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Host transcription factor Rpb11p affects tombusvirus replication and recombination via regulating the accumulation of viral replication proteins

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Abstract

Previous genome-wide screens identified over 100 host genes whose deletion/down-regulation affected tombusvirus replication and 32 host genes that affected tombusvirus RNA recombination in yeast, a model host for replication of *Tomato bushy stunt virus* (TBSV). Down-regulation of several of the identified host genes affected the accumulation levels of p33 and $p92^{pol}$ replication proteins, raising the possibility that these host factors could be involved in the regulation of the amount of viral replication proteins and, thus, they are indirectly involved in TBSV replication and recombination. To test this model, we developed a tightly regulated expression system for recombinant p33 and $p92^{pol}$ replication proteins in yeast. We demonstrate that high accumulation level of p33 facilitated efficient viral RNA replication, while the effect of p33 level on RNA recombination was less pronounced. On the other hand, high level of $p92^{pol}$ accumulation promoted TBSV RNA recombination more efficiently than RNA replication. As predicted, Rpb11p, which is part of the polII complex, affected the accumulation levels of p33 and $p92^{pol}$ as well as altered RNA replication and recombination. An in vitro assay with the tombusvirus replicase further supported that Rpb11p affects TBSV replication and recombination only indirectly, via regulating p33 and $p92^{pol}$ levels. In contrast, the mechanism by which Rpt4p endopeptidase/ATPase and Mps1p threonine/tyrosine kinase affect TBSV recombination is different from that proposed for Rpb11p. We propose a model that the concentration (molecular crowding) of replication proteins within the viral replicase is a factor affecting viral replication and recombination.

Keywords: Tomato bushy stunt virus; Cucumber necrosis virus; RNA replication; RNA recombination; Host factor; Yeast

Introduction

RNA viruses depend on their hosts during their replication in the infected cells to produce viral proteins. Also, the host cells provide membranes and ribonucleotides for RNA synthesis. In addition, RNA viruses actively recruit host proteins to facilitate the replication process (Ahlquist et al., 2003; Buck, 1996; Lai, 1998; Nagy and Pogany, 2006; Noueiry and Ahlquist, 2003; Osman and Buck, 1997; Shi and Lai, 2005). Viral replication proteins and the viral RNA together with host proteins assemble viral replicase complexes on selected host membranes that lead to efficient viral RNA synthesis. Current major research efforts aim at cataloging host proteins that affect virus replication by providing direct functions for the replication process, influencing the amounts of viral replication proteins, or participating in antiviral mechanisms. Accordingly, genome-wide screens of model *Saccharomyces cerevisiae* host for RNA viruses, such as *Brome mosaic virus* (BMV) and *Tomato bushy stunt virus* (TBSV) as well as proteomic analysis of viral replicases identified a large number of host proteins affecting RNA virus replication (Jiang et al., 2006; Kushner et al., 2003; Panavas et al., 2005b; Serva and Nagy, 2006). Altogether, these studies revealed the complex nature of host–virus interactions.

In addition to replication, viruses also undergo RNA recombination, a process that leads to joining of noncontiguous RNA segments (Lai, 1992; Nagy and Simon, 1997; Worobey and Holmes, 1999). RNA recombination is a mechanism that accelerates evolution and adaptation of positive-strand RNA viruses and the emergence of new viral variants (Lai, 1992; Nagy and Simon, 1997; Worobey and Holmes, 1999; Zimmern, 1988). RNA recombination could also increase the fitness of RNA viruses by eliminating deleterious mutations introduced by the error-prone viral RNA-dependent RNA polymerase (RdRp).

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RNA recombinants can often be found in natural infections in plants and animals and interviral recombinants between different viruses and recombinants between host sequences and viruses have also been isolated (Aaziz and Tepfer, 1999; Bonnet et al., 2005; DeStefano et al., 1994; Desvoyes and Scholthof, 2002; Froissart et al., 2005; Gibbs and Weiller, 1999; Greene and Allison, 1994; Moonan et al., 2000; Revers et al., 1996; Tan et al., 2004; Vigne et al., 2005; Worobey and Holmes, 1999). Because RNA recombination can greatly increase viral genome variability, it is a major threat to compromise current antiviral strategies (Keese and Gibbs, 1993; Lai, 1992; Roossinck, 2003; Worobey and Holmes, 1999).

Mechanistic studies on RNA recombination in cell-free systems based on purified recombinant viral RdRps of BMV, *Cucumber mosaic virus*, *Bovine viral diarrhea virus*, *Hepatitis C virus*, *Turnip crinkle virus* and partially purified *Cucumber necrosis virus* (CNV, a tombuvirus) replicase preparations revealed a replicase-driven template-switching (TS) as the dominant mechanism in RNA recombination (Cheng and Nagy, 2003; Cheng et al., 2005; Kim and Kao, 2001). The TS mechanism is further supported by results obtained with mutants within the viral replication genes that influenced the frequency of recombination and the sites of recombination junctions in vivo (Figlerowicz et al., 1997; Nagy et al., 1995; Panaviene and Nagy, 2003). Moreover, the role of the sequence/structure of the viral RNA in RNA recombination has also been extensively studied (Kim and Kao, 2001; Nagy and Bujarski, 1993, 1996, 1997; Nagy et al., 1998; Wierzchoslawski et al., 2003). Most recombination junctions were found within noncoding regions that include *cis*-acting replication elements, such as promoters for complementary RNA synthesis, subgenomic promoters, replication enhancers and internal replication elements (Nagy and Simon, 1997).

In contrast with our rapidly deepening understanding of the contribution of viral factors to viral RNA replication and recombination, our knowledge on the role of host proteins/factors in viral RNA replication/recombination is poor. A genetically tractable model system, based on a plant tombusvirus and yeast, has been developed to facilitate studies on replication and recombination (Panavas and Nagy, 2003; Pantaleo et al., 2003). In the model system based on TBSV, the viral repRNA is expressed from the galactose/glucose inducible/repressible *GAL1* promoter, whereas the two viral replication proteins, termed p33 and p92^{pol}, are expressed separately from expression plasmids via the constitutive *ADH1* promoter (Panavas and Nagy, 2003; Panaviene et al., 2004). Induction of repRNA expression leads to the assembly of the viral replicase complex consisting of p33, p92^{pol} replication proteins and Ssa1/2p, Tdh2/3p and Pdc1p host proteins on peroxisomal membranes (Panavas et al., 2005a; Serva and Nagy, 2006). Recent systematic screens of the yeast knock out (YKO) library containing 80% and the essential gene library (yTHC) containing ~15% of all the yeast genes, respectively, led to the identification of 126 and 32 genes, which affected tombusviral RNA replication and recombination, respectively (Jiang et al., 2006; Panavas et al., 2005b; Serviene et al., 2005, 2006). More detailed analysis of the mechanism of suppression of viral RNA recombination has been performed with one of the identified genes, namely *XRN1* (Cheng et al., 2006). This work revealed that Xrn1p, which is an evolutionary conserved 5′–3′ exoribonuclease, inhibited viral RNA recombination by rapid and selective degradation of viral RNA recombination intermediates from wt yeast cells (Cheng et al., 2006). The actual roles of the other identified yeast genes in viral RNA recombination are currently unknown.

Previous work has defined that down-regulation of several yeast genes, which affected viral RNA recombination and/or viral RNA replication, also affected the accumulation level of p33 and p92^{pol} replication proteins (Jiang et al., 2006; Panavas et al., 2005b; Serviene et al., 2005, 2006). Therefore, it is conceivable that several of these host genes might have only indirect effect on viral RNA replication/recombination by regulating the amount of viral replication proteins. This model was tested in the current work by developing a tightly regulated expression system for p33, p92^{pol} and the viral replicon (rep)RNA. The results demonstrate that the amount and ratio of p33 and p92^{pol} replication proteins play a significant role in viral RNA replication and recombination, whereas the amount of the replicon RNA was less important. Comparison of these data with another data set obtained via down-regulation of three host protein levels revealed that Rpb11p transcription factor, unlike Rpt4p endopeptidase or Msp1p kinase, affected viral RNA replication and recombination by regulating the accumulation level of p33 and p92^{pol}. Altogether, the data will be very useful for determination of direct or indirect effects of the more than 100 host proteins identified in previous genomic screens for tombusvirus replication and recombination.

Results

Development of tightly regulated expression system for tombusvirus replication proteins and the repRNA in yeast

Systematic screens of 95% of yeast genes for the effect on TBSV RNA replication and recombination have led to the identification of 126 and 32 genes, respectively (Jiang et al., 2006; Panavas et al., 2005b; ; Serviene et al., 2005, 2006). Deletion or down-regulation of several of the identified host genes affected the accumulation levels of p33 and p92^{pol} replication proteins, raising the possibility that these host factors could be involved in the regulation of the amount of viral replication proteins via regulating protein expression and/or degradation and, thus, these factors are indirectly involved in TBSV replication and recombination.

