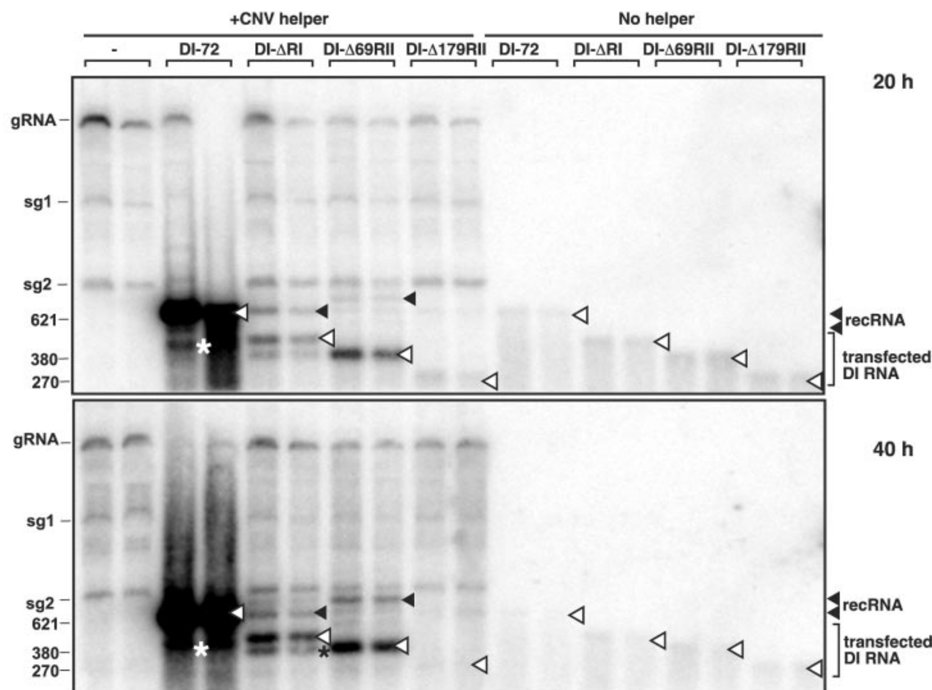

FIG. 4. *N. benthamiana* protoplasts also support the generation of viral recombinants from 3′Fr RNAs. Three representative 3′Fr RNAs (missing RI and portions of RII, as indicated in their names) were coelectroporated with CNV helper virus into protoplasts. Northern blot analyses of the total RNAs obtained at 20 h (top) and 40 h (bottom) based on RIII-specific probes revealed two of the electroporated 3′Fr RNAs (open arrowheads) replicated in the presence of the helper virus (left side of the image). The newly generated recombinants are indicated by black arrowheads. Asterisks indicate partial degradation products (DI-Δ69RII-like RNAs). Note that the electroporated 3′Fr RNAs show similar levels of stability in the absence of replication (no helper virus; right side of the image).

Ngl2p endoribonuclease can generate 3′Fr RNAs in vivo and in vitro. Based on the detection of nonoverlapping 5′Fr and 3′Fr RNAs from the full-length DI-72 replicon RNA (Fig. 1 and 2), we predicted that a putative endoribonuclease might be involved in selective cleavage of the full-length replicon RNA. To this end, we selected Ngl2p and Rny1p from a small group of known cytoplasmic endoribonucleases for further studies, because they are abundant and located in the cytoplasm (9). The expression of Ngl2p in *ngl2*Δ mutant yeast with replicating DI-72 replicon RNA resulted in an increased level of RI-containing degradation products (i.e., 5′Fr), by sevenfold (Fig. 5), and of the 3′Fr products, by twofold (Fig. 5B), whereas the expression of Rny1p endoribonuclease had no significant effect on DI RNA degradation (not shown). The observed partially degraded products in the Ngl2p-expressing strain were comparable in size to the previously characterized 5′Fr RNAs (Fig. 2 and 5), supporting the model that Ngl2p is involved in selective degradation of DI-72 replicon RNA that results in 5′Fr and 3′Fr RNAs (Fig. 5). Overexpression of Ngl2p in *xrn1*Δ mutant cells increased the total amount of 3′ degradation products (3′Fr) of DI-72 replicon RNA more than threefold. The increased levels of 5′Fr and 3′Fr RNAs in yeast expressing Ngl2p are consistent with the model that Ngl2p is involved in TBSV RNA degradation in yeast. In addition, cleavage of the full-length DI-72 replicon RNA by Ngl2p likely generates 3′Fr RNAs, which could then stimulate RNA recombination if saved from rapid degradation (i.e., in *xrn1*Δ mutant cells).