To test the above model, first we had to examine the effects of p33 and p92^{pol} and the repRNA transcript levels on TBSV replication and recombination in yeast. These replication and recombination assays required the development of a tightly regulated expression system for the separate expression of p33 and p92^{pol} and the repRNA transcripts (Fig. 1A). Namely, the p33 open reading frame (ORF) was placed behind the copper-regulated *CUP1* promoter (Mascorro-Gallardo et al., 1996). The p92^{pol} ORF was placed behind the titratable tet promoter (i.e.,

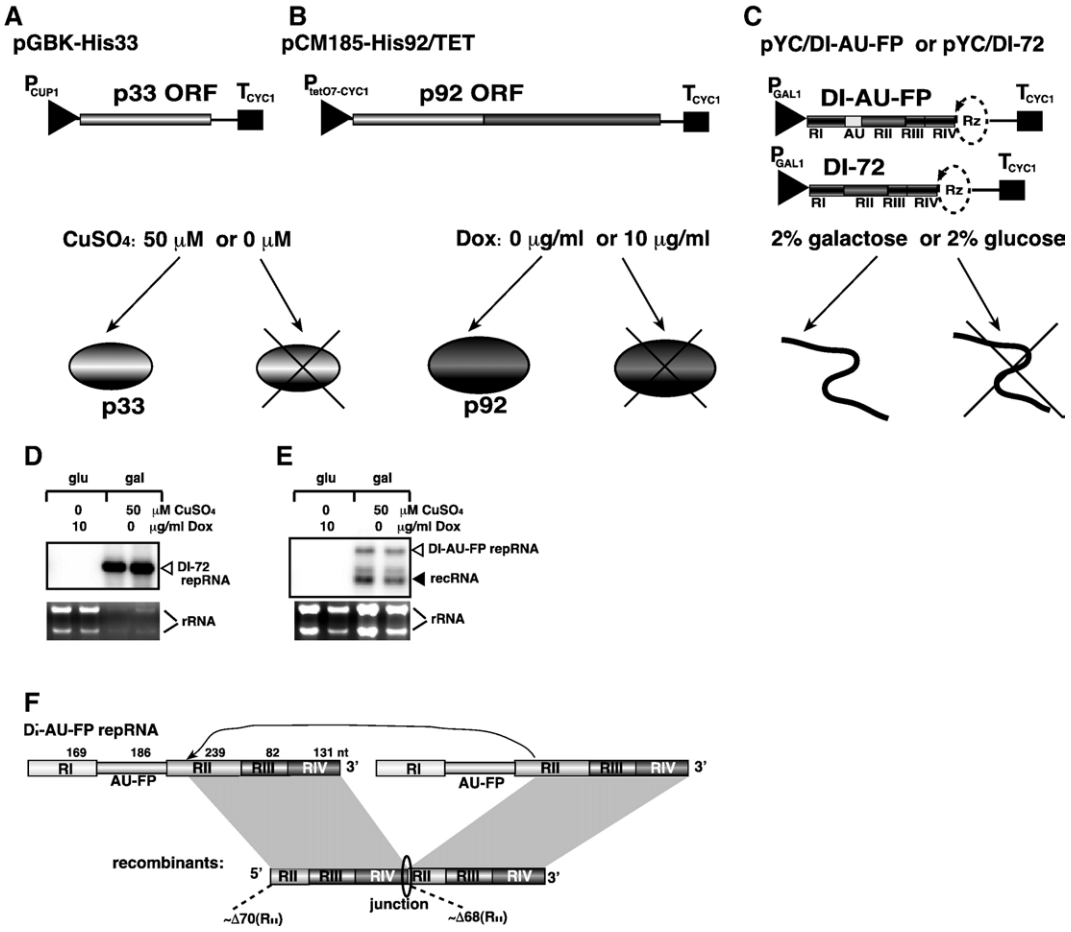


Fig. 1. Tightly regulated expression of tombusvirus p33 and p92^{pol} replication proteins and the repRNA from expression plasmids in yeast. (A–C) Schemes of expression strategies based on the controllable *CUP1*, tet and *GAL1* promoters. The conditions for maximum and minimum (protein or RNA products crossed with X) levels of expression are indicated. (D) Simultaneous down-regulation of p33 and p92^{pol} replication proteins and the repRNA eliminates DI-72 repRNA replication and (E) DI-AU-FP RNA recombination in yeast. Samples on the right side of each panel show the positive controls when all three viral components are simultaneously expressed. Ribosomal RNA is shown as a gel loading control. (F) Schematic representation of RNA recombinants obtained with the DI-AU-FP repRNA. Recombination events form a dimer from two 5′ truncated DI-AU-FP repRNAs as shown. These recombinants were characterized extensively in prior publications (Serviene et al., 2006; Serviene et al., 2005).

tetO$_7$-CYC1-TATA, Fig. 1B), which can be down-regulated by the addition of various amounts of doxycycline to the growth medium (Gari et al., 1997; Mnaimneh et al., 2004). Moreover, the repRNA was expressed from the galactose/glucose induced/suppressed *GAL1* promoter (Fig. 1C). This arrangement allowed us to separately regulate the expression of p33, p92^{pol} and the repRNA. Accordingly, growing yeast in a medium suppressing all three promoters led to undetectable repRNA accumulation (Fig. 1D) and TBSV RNA recombinant formation (Fig. 1E). Conversely, replication of repRNA and the accumulation of recombinants were easily detectable under inducing conditions (Figs. 1D–E).

The generated recombinants were partially truncated repRNA dimers, similar to those published earlier (Fig. 1F) (Serviene et al., 2005, 2006). Overall, suppression of all three promoters during yeast propagation prevented TBSV replication/recombination prior to separate inductions of p33, p92^{pol} and the repRNA transcripts. Thus, this assay was suitable to dissect the individual contribution of these viral factors to replication and recombination by preventing TBSV replication/recombination during pre-culturing of yeast cells.

High level of p33 expression facilitates TBSV replication and recombination

To test the effect of p33 accumulation level on TBSV replication, we pre-grew yeast cells under fully suppressive condition (2% glucose, no copper and 10 μg/ml doxycycline, see Materials and methods section), followed by induction of TBSV replication by replacing glucose with galactose and omitting doxycycline from the growth medium. This resulted in maximum level of expression of the p92^{pol} and the DI-72 repRNA transcript, whereas the expression of p33 was regulated by altering the concentration of copper ions between 50 and 0 μM (Fig. 2A). The level of repRNA accumulation was measured via Northern blotting of the total RNA extract obtained from yeast 17 h after the induction of repRNA replication (Figs. 2A and C), whereas the accumulation level of

p33 was estimated by Western blotting (Fig. 2E). The data revealed close correlation between repRNA accumulation and p33 accumulation level. The lowest level of repRNA accumulation (20%) was observed when yeast was grown without adding extra copper to the medium (0 μM), which resulted in ~15% p33 accumulation (Fig. 2E). The "leaky" expression from *CUP1* promoter without added copper is likely due to the presence of trace amount of copper (3 μM) in standard yeast nitrogen base used for preparation of minimal media. When we used copper-free medium, we found that yeast grew poorly on the minimal media (not shown), preventing us to completely shut down p33 expression. Nevertheless, the tight correlation between p33 levels and repRNA accumulation suggests that p33 is a limiting factor in TBSV replication.

Since changing the amount of p33 in yeast also affects the ratio of p33/p92^{pol}, which in turn might influence TBSV replication, we performed experiments using 0.08 μg/ml doxycycline to reduce the level of p92^{pol} to ~50% of that observed in cells grown in the absence of doxycycline (Fig. 2A, left panel), whereas p33 levels were controlled by addition of various amounts of copper as above. We found that ~80% reduction in p33 accumulation resulted in ~80% reduction in repRNA accumulation (Fig. 2A, right panel), similar to the picture obtained with higher accumulation of p92^{pol} (Fig. 2A, left panel). Since the ratio of p33/p92^{pol} was different in the two experiments, we conclude that the ratio of the replication proteins is not a major factor in TBSV replication when p92^{pol} is present at either 100% or ~50% levels.

We used a similar approach to study the effect of p33 accumulation on TBSV recombination. The major difference from the above replication studies was the use of DI-AU-FP repRNA, which carries an AU-rich recombination hotspot sequence (Shapka and Nagy, 2004) to promote the accumulation of RNA recombinants (Fig. 1C). Briefly, the amount of p33 was regulated by altering the concentration of copper ions between 50 and 0 μM (Fig. 2B) followed by measuring the levels of repRNA and recRNA accumulation by Northern blotting of the total RNA extract obtained from yeast 17 h after the induction of DI-AU-FP repRNA replication (Figs. 2B and D). In order to identify changes in RNA recombination, we estimated the recRNA/repRNA ratio instead of measuring recRNA levels. This is because replication of the repRNA can affect recombination since ultimately the repRNA provides the template/substrate for recombination. Thus, higher repRNA levels favor recombination (Serviene et al., 2005, 2006; Shapka and Nagy, 2004). Therefore, recRNA/repRNA ratio is helpful to identify factors affecting recombination directly, not indirectly via affecting replication (Serviene et al., 2005, 2006).

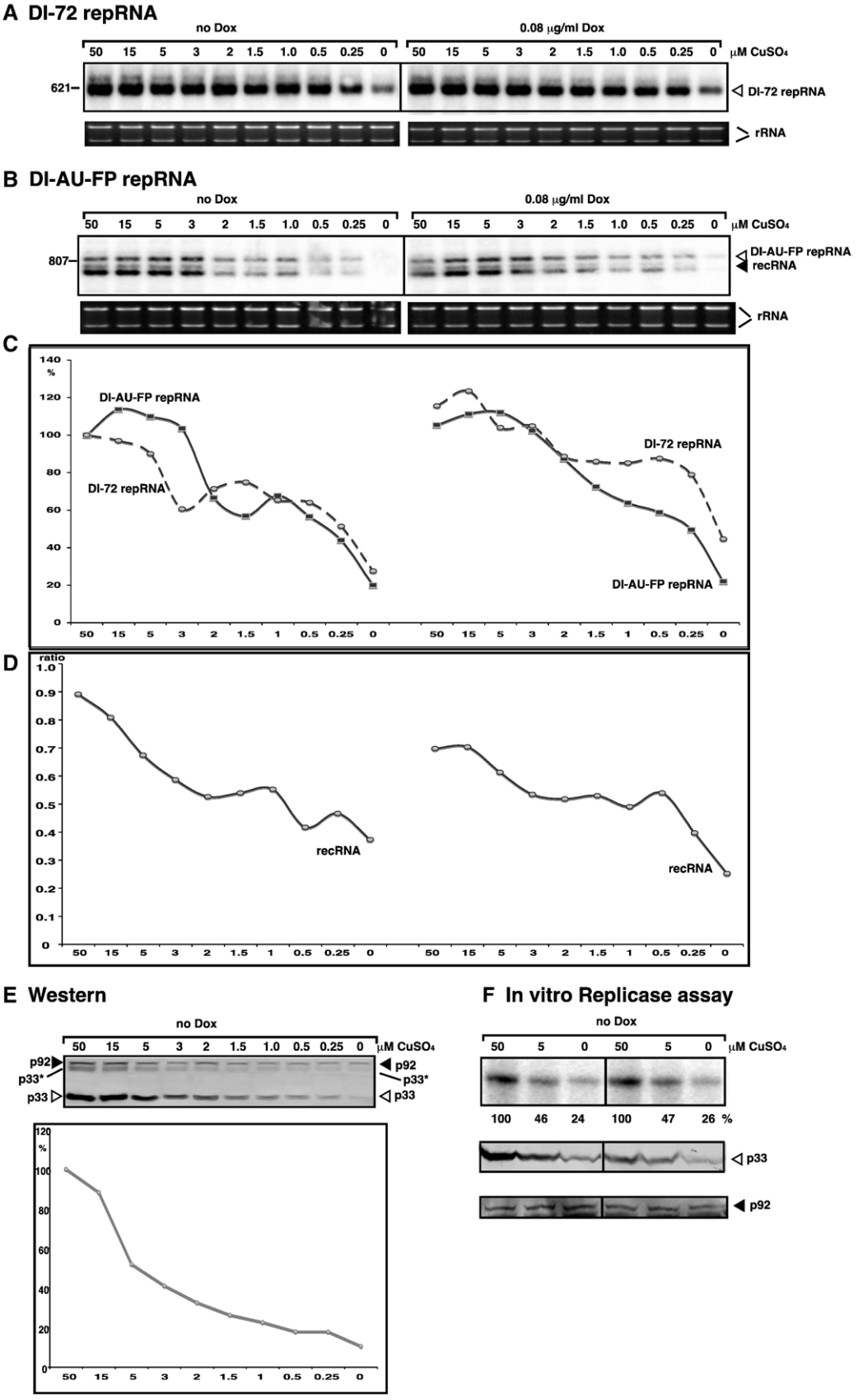
The recRNA/repRNA ratio revealed that higher level of p33 accumulation promoted RNA recombination. For example, a 7-fold increase in p33 accumulation led to a 2.5-fold increase of recRNA/repRNA ratio (compare data obtained after induction of p33 with 50 and 0 μM copper, respectively; Figs. 2D–E). The lowest recRNA/repRNA ratio was observed (Fig. 2D, left panel) when yeast was grown without adding extra copper to the medium (0 μM), which resulted in ~15% p33 accumulation (Fig. 2E).

To test if the ratio of p33/p92^{pol} can affect TBSV recombination, we performed experiments using 0.08 μg/ml doxycycline to reduce accumulation of p92^{pol} to ~50%, while p33 levels were controlled by addition of various amounts of copper as above. We found that the high ratio of p33/p92^{pol} reduced TBSV recombination by ~20% (compare Fig. 2D right graph with the left graph). Therefore, the ratio of p33/p92^{pol} within the viral replicase is likely an important factor in RNA recombination.

To test if the amount of p33 can affect the activity of the tombusviral replicase directly, we obtained replicase preparations from yeast expressing decreasing amounts of p33 in combination with constant p92^{pol} and DI-72 repRNA levels. The activity of the replicase in the membrane-enriched fraction, which contains the endogenous (co-purified) template RNA, was tested by adding ^{32}P-labeled UTP together with the other three unlabeled ribonucleotides (Panaviene et al., 2004; Stork et al., 2005). The in vitro replicase assay revealed that the replicase preparation with the highest amount of p33 was the most active, whereas the replicase with the lowest amount of p33 was ~4-fold less active (Fig. 2F). The amount of p92^{pol} in the replicase preparations was comparable (Fig. 2F). Altogether, these data support the model that high amount of p33 stimulates the activity of the tombusvirus replicase and p33 is present in limiting amount (i.e., more p33 results in more active replicase). The data from the in vitro replicase assay support the model that the primary function of high level of p33 is in viral RNA replication, not in the stabilization of the repRNA. Thus, host factors that can boost the production of p33 in cells will likely stimulate tombusvirus replication. In summary, within the range of p33 concentration tested, the rate of replication increased 5–6-fold, whereas the rate of recombination only increased ~2-fold.

High level of p92^{pol} expression facilitates TBSV recombination

To test the effect of p92^{pol} accumulation level on TBSV replication, we pre-grew yeast cells under fully suppressive condition, followed by induction of TBSV replication by replacing glucose with galactose and adding 50 μM copper to the growth medium. This resulted in maximum levels of expression of p33 and DI-72 repRNA, while the expression of p92^{pol} was regulated by altering the concentration of doxycycline between 0 and 0.45 μg/ml (Fig. 3A). The lowest level of repRNA accumulation (~50%, Fig. 3C, left graph) was observed when yeast was grown in the presence of doxycycline (0.45 μg/ml), which resulted in undetectable p92^{pol} accumulation (Fig. 3E). The obtained data revealed correlation between repRNA accumulation and p92^{pol} accumulation level. However, ~10-fold reduction of p92^{pol} accumulation led only to 2–2.5× decrease in repRNA accumulation, suggesting that p92^{pol} level has only moderate effect on RNA replication. Also, reduction of p92^{pol} amount had only moderate effect on repRNA accumulation when the p33/p92^{pol} ratio was changed (via reducing p33 expression to ~50% by adding 5 μM copper in the media, Figs. 3A–C, right panel). Overall, it seems that TBSV replication is less


A DI-72 repRNA
no Dox
0.08 μg/ml Dox
50 15 5 3 2 1.5 1.0 0.5 0.25 0
μM CuSO4
621
DI-72 repRNA
rRNA
B DI-AU-FP repRNA
no Dox
0.08 μg/ml Dox
50 15 5 3 2 1.5 1.0 0.5 0.25 0
μM CuSO4
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DI-AU-FP repRNA
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DI-72 repRNA
DI-AU-FP repRNA
D
ratio
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recRNA
E Western
no Dox
50 15 5 3 2 1.5 1.0 0.5 0.25 0
μM CuSO4
p92
p33*
p33
F In vitro Replicase assay
no Dox
50 5 0 50 5 0
μM CuSO4
100 46 24 100 47 26 %
p33
p92

affected by down-regulation of p92^{pol} expression than by down-regulation of p33 level.

To study the role of p92^{pol} level in TBSV recombination, we measured the recRNA/repRNA ratio under decreasing levels of p92^{pol} expression (Figs. 3B and D). We found that the highest level of p92^{pol} expression increased recRNA/repRNA ratio by 3- to 4-fold when compared to the lowest p92^{pol} expression level (Figs. 3D and E). The lowest recRNA/repRNA ratio was observed (Fig. 3D) when yeast was grown with the highest amount of doxycycline (0.45 μg/ml). Thus, recombination is greatly affected by the amount of p92^{pol} available.

To test if reduced ratio of p33/p92^{pol} can affect TBSV recombination, we performed experiments using 5 μM copper to reduce p33 accumulation to ~50% (Figs. 3B and D, right panels), while p92^{pol} levels were regulated by the addition of various amounts of doxycycline as above. We found that the lower ratio of p33/p92^{pol} reduced TBSV recombination by 20% (compare Fig. 3D, graphs on the left and right). In summary, within the range of p92^{pol} concentration tested, the rate of replication increased only ~2-fold, whereas the rate of recombination increased ~3–4-fold.

The effect of p33/p92^{pol} ratio on TBSV replication and recombination

To test the effect of changing the ratio between p33/p92^{pol} on replication more extensively than in the above experiments, we have conducted a set of experiments in which we changed p92^{pol} and p33 levels simultaneously. Briefly, p92^{pol} was expressed from the highest amount to the lowest amount, whereas p33 was expressed from the smallest amount to the highest amount (Fig. 4A). In this experiment, the TBSV repRNA accumulation did not correlate with p33 level (Fig. 4B). The highest level of TBSV accumulation was observed with yeast grown in a medium containing 0.1–0.2 μg/ml doxycycline and 0.5–1.5 μM $CuSO_4$, which resulted in ~10-fold more p33 than p92^{pol} (Figs. 4A and F). The lowest replication was observed when p33 and p92^{pol} were expressed in 1:1 ratio (0 μg/ml doxycycline and 0 μM $CuSO_4$, Figs. 4A and E). Overall, these experiments revealed that the ratio between p33/p92^{pol} is important in TBSV replication and the optimal level of TBSV replication requires at least ~10-fold more p33 than p92^{pol}.