To test if Ngl2p can also cleave DI-72 RNA in vitro, we affinity purified Ngl2p from yeast cells overexpressing FLAG-tagged Ngl2p (see Materials and Methods). After the nuclease assay (see Materials and Methods), we performed in vitro reverse transcription with a ^{32}P-labeled primer, which was complementary to RIII(+), on the Ngl2p-treated DI-72(+) RNA template (Fig. 6, lane 1). Based on the sizes of the cDNA fragments separated in sequencing gels, we concluded that Ngl2p could cleave DI-72(+) RNA in vitro, resulting in three major and additional minor cleavage products within RI and RII (Fig. 6, lane 1). In conclusion, these in vitro and in vivo experiments support the model that the Ngl2p endoribonuclease can specifically cleave DI-72(+) RNA, which could then result in 3′Fr RNA substrates for RNA recombination.

Redundant endoribonucleases are involved in cleavage of the replicon RNA in yeast. To test if Ngl2p is the only endoribonuclease of yeast capable of cleaving the replicon RNA, we tested viral RNA degradation in *ngl2*Δ mutant cells (Fig. 5). These experiments demonstrated that *ngl2*Δ mutant cells, similar to the parental yeast cells, produced the characteristic RI-sized cleavage products of the DI-72 replicon RNA, suggesting that yeast has functionally redundant endoribonucleases (possibly Ngl1p, Ngl3p, and others). In addition, we have previously detected viral RNA recombinants at close to the parental level in *ngl2*Δ mutant cells (43). Therefore, we concluded that Ngl2p and additional endoribonucleases are likely involved in viral RNA degradation/recombination in yeast. The combined activity of these endoribonucleases produces 5′Fr and 3′Fr RNAs (the latter are the recombination substrates), leading to rapid recombinant accumulation in *xrn1*Δ mutant cells.

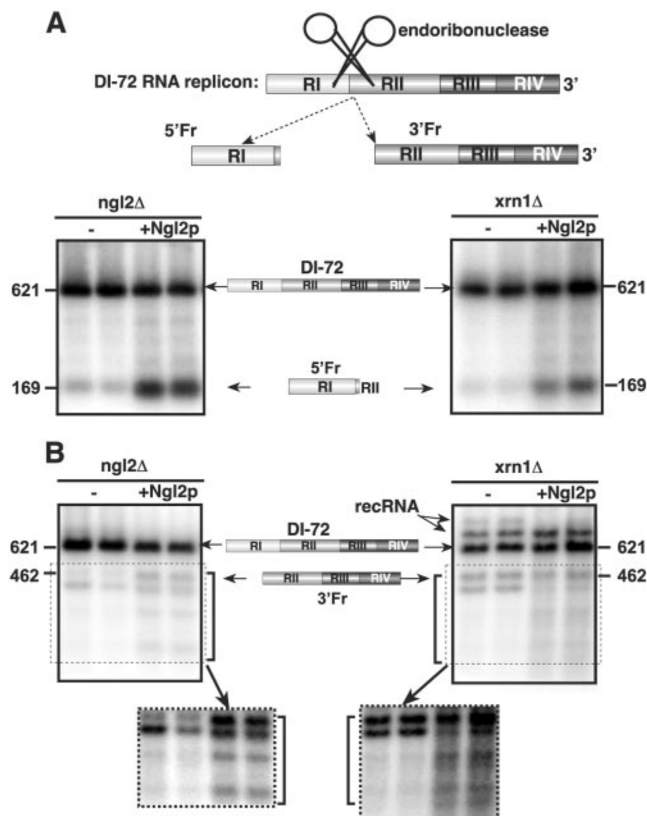

FIG. 5. Expression of Ngl2p endoribonuclease leads to increased levels of 5′Fr and 3′Fr RNAs in yeast. (A) Northern blot analyses of total RNA extracts obtained from either the *ngl2*Δ or *xrn1*Δ mutant strain not expressing (−) or expressing Ngl2p (in addition to p33, p92, and DI-72 RNA). The probe was specific for RI (i.e., 5′Fr RNA). The positions of the expressed DI-72 replicon RNA and the generated 5′Fr RNAs are shown. (B) Northern blot analyses of the above RNA samples with a probe specific for RIII (i.e., 3′Fr RNA). Part of the gel is shown after a longer exposure to visualize less intense bands on the blot. See above for additional description.