We also tested the effect of p33 and p92^{pol} ratio on TBSV recombination using a similar approach as described above. Recombination was the highest when p33 was present in low amount, whereas p92^{pol} was close to its maximum level under the experimental conditions (0–0.10 μg/ml doxycycline). The lowest recombination level was observed with high p33/low p92^{pol} levels (Figs. 4D and E). Collectively, these experiments further support that the ratio between p33/p92^{pol} is an important factor in TBSV recombination, although the effect was less pronounced than that observed in case of high level of p33 in combination with high level of p92^{pol} (Figs. 3B, D and E).

Limited effect of initial repRNA level on TBSV replication and recombination

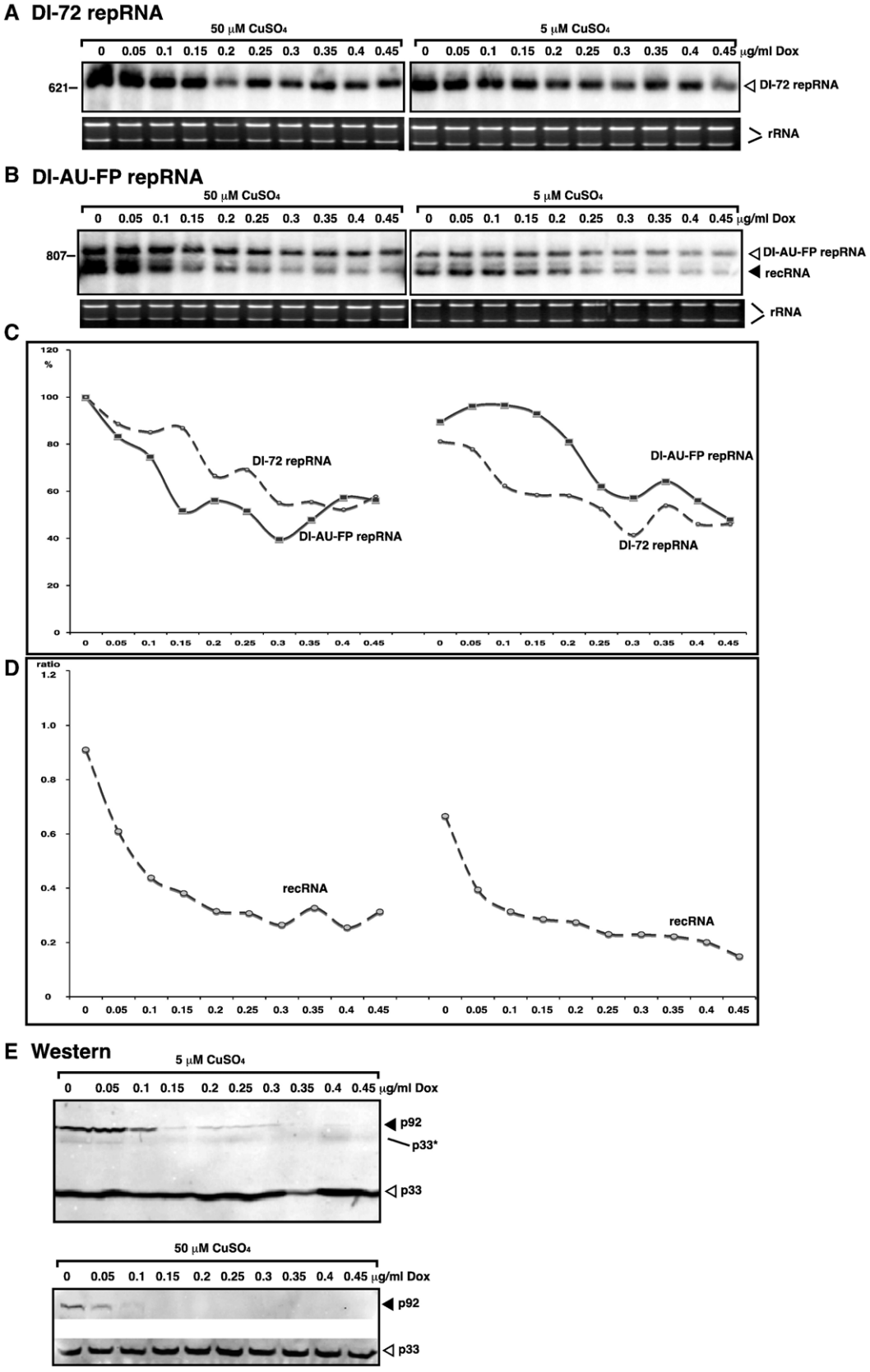
To test if the initial level of DI-AU-FP repRNA affects replication, we placed its cDNA behind the copper-regulated *CUP1* promoter (Fig. 5A), whereas p33 and p92^{pol} were expressed from *ADH1* promoters. This arrangement allowed the down-regulation of DI-AU-FP repRNA expression from 100 to 30% in yeast tested in the absence of replication (when no p92^{pol} was expressed, Fig. 5F). However, the level of replication of DI-AU-FP repRNA or the ratio of the recRNA/repRNA did not change significantly in yeast expressing reduced amount of DI-AU-FP transcripts (50–0 μM $CuSO_4$, Figs. 5B–E). These data support the model that the amount of the original DI-AU-FP RNA transcripts has only minor effect on subsequent replication and recombination within the tested RNA transcript range (30–100%).

Down-regulation of Rpb11p decreases levels of p33 and p92^{pol} replication proteins and affects TBSV replication and recombination

To test if host proteins could affect TBSV replication and recombination by regulating p33 and/or p92^{pol} levels, first we selected the essential protein Rpb11p (Jiang et al., 2006; Serviene et al., 2006). Our simple model was that down-regulation of Rpb11p, which is part of pol II complex involved in synthesis of mRNAs (Hampsey, 1998), could affect the production of p33 and p92 mRNAs from the expression plasmids. The resulting reduced levels of p33 and p92^{pol} then likely inhibit TBSV replication and recombination, based on our data with regulated

Fig. 2. The effect of p33 level on TBSV repRNA accumulation and recombination in yeast. (A) RNA blot analysis of accumulation of the DI-72 repRNA 17 h after induction of transcription by galactose (repRNA transcription was shut down by changing to a medium containing 2% glucose after 6 h). The expression level of p33 is regulated by various amounts of $CuSO_4$ as shown, whereas p92^{pol} was expressed at the maximum level (panel on the left) or ~50% level (0.08 μg/ml doxycycline, panel on the right). Ethidium-bromide stained gels of ribosomal RNA show the loading accuracy. (B) RNA blot analysis of accumulation of the DI-AU-FP repRNA and the de novo recRNAs. See further details in panel A. The scheme of a representative recRNAs is shown in Fig. 1F. (C) Relative accumulation of DI-72 and DI-AU-FP repRNAs under the conditions described in panels A and B. RepRNA accumulation at the highest p33 level was chosen as 100%. Each experiment was repeated at least three times. (D) The calculated ratio of recRNA/repRNA obtained in yeast expressing DI-AU-FP repRNA under the conditions described in panel B. (E) Western blot analysis with anti-His antibody to determine the His-tagged p33 and p92^{pol} accumulation in yeast grown under the same conditions as in panel A. Note that p33 forms a heat/SDS-resistant dimer, marked with an asterisk next to the blot. The accumulation level of p33 monomer is shown in the graph. The highest p33 level obtained with 50 μM $CuSO_4$ was chosen as 100%. (F) The effect of p33 levels on the tombusvirus replicase activity in vitro. The membrane-enriched (ME) fraction containing the viral replicase bound to the repRNA template was isolated (Panaviene et al., 2004), followed by an in vitro assay in the presence of ^{32}P-UTP and other ribonucleotides. The obtained replicase products (top panel showing DI-72 repRNA) were analyzed in a denaturing gel and quantified with a phosphorimager. The replicase activity obtained with the most p33 (induced in the presence of 50 μM $CuSO_4$) was chosen as 100%. The levels of p33 and p92^{pol} replication proteins in the ME fractions were estimated with Western blotting. Each experiment was done three times.

p33 and p92^{pol} expression (Figs. 2 and 3). To test this model, we down-regulated Rpb11p expression from the tet promoter by adding increasing amount of doxycycline (Fig. 6A). It is known that doxycycline does not affect p33 and p92^{pol} expression in the parental yeast strain (Jiang et al., 2006; Serviene et al., 2006). It is also worth noting that we used DI-AU-FP repRNA in these and



subsequent experiments to allow for simultaneous measurements on replication and recombination. We have demonstrated before that DI-AU-FP repRNA is suitable for replication studies, since host proteins that affected the accumulation of DI-AU-FP repRNA also changed the accumulation of DI-72 repRNA to similar extent (Jiang et al., 2006).