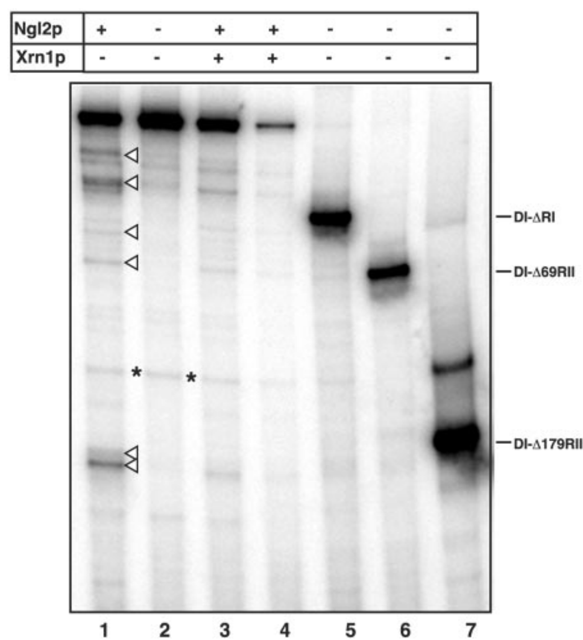

FIG. 6. Ngl2p-cleaved DI-72(+) replicon RNA is sensitive to Xrn1p. The full-length DI-72 replicon RNA carrying 5′ triphosphate was treated with affinity-purified Ngl2p, followed by treatment with Xrn1p for 10 and 30 min (lanes 3 and 4, respectively). Reverse transcription in the presence of a ^{32}P-labeled primer was used to detect partially degraded RNAs with different 5′ termini. The partial Ngl2p cleavage products are marked with arrowheads, whereas RT pausing sites are shown with asterisks. The three samples on the right are molecular size markers.

Xrn1p exoribonuclease can rapidly degrade 3′Fr RNAs in vitro and in vivo. Based on (i) the abundance of the full-length DI-72 RNA and its 5′ fragments generated by endoribonuclease cleavage (Fig. 5) in both *ngl2*Δ and *xrn1*Δ mutant cells and (ii) the abundance of 3′Fr RNAs only in *xrn1*Δ mutant cells, but not in the parental cells (Fig. 1C), we predicted that Xrn1p is likely involved in rapid degradation of 3′Fr RNAs but that the full-length DI-72 RNA might only be slowly degraded by Xrn1p.

To test the activity of Xrn1p on DI-72(+) RNA templates, we affinity purified Xrn1p as a TAP fusion protein from yeast cells (Fig. 7D) (39). In addition, we also used metal-affinity-purified Xrn1p provided by A. W. Johnson. The obtained Xrn1p preparations were used to treat ^{32}P-labeled, in vitro-transcribed DI-72-derived RNAs (Fig. 7). These in vitro experiments demonstrated that 3′Fr RNA, which carried a monophosphate (the expected product of an endonuclease cleavage) at its 5′ end, was quickly degraded by Xrn1p (Fig. 7A). In contrast, full-length DI-72(+) RNA, regardless of the presence of either mono- or triphosphate at its 5′ end, was more resistant to Xrn1p under the conditions tested (Fig. 7B and C). The significant degree of resistance to Xrn1p-mediated degradation by the full-length DI-72(+) RNA and the 5′Fr RNA could be due to the highly structured nature of RI(+) (40), which is present in both RNAs but not in 3′Fr RNA. It is established that Xrn1p activity is inhibited by stable secondary structures in the RNA substrate (47). We concluded based on the in vitro experiments described above that Xrn1p is capable of rapidly degrading the 3′Fr RNA, but not the full-length DI-72 RNA.