At the highest Rpb11p expression level (0 μg/ml doxycycline), we observed the highest replication and recombination rates as well as the most abundant p33 and $p92^{pol}$ expression. Down-regulation of Rpb11p expression by doxycycline led to rapid decrease in $p92^{pol}$ and, to a lesser extent, p33 levels (Fig. 6C). Interestingly, repRNA accumulation and the ratio of recRNA/repRNA also decreased gradually (Figs. 6B–D). These data demonstrate a close correlation between replication protein levels and repRNA accumulation and the ratio of recRNA/repRNA, as predicted based on our findings that the levels of replication proteins play a major role in TBSV RNA accumulation and recombination.

The test if down-regulation can affect the activity of the tombusviral replicase, we obtained replicase preparations from yeast with different levels of Rpb11p expression. The activity of the replicase in the membrane-enriched fraction revealed that the replicase preparation with the lowest Rpb11p expression was the least active (Fig. 6E). Interestingly, the amount of p33 and $p92^{pol}$ within the replicase preparations decreased closely following down-regulation of Rpb11p expression (due to repression by doxycycline, Fig. 6E). Altogether, these data strongly support the model that Rpb11p regulates the expression of p33 and $p92^{pol}$, which then leads to changes in the replicase activity as predicted based on the above expression experiments (Figs. 2–4).

Down-regulation of Rpt4p and Mps1p affects TBSV replication and recombination without changing the levels of p33 and $p92^{pol}$ replication proteins

To test the generality of the mechanism of host protein affecting TBSV replication and recombination via regulating the levels of the viral replication proteins, we examined the effect of down-regulation of two other host proteins, namely Rpt4p and Mps1p, on replication and recombination. We chose these two host proteins because down-regulation of Rpt4p and Mps1p essential proteins was previously found to affect TBSV recombination (i.e., reduced the ratio of recRNA/repRNAs), similar to that observed with Rpb11p (Serviene et al., 2006). Gradual down-regulation of Rpt4p, which is an endopeptidase/ATPase involved in the degradation of ubiquitinated substrates (McDonald et al., 2002), reduced the ratio of rec/repRNAs (Figs. 7A, D), albeit the accumulation of repRNA did not change significantly (Figs. 7A, C). However, the expression levels of p33 and $p92^{pol}$ replication proteins did not change when Rpt4p expression was down-regulated (Fig. 7B). These data suggest that Rpt4p does not affect TBSV recombination via altering the expression levels of the viral replication proteins.

Comparable experiments with Mps1p (Jiang et al., 2006; Serviene et al., 2006), a threonine/tyrosine kinase, showed altered RNA recombination (reduced ratio of recRNA/repRNA) when Mps1p expression was down-regulated (Figs. 8A, D). Conversely, the level of repRNA accumulation increased (Figs. 8A, C). The expression level of p33 and $p92^{pol}$ was also increased in the presence of low amounts of doxycycline (0.1–0.4 μg/ml) and decreased with high level of doxycycline (1.0 μg/ml) (Fig. 8B). Overall, we did not find correlation between replication protein levels and the ratio of recRNA/repRNAs or repRNA accumulation. Therefore, the results with Mps1p, similar to Rpt4p, do not fit the model that Mps1p and Rpt4p affect TBSV recombination via regulating the levels of the replication proteins in yeast. Collectively, we suggest that only a fraction of host proteins affect TBSV replication and recombination via regulating the levels of the viral replication proteins, whereas other host proteins act on TBSV replication/recombination via different mechanisms.

Discussion

Host proteins likely affect RNA virus replication and recombination via several different ways/mechanisms (Ahlquist et al., 2003; Cheng et al., 2006; Jiang et al., 2006; Lai, 1998; Noueiry and Ahlquist, 2003; Serviene et al., 2005, 2006; Shi and Lai, 2005). A possibly common mechanism for a host factor to affect tombusvirus replication/recombination is to regulate the amounts of p33 and/or $p92^{pol}$ replication proteins and the initial transcribed repRNA (which are produced in yeast via plasmid-based transcription). Therefore, to advance our understanding of the roles of host and viral proteins in tombusvirus replication, we have studied two major questions in this work. First, we wanted to define the effects of the tombusviral p33 and $p92^{pol}$ replication proteins and the initial level of the repRNA template on TBSV replication and recombination. Second, we wanted to test if three of the previously identified host proteins could affect TBSV replication and recombination indirectly, via regulating the levels of tombusviral p33 and $p92^{pol}$ replication proteins. Development of a tightly regulated expression system was critical in these experiments (Fig. 1). Accordingly, we have

Fig. 3. The effect of $p92^{pol}$ level on TBSV repRNA accumulation and recombination in yeast. (A) RNA blot analysis of accumulation of the DI-72 repRNA 17 h after induction of transcription by galactose. The expression level of $p92^{pol}$ is negatively regulated by various amounts of doxycycline as shown, whereas p33 was expressed at the maximum level (50 μM $CuSO_4$, panel on the left) or ~50% level (5 μM $CuSO_4$, panel on the right). Each experiment was repeated at least three times. See further details in the legend to Fig. 2A. (B) RNA blot analysis of accumulation of the DI-AU-FP repRNA and the de novo recRNAs. See further details in panels A–B. (C) Relative accumulation of DI-72 and DI-AU-FP repRNAs under the conditions described in panels A–B. RepRNA accumulation at the highest $p92^{pol}$ level (no doxycycline) was chosen as 100%. The horizontal line represents the concentration of the doxycycline in the yeast growth medium. (D) The ratio of recRNA/repRNA obtained in yeast expressing DI-AU-FP repRNA under the conditions described in panel B. The horizontal line represents the concentration of the doxycycline in the yeast growth medium. (E) Western blot analysis with anti-His antibody to determine the p33 and $p92^{pol}$ accumulation in yeast grown under the same conditions as in panel A. See further details in the legend to Fig. 2.

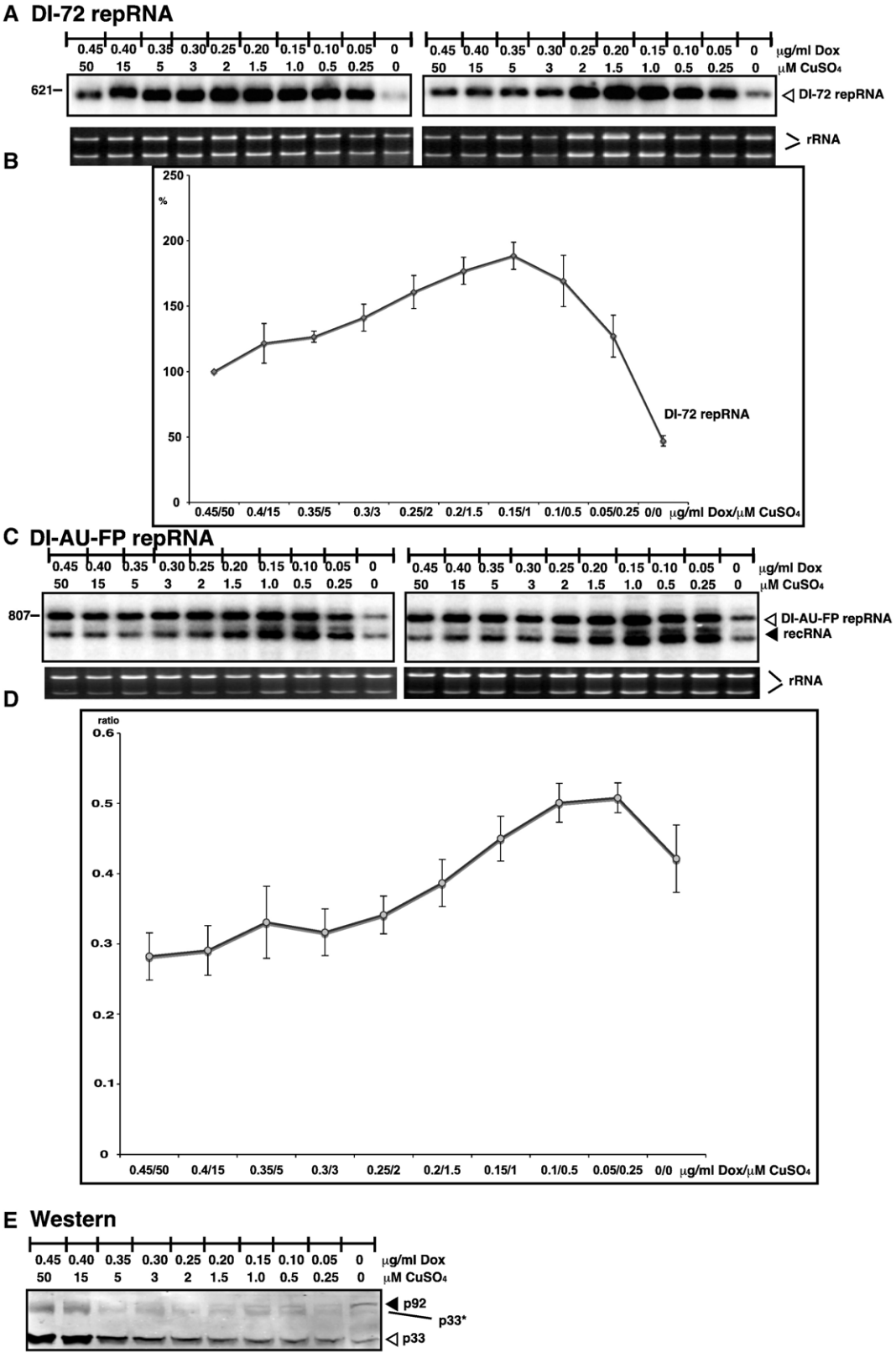


Fig. 4. The effect of simultaneous regulation of p33 and p92^{pol} accumulation on TBSV repRNA accumulation and recombination in yeast. The details for (A, B) RNA blot analysis, (C, D) graphs and the Western blot analysis (E) are the same as described in the legend to Fig. 2.

not detected TBSV replication and recombination prior to inducing the expression of the replication proteins and the repRNA in our system (Fig. 1D). In addition, the individual regulation of p33 and $p92^{pol}$ replication proteins and the repRNA transcript facilitated the studies on the contribution of these viral factors to TBSV replication and recombination.

The amount of p33 is a major determinant of TBSV RNA replication

Regulated expression of p33 replication protein revealed a correlation between p33 level and TBSV replication with both DI-72 and DI-AU-FP repRNAs (Figs. 2A, C). Ten-fold higher level of p33 expression led to ~5–6-fold more robust repRNA replication, suggesting that p33 is present in limiting amount during replication. The stimulatory effect of p33 is direct since replicase preparations containing higher amounts of p33 showed increased RNA synthesis activity in vitro (Fig. 2F). The effect of $p92^{pol}$ level on repRNA accumulation was less dramatic (only 2-fold difference between the highest and the lowest repRNA accumulation levels, Figs. 3A, C) than the effect of p33. The only known function of $p92^{pol}$ is its RdRp function (White and Nagy, 2004), so it is possible that $p92^{pol}$ needs to be present in small amount within the replicase in comparison with p33, which has multiple functions (McCartney et al., 2005; Monkewich et al., 2005; Nagy and Pogany, 2006; Panavas et al., 2005a; Panaviene et al., 2003; Panaviene and Nagy, 2003; Pogany et al., 2005; Shapka et al., 2005; Stork et al., 2005). Accordingly, $p92^{pol}$ is present in 10–20-fold lower amount in the tombusviral replicase than p33 (Panaviene et al., 2004; Scholthof et al., 1995). In addition to the absolute amounts, the ratio of p33 and $p92^{pol}$ also affected replication (Fig. 4). The $p33/p92^{pol}$ ratio, however, had somewhat moderate effect (maximum ~3-fold differences) on TBSV replication when compared with p33 level (~5–6-fold difference). The data suggest that 10:1 $p33/p92^{pol}$ or higher ratio is optimal for repRNA replication (Fig. 4). However, the conclusions regarding the $p33/p92^{pol}$ ratio are hindered by the difficulty to detect $p92^{pol}$ in samples with low $p92^{pol}$ expression. Nevertheless, it seems that the ratio of $p33/p92^{pol}$ can be one of the factors affecting TBSV replication.

Similar to the above results on the dependence of TBSV repRNA accumulation on the p33 auxiliary protein levels, the distantly related BMV RNA replication also showed great dependence on the level of the auxiliary replication protein, termed 1a, whereas the effect of 2a RdRp protein level was less significant (Rao, 2006). Studies on regulated expression of replication proteins of *Carnation Italian ringspot virus* (CIRV), a related tombusvirus, which replicates on mitochondrial membrane surfaces, revealed that the p36 replication protein was needed above "threshold" level for CIRV DI RNA replication in yeast (Pantaleo et al., 2004). On the contrary, DI RNA replication was proportional to p95 RdRp protein level. Comparison of these and the above data on TBSV repRNA replication suggests that TBSV and CIRV RNAs show difference in their dependence on the replication protein levels during RNA replication.

In contrast with the direct effect of p33 and $p92^{pol}$ levels on TBSV replication, the amount of the original repRNA transcripts generated from the plasmid DNA did not affect repRNA accumulation (Fig. 5). The likely reason for this is that robust replication, which is a feature of tombusviruses, might be able to generate plenty of templates from the initial templates that will be present in excess amount in comparison with the limiting amount of viral replicase.

The amount of $p92^{pol}$ is a major determinant of RNA recombination

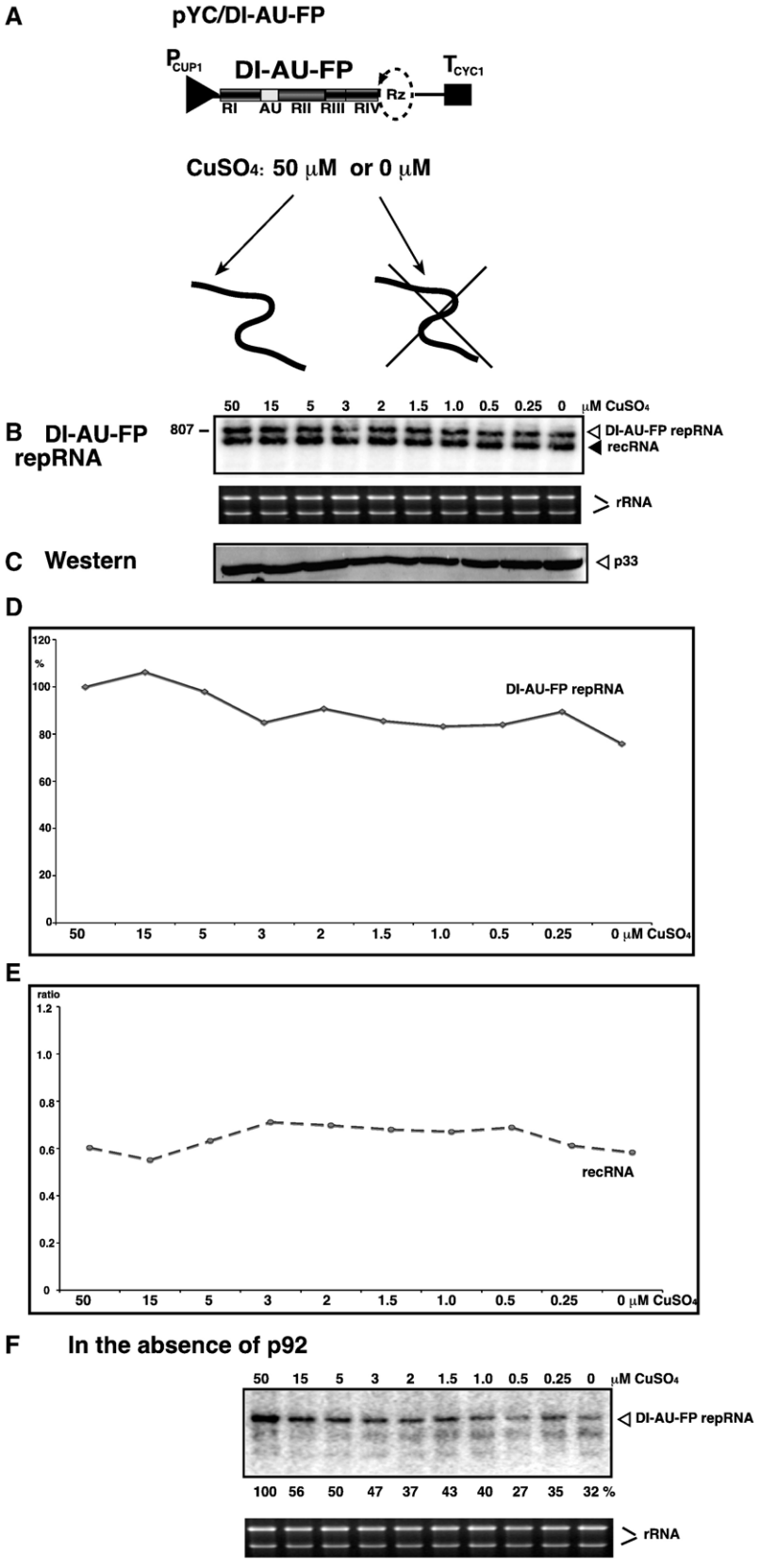
One of the surprising findings in this work is the pronounced effect of $p92^{pol}$ level on TBSV recombination. Yeast expressing the highest level of $p92^{pol}$ supported TBSV recombinants at a ~3- to 4-fold higher level (based on the ratio of recRNA/repRNA) than cells expressing the lowest level of $p92^{pol}$ (Fig. 3D). Interestingly, the ratio of recRNA/repRNA also increased with high level of p33, suggesting that p33 promotes RNA recombination. However, the p33 level or the $p33/p92^{pol}$ ratio had somewhat moderate effects (maximum ~2–3-fold differences) in comparison with the effect of $p92^{pol}$. The initial repRNA transcript level might not be critical for RNA recombination because subsequent robust replication might provide enough templates for additional rounds of replication and recombination. It is likely that, unlike the RNA template, other viral or host factors are available in limited amounts during replication and recombination. Altogether, these observations suggest that host factors, which promote high-level expression of $p92^{pol}$, could also facilitate the accumulation of TBSV recombinants, while changing the p33 level or the $p33/p92^{pol}$ ratio could promote recombination to a lesser extent. Collectively, the data presented in this paper show that p33 level seems to affect mainly replication, whereas $p92^{pol}$ level has a major effect on RNA recombination under the experimental conditions tested.