To test if Xrn1p could degrade DI-72(+) RNA after cleavage with Ngl2p, we treated DI-72(+) RNA with purified recombinant Ngl2p and Xrn1p, followed by primer extension with RT (Fig. 6, lanes 3 and 4). As expected, the amounts of Ngl2p-cleaved DI-72(+) RNA products were decreased in the presence of Xrn1p. Overall, the data from the in vitro experiments are consistent with the model that Xrn1p is capable of rapidly degrading endoribonuclease-cleaved replicon RNA products.

To examine if 3′Fr RNA is more stable in *xrn1*Δ mutant cells than in parental yeast cells, we expressed DI-Δ69RII RNA from the galactose-inducible GAL1 promoter in the absence of p33/p92 replication proteins in the above yeast strains (43). The stability of DI-Δ69RII was then determined by measuring the half-lives of the RNA transcripts in the absence of replication. As expected, the half-life of DI-Δ69RII RNA increased almost threefold in *xrn1*Δ mutant cells compared to the parental yeast cells (Fig. 8). The increased half-life of the recombination substrate in *xrn1*Δ mutant cells supports our model that

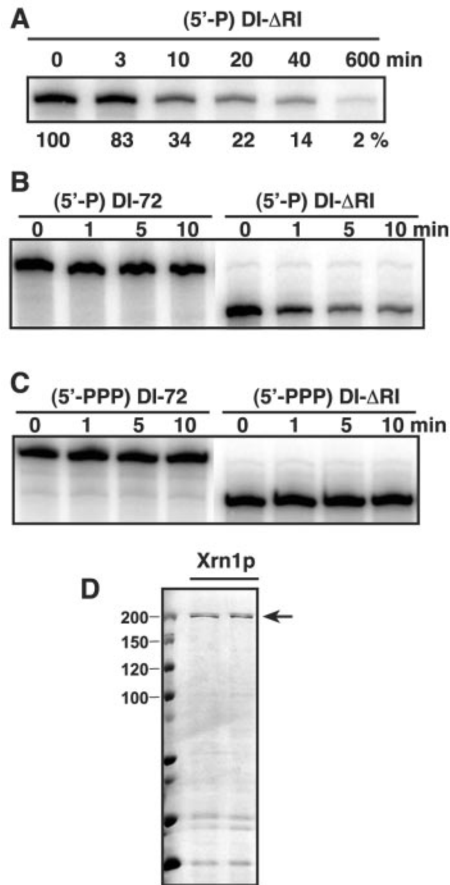

FIG. 7. Degradation of replicon RNAs by Xrn1p in vitro. (A) Demonstration of the exoribonuclease activity of affinity-purified Xrn1p in vitro. The template was ^{32}P-labeled DI-ΔRI carrying a monophosphate at the 5′ end. A comparison of the sensitivities of the full-length DI-72 and DI-ΔRI replicon RNAs carrying either 5′ monophosphate (B) or 5′ triphosphate (C) to degradation by Xrn1p in vitro was performed. The time of treatment with Xrn1p is shown in minutes at the top. (D) Silver-stained SDS-PAGE analysis of affinity-purified Xrn1p preparations obtained from yeast expressing Xrn1p-TAP.

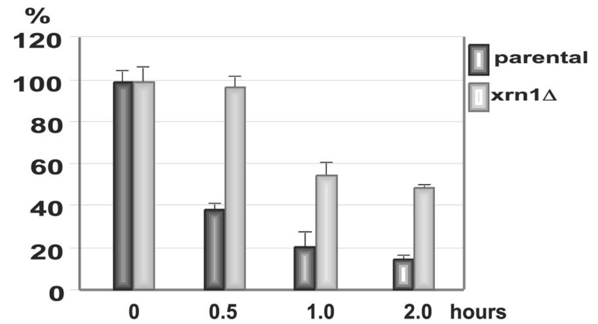

FIG. 8. Increased stability of 3′Fr RNAs in *xrn1*Δ mutant cells. The 5′-truncated DI-Δ69RII RNA, which represents a partially degraded DI-72 RNA product (Fig. 2), was expressed in the *xrn1*Δ mutant and parental (wt) yeast strains from the *GAL1* promoter (in the absence of p92), followed by the suppression of RNA synthesis by glucose. RNA samples were taken at various time points (as shown) and analyzed by Northern blotting. The intact DI-Δ69RII RNA was quantified and shown as a percentage of the RNA level detected at time zero.

the abundant recombinationally active templates are more stable and thus more available to increase the frequency of recombination in *xrn1*Δ mutant cells than in the parental yeast cells.

Xrn1p is a suppressor of viral RNA recombination in vivo. To test if Xrn1p can suppress TBSV RNA recombination, we expressed Xrn1p from a plasmid in *xrn1*Δ mutant cells that also coexpressed DI-72(+) replicon RNA and the p33/p92 replication proteins (Fig. 9A). The expression of Xrn1p in *xrn1*Δ mutant cells reduced the amount of recombinant RNAs three- to fourfold as well as decreasing the amount of 3′Fr-like, partially degraded replicon RNAs three- to fourfold (Fig. 9B). In contrast, Xrn1p did not reduce the level of DI-72 replicon RNA. Similarly, the expression of Xrn1p inhibited the accumulation of recombinant RNAs 12-fold and of partially degraded 3′Fr products 6-fold in *xrn1*Δ mutant cells expressing the highly recombinogenic DI-AU-FP RNA (Fig. 9B). Altogether, these data establish that Xrn1p inhibits the accumulation of both viral recombinant RNAs and partial viral RNA degradation products, thus acting as a suppressor of viral RNA recombination.

DISCUSSION

In contrast to recent advances in our understanding of the roles of host factors in RNA virus replication (2, 19, 31), the roles of host proteins in viral RNA recombination are currently unknown. However, host factors might affect RNA recombination in multiple ways by (i) influencing the quantity of viral RNA substrates available in cells, which is known to affect the frequency of recombination (5, 18, 24); (ii) changing the characteristics of the viral replicase, which are proposed to drive most RNA recombination events (10, 11, 25, 33); or (iii) other still undiscovered mechanisms.

In this paper, we demonstrate that the *XRN1* gene, which was identified previously during a systematic genome-wide

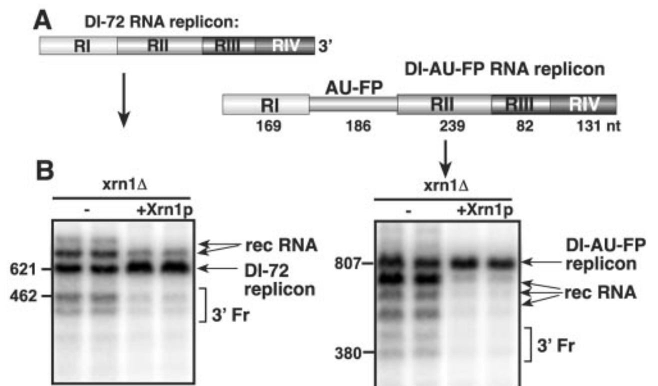

FIG. 9. Expression of Xrn1p in *xrn1*Δ mutant strain inhibits recombination. (A) The coexpressed replicon RNA was either DI-72 or DI-AU-FP replicon RNA, as shown schematically. These cells also expressed p33 and p92. (B) Northern blot analysis was done as described in the legend to Fig. 1C with an RIII-specific probe. The DI-72 and DI-AU-FP replicon RNAs, the recombinants, and partially degraded 3′Fr RNAs are marked. Note that the recombinant RNAs are shorter than the DI-AU-FP replicon RNA due to the deletion of the 5′-terminal ~350 to 400 nt in the 3′Fr recombination substrate (bracketed).