A model for a role of molecular crowding within the viral replicase in replication and recombination

The detailed analysis of the effect of the amount and ratio of p33 and $p92^{pol}$ in the tombusvirus replicase on replication and recombination revealed that the local concentration (crowding) of the replication proteins in the viral replicase might be a factor in replication/recombination. The replicase complexes obtained from yeast producing more p33 were more active than those containing less p33 (Figs. 2A–C), suggesting that the amount of p33 directly affects replicase activity. This observation supports the role of p33 as an organizer of the viral replicase complex. In addition, we propose that high concentration of p33 in yeast might increase TBSV replication by increasing the efficiency of template RNA recruitment to the site of replication. Indeed, one of the major functions of p33 is to bind specifically to a central stem–loop structure in the plus-stranded repRNA to facilitate template selection/recruitment (Monkewich et al., 2005; Pogany et al., 2005). Alternatively, individual replication complexes with abundant p33 might have higher RNA synthesis

activities than replicases with lower p33 contents. Accordingly, addition of recombinant p33 preparations to in vitro replicase assays increased RNA synthesis (Stork and Nagy, unpublished).

Interestingly, the individual sizes of spherules occurring in tombusvirus-infected cells are somewhat variable, which could be due to their different p33 content. These spherules are the



sites of tombusvirus replication (McCartney et al., 2005). We propose that molecular crowding within the replicase complexes (a separate spherule could be regarded as one functional replicase complex) might be affected by the available amount of p33 in cells.

In addition to p33, the amount of $p92^{pol}$ within the replicase seems to be a major factor in RNA recombination (Fig. 3). We propose that the amount of $p92^{pol}$ (i.e., how crowded $p92^{pol}$ is within the replicase) could likely affect the frequency of replicase-driven template-switching in yeast cells. It is possible that individual $p92^{pol}$ molecules might have a much better chance to collide with one another on the same RNA template when present at high local concentration during RNA synthesis. The collision between $p92^{pol}$ molecules working on the same template then could halt RNA synthesis, which would favor template-switching (Cheng and Nagy, 2003; Cheng et al., 2005; Kim and Kao, 2001; Nagy and Simon, 1997). Our model is further supported by previous observations that recombinant RNA-dependent RNA polymerase (RdRp) preparations for different viruses showed remarkably high recombination frequencies in vitro when compared with purified replicase complexes (which also contain other auxiliary proteins) (Cheng and Nagy, 2003; Cheng et al., 2005; Kim and Kao, 2001; Nagy and Simon, 1997). The high recombination frequency supported by high level of RdRp proteins might be a reason that plus-stranded RNA viruses keep the level of their RdRps low within the viral replicase complexes (10–20:1 ratio of p33 and $p92^{pol}$ in tombusviruses). Based on our "replicase molecular crowding" model, we predict that various hosts can likely affect RNA virus replication and recombination by influencing the production of viral replication proteins, thus affecting the activity of the viral replicase.

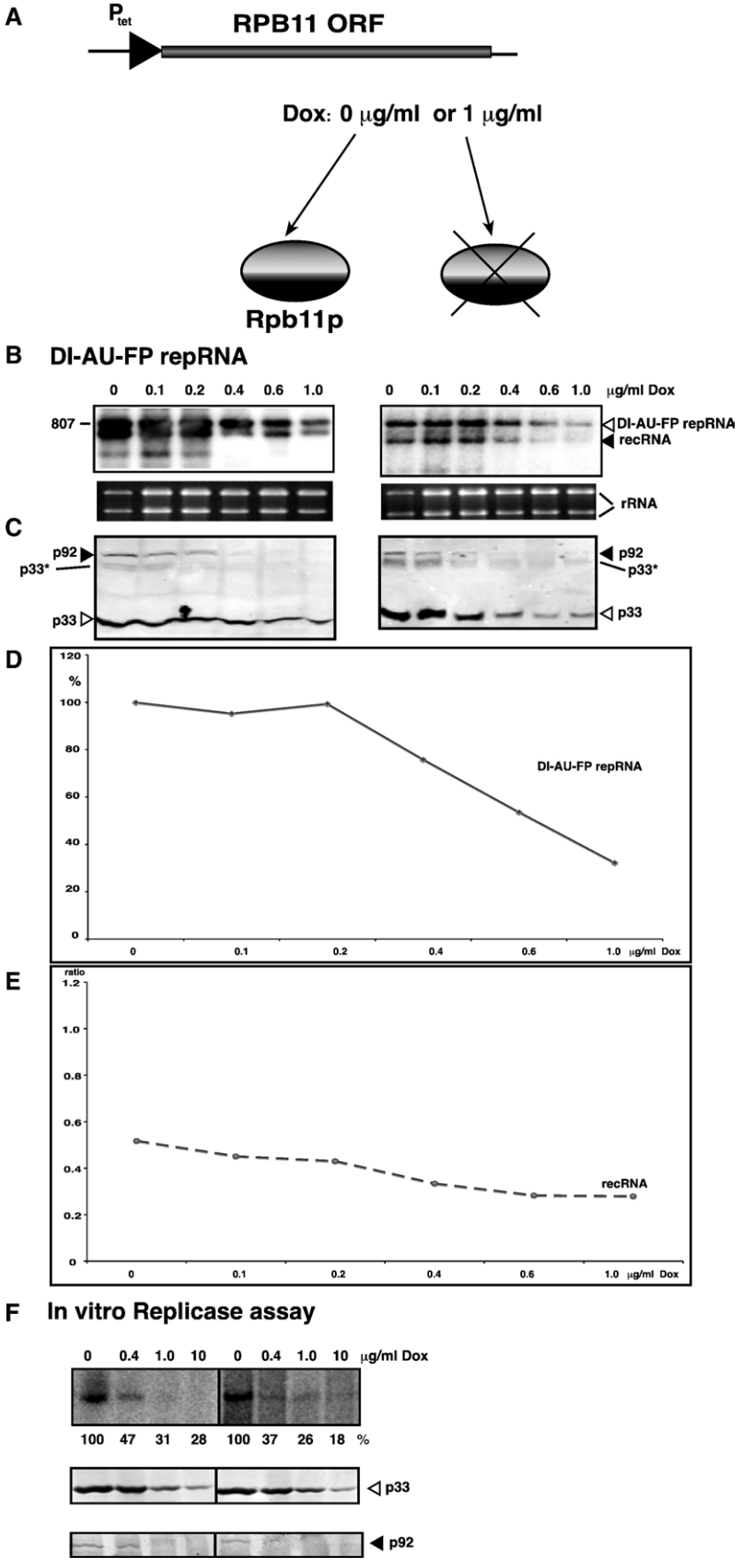
Rpb11p affects TBSV replication and recombination by regulating the accumulation of p33 and $p92^{pol}$ replication proteins

Prior genome-wide screens have led to the identification of a large number of host factors that affected tombusvirus replication/recombination in yeast (Jiang et al., 2006; Panavas et al., 2005b; Serviene et al., 2005, 2006). A major challenge is to determine what are the viral/host targets of these host factors and the mechanisms by which they affect tombusvirus replication/recombination. Various host proteins could affect the accumulation of p33 and $p92^{pol}$ replication proteins by (i) affecting transcription from the launching plasmids; and (ii) affecting the stability of p33 and $p92^{pol}$ in the cells. Among the identified host proteins affecting TBSV replication/recombination, we predicted that Rpb11p might be involved in production of p33 and $p92^{pol}$ due to its role in mRNA synthesis. Rpb11p is part of the pol II complex (Hampsey, 1998), which is likely involved in synthesis of p33/p92 mRNAs from the expression plasmid DNAs. If this is the case, then down-regulation of Rpb11p should reduce the production of p33/$p92^{pol}$ proteins from the decreased amount of p33 and p92 mRNAs synthesized from the expression plasmids. Accordingly, the amounts of both p33 and $p92^{pol}$ decreased in yeast grown in the presence of increasing concentration of doxycycline (which down-regulated Rpb11p levels in our assay). As predicted, TBSV replication and RNA recombination (based on the ratio of recRNA/repRNA) decreased ~2-fold in yeast expressing reduced amount of Rpb11p. Based on our p33 and $p92^{pol}$ data from Figs. 2 and 3, the simplest interpretation of TBSV replication/recombination data obtained with *tet-RPB11* yeast (which allows for controlled Rpb11p expression) is that Rpb11p is only indirectly involved in these processes by controlling the amount of p33/$p92^{pol}$ available in yeast.