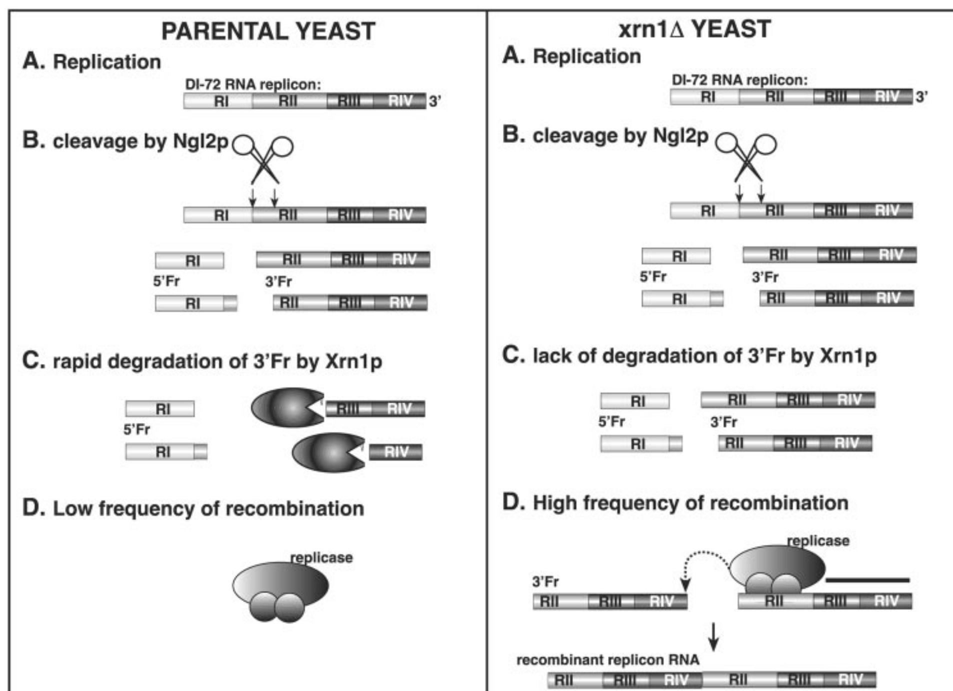

FIG. 10. Model of the suppression of tombusviral RNA recombination by Xrn1p in yeast. (A) DI-72 replicon RNA replicates in the parental strain (left panel) or in the *xrn1*Δ mutant strain (right panel). (B) During or after the replication of DI-72 replicon RNA, a host endoribonuclease such as Ngl2p cleaves some of the viral RNA (marked by arrows), producing 5′Fr and 3′Fr RNAs. (C) These cleaved RNA products will then be rapidly removed by the host Xrn1p exoribonuclease in the parental strain. In contrast, the 5′Fr and 3′Fr RNAs are only slowly degraded in the *xrn1*Δ mutant strain (right panel). (D) Rapid removal of the recombination substrates inhibits viral RNA recombination in the parental strain. However, the abundant 3′Fr RNAs will participate in replicase-driven recombination events in the *xrn1*Δ mutant strain. Note that 5′Fr RNAs lack important *cis*-acting replication sequences (34, 37, 38) and do not seem to participate in RNA recombination.

screen of yeast genes affecting tombusviral RNA recombination (43), plays a central role in viral RNA recombination by controlling the amounts of partially degraded RNA substrates available for recombination in cells. Based on multiple in vivo and in vitro assays, we propose that Xrn1p reduces the recombination frequency by rapidly degrading the preferred recombination substrates, which are 5′-truncated replicon RNAs, termed 3′Fr RNAs. Indeed, previous studies revealed that (i) RII of DI-72 RNA is a recombination hot spot (7, 33, 44), (ii) 5′-truncated TBSV RNAs are active in RNA recombination in plant protoplasts (49), and (iii) the tombusvirus replicase favors end-to-end template switching in vitro (5). All of the above data support our model that partially degraded 3′Fr RNAs promote efficient RNA recombination by the tombusvirus replicase due to the exposure of the RII recombination hot spot in the 5′-truncated RNAs. Accordingly, overexpression of the 3′Fr RNAs in yeast or in plant protoplasts, which likely overwhelmed the host RNA degradation machinery (which is based on Xrn1p in yeast [16, 36] and Xrn4p in plants [45]), led to efficient viral recombination (Fig. 3 and 4). This suggests that high levels of recombination substrates are sufficient to produce viral RNA recombinants in a wt or mutant host background. In contrast, we found that the full-length DI-72 replicon RNA was more resistant to Xrn1p degradation in vitro, suggesting that Xrn1p selectively degrades partially cleaved and/or incomplete replicon RNAs. In addition, the above data suggest that the highly structured RI sequence present at the 5′ end of the TBSV replicon RNA (40, 41) seems to be partially resistant to Xrn1p-mediated 5′-3′ RNA degradation. Therefore, we propose that Xrn1p affects replication/recombination of the TBSV replicon by (i) preferably degrading partial RNA degradation products, (ii) reducing their chances of participating in RNA recombination, and (iii) maintaining the most stable full-length DI-72 replicon RNA.

The vastly increased accumulation of viral RNA recombinants in *xrn1*Δ mutant cells in comparison with parental yeast cells and the inhibition of recombinant RNA accumulation in *xrn1*Δ mutant cells expressing Xrn1p strongly establish that Xrn1p is a potent suppressor of the viral RNA recombination process. Because a similar 5′-3′ exoribonuclease, named Xrn4p, with comparable functions has been identified and characterized from *Arabidopsis* (45), it is possible that the Xrn4p exoribonuclease plays analogous roles in plants in tombusvirus recombination and possibly in that of other RNA viruses, too.

This work also reveals that Xrn1p does not work alone in viral RNA degradation and recombination. This conclusion is supported by the abundance of partially degraded replicon RNA products, which represented both 5′ and 3′ fragments, in *xrn1*Δ mutant cells (Fig. 1). The nonoverlapping nature of the 5′ and 3′ fragments accumulating in *xrn1*Δ mutant cells supported the involvement of a host endoribonuclease(s) that might cleave the full-length replicon RNA prior to further digestion by Xrn1p. Accordingly, overexpression of the Ngl2 endoribonuclease resulted in increased amounts of partially degraded replicon RNAs in vivo (Fig. 5). Moreover, purified Ngl2p was able to cleave the replicon RNA in vitro, generating

cleavage products of the expected sizes. These observations are consistent with the role of Ngl2p in tombusviral RNA degradation. However, Ngl2p is not essential in the viral RNA degradation and recombination processes, because partially degraded products were also observed in *ngl2*Δ mutant cells (Fig. 5B). This is likely due to the redundancy of cytoplasmic endoribonucleases, such as Ngl1p and Ngl3p, in yeast. Overall, the presented data promote central roles for host exo- and endoribonucleases in viral RNA recombination.

Model for the roles of host ribonucleases in viral RNA recombination. Based on our in vivo and in vitro studies, we propose that Ngl2p and similar endoribonucleases, together with the Xrn1p 5′-3′ exoribonuclease, affect viral RNA recombination. First, selective cleavage of the full-length DI-72(+) RNA within the RII sequence by Ngl2p produces 5′ and 3′ fragments, the latter of which is important for RNA replication/recombination. Subsequently, the 3′ fragment of DI-72 RNA could be quickly degraded by Xrn1p in the parental yeast strain, thus reducing its chance to participate in RNA recombination events (Fig. 10). Thus, Xrn1p basically acts as a suppressor of RNA recombination. In the absence of Xrn1p, however, the 3′ fragment (i.e., 5′-truncated RNA) of the replicon RNA accumulates to higher levels due to (i) ongoing Ngl2p cleavage of the full-length replicon RNA, (ii) replication of the generated 5′-truncated replicon RNA, and (iii) a lack of Xrn1p-mediated degradation of the 5′-truncated replicon RNA. The resulting increased amount of 5′-truncated replicon RNA could then serve as an efficient template for RNA recombination due to exposure of the RII recombination hot spot. Overall, our model predicts that the combined activities of the Ngl2p and Xrn1p ribonucleases control the amounts of available recombination substrates and thus the frequency of RNA recombination events and the in vivo stabilities of the recombinants (43).

General conclusions. We have dissected for the first time the roles of host factors in viral RNA recombination, which is a major process in virus evolution. This study establishes that the Xrn1p 5′-3′ exoribonuclease acts as a suppressor of viral RNA recombination. In addition, the combined activities of the Ngl2p endoribonuclease and Xrn1p control the amounts of partially degraded replicon RNAs, which affect RNA recombination by serving as substrates. Thus, our results suggest that host-mediated viral RNA turnover is a major factor in viral RNA recombination.

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