Host proteins could affect TBSV replication and recombination without regulating the amounts of p33 and $p92^{pol}$ replication proteins

To see how general is the mechanism that host factors affect replication and recombination via regulating p33/$p92^{pol}$ accumulation levels, we also tested the effect of two additional host proteins. These included Rpt4p endopeptidase/ATPase (involved in the degradation of ubiquitinated substrates) and Mps1p threonine/tyrosine kinase, whose down-regulation also decreased TBSV recombination (Serviene et al., 2006). Down-regulation of either Rpt4p or Mps1p (with 0.1–0.6 μg/ml doxycycline), however, did not reduce the amount of p33 and $p92^{pol}$ in yeast (Figs. 7 and 8), yet it resulted in 2–3-fold reduced recombination (reduced recRNA/repRNA ratio). Also, TBSV replication was not changed in case of Rpt4p (Fig. 7) or increased for Mps1p (Fig. 8) when the expression of these genes was down-regulated. Therefore, the mechanism by which Rpt4p endopeptidase/ATPase and Mps1p threonine/tyrosine kinase affect TBSV recombination must be different from that proposed for Rpb11p. The specific effect of Rpt4p only on TBSV recombination, but not on replication, suggests that this protein could be directly involved in RNA recombination. On the contrary, down-regulation of Mps1p increased RNA replication, whereas it decreased RNA recombination (Fig. 8). These data suggest that Mps1p could affect the activity/precision of the tombusvirus replicase. Further studies will be

Fig. 5. The effect of initial transcript level of TBSV repRNA on repRNA accumulation and recombination in yeast. (A) A scheme for expression of DI-AU-FP repRNA from *CUP1* promoter in yeast. (B) RNA blot analysis of accumulation of the DI-AU-FP repRNA 17 h after induction of transcription by various amounts of $CuSO_4$ as shown. The expression of p33 and $p92^{pol}$ was done from *ADH1* promoters. Ethidium-bromide stained gel shows the loading accuracy. The recRNA emerged in yeast is depicted with a black arrowhead. See further details in Fig. 2. (C) Western blot analysis with anti-His antibody to determine the p33 accumulation in yeast grown under the same conditions as in panel B. (D) Relative accumulation of DI-72 and DI-AU-FP repRNAs under the conditions described in panels A and B. RepRNA accumulation at the highest p33 level was chosen as 100%. Each experiment was repeated at least three times. (E) The ratio of recRNA/repRNA obtained in yeast expressing DI-AU-FP repRNA under the conditions described in panel B. The experiment was repeated twice. (F) Northern blot analysis of the expression level of DI-AU-FP from the *CUP1* promoter in the presence of variable amount of $CuSO_4$ is shown. Yeast did not express $p92^{pol}$, thus the repRNA accumulated only via transcription. The yeast sample with the highest $CuSO_4$ was chosen as 100%.


A
P tet
RPB11 ORF
Dox: 0 μg/ml or 1 μg/ml
Rpb11p
B DI-AU-FP repRNA
0 0.1 0.2 0.4 0.6 1.0
0 0.1 0.2 0.4 0.6 1.0 μg/ml Dox
807
DI-AU-FP repRNA
recRNA
rRNA
C
p92
p33*
p33
p92
p33*
p33
D
120
%
100
80
60
40
20
0
DI-AU-FP repRNA
0 0.1 0.2 0.4 0.6 1.0 μg/ml Dox
E
ratio
1.2
1.0
0.8
0.6
0.4
0.2
0
recRNA
0 0.1 0.2 0.4 0.6 1.0 μg/ml Dox
F In vitro Replicase assay
0 0.4 1.0 10 0 0.4 1.0 10 μg/ml Dox
100 47 31 28 100 37 26 18 %
p33
p92

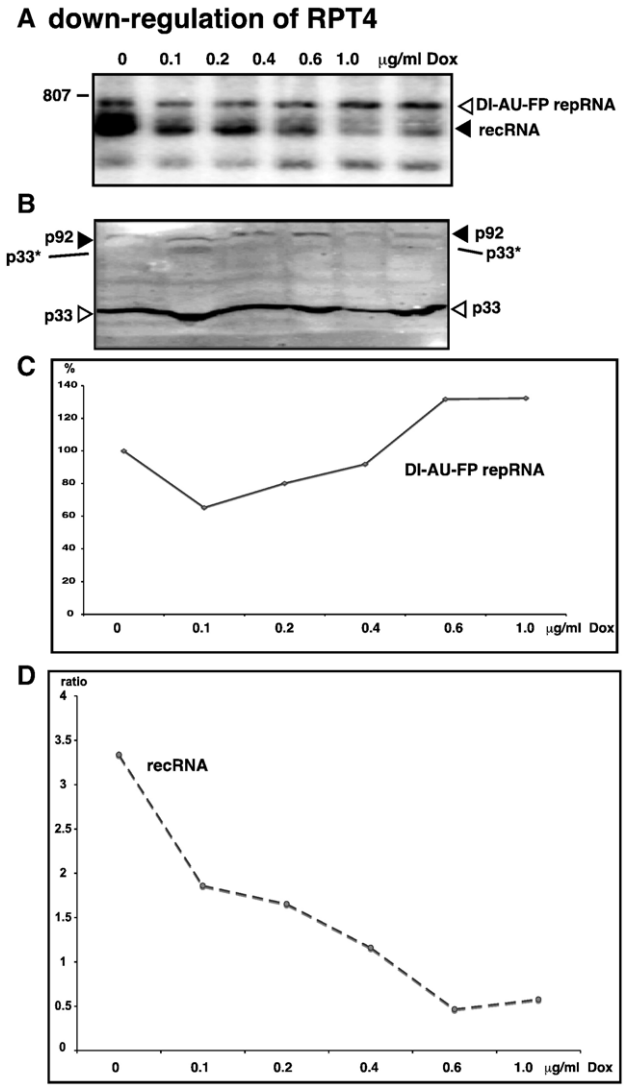


Fig. 7. The effect of down-regulation of Rpt4p on the expression levels of p33 and $p92^{pol}$ and on TBSV repRNA accumulation and recombination in yeast. See further details in Fig. 6.

needed to dissect the detailed roles of Rpt4p and Mps1p in TBSV recombination and replication.

As shown by the above examples with three host proteins, our approach to try to explain the effect of host proteins on TBSV replication/recombination via altering the amounts and/or ratio of p33 and $p92^{pol}$ replication proteins will likely be useful to further characterize the direct or indirect roles of the more than 100 identified host proteins from previous genome-wide screens. This could help narrowing future efforts on host proteins directly involved in TBSV replication and recombination.

Materials and methods

Yeast strains and expression plasmids

S. cerevisiae strain INVSc1 was obtained from Invitrogen. The yTHC yeast strains expressing *RPB11*, *RPT4* and *MPS1* from the doxycycline regulated promoter were obtained from Open Biosystems.

The expression plasmids pGBK-His33 [expressing a 6xHis-tagged CNV p33 under the control of the *ADH1* promoter], pGAD-His92 (expressing a 6xHis-tagged CNV p92 gene p33 under the control of the *ADH1* promoter), pYC/DI-AU-FP and pYC/DI-72 (expressing TBSV DI-72 RNA or the DI-AU-FP RNA respectively, under the control of the *GAL1* promoter) have previously been described (Panavas and Nagy, 2003; Panaviene et al., 2004; Serviene et al., 2006).

To express p92 protein from a tetracycline/doxycycline regulatable promoter, we generated pCM185-His92/TET. First, pCM185 was obtained from EUROSCARF (Frankfurt, Germany). pCM185 contains a *TRP1* marker, a tetracycline inducible repressor (tetR) and seven tetracycline operator boxes ($tetO_7$) upstream of a CYC1 TATA promoter (Gari et al., 1997). Second, pCM185 was digested with *Bam*HI and *Pst*I and ligated with a *Bgl*II and *Pst*I digested PCR product of p92 ORF with 6xHis-tag, amplified with primes #1402 (CGGGCAGATCTTACCATGGGGGGTTC) and #992B (GAGCTGCAGCTATTTCACACCAAG) from pGAD-His 92.

To obtain pGBK-His33/CUP1, the *CUP1* promoter sequence was amplified by PCR with primers #1598 (GCGTGTACAGGATCCCATTACCGACAT) and #1600 (GCGCCATGGAATTCGTTACAGTTTGT) using pSAL1 plasmid (obtained from Piia Leskinen) (Mascorro-Gallardo et al., 1996) as a template. The resulting PCR product and the plasmid pGBK-His33 (Panaviene et al., 2004) were digested with *Nco*I and *Bsp*1407I followed by ligation.

To express DI-AU-FP RNA from the *CUP1* promoter, we constructed pYC/DI-AU-FP/CUP1 via PCR amplification of the *CUP1* promoter sequence with the primers #1779 (CGCGGAATTCGACATTTGGGCGCTATACGTGCATATGT) and #1780 (CGCGCTCGAGTACAGTTTGTTTTTCTTAATATCTATTTCGA). This was followed by digestion with *Xho*I and

Fig. 6. The effect of down-regulation of Rpb11p on the expression levels of p33 and $p92^{pol}$ and on TBSV repRNA accumulation and recombination in THC yeast collection. (A) A schematic representation of the strategy used to down-regulate Rpb11p by doxycycline. Note that expression of *RPB11* takes place from the original chromosomal location in the THC yeast collection. (B) RNA blot analysis of accumulation of the DI-AU-FP repRNA and the recRNAs 17 h after the start of induction of transcription by galactose (the induction was done at the first 6 h). The amount of doxycycline used to down-regulate Rpb11p is shown on top. Both p33 and $p92^{pol}$ were expressed from the *ADH1* promoter. See further details in Fig. 2. (C) Western blot analysis with anti-His antibody to determine the p33 and $p92^{pol}$ accumulation in yeast grown under the same conditions as in panel B. (D) Relative accumulation of DI-AU-FP repRNA under the conditions described in panel B. RepRNA accumulation at the highest Rpb11p level (no doxycycline) was chosen as 100%. Each experiment was repeated at least three times. (E) The ratio of recRNA/repRNA obtained in yeast expressing DI-AU-FP repRNA under the conditions described in panel B. (F) The effect of down-regulation of Rpb11p on the tombusvirus replicase activity in vitro. The membrane-enriched (ME) fraction containing the viral replicase bound to the repRNA template was tested in vitro as described in the legend to Fig. 2F. Each experiment was done in three times.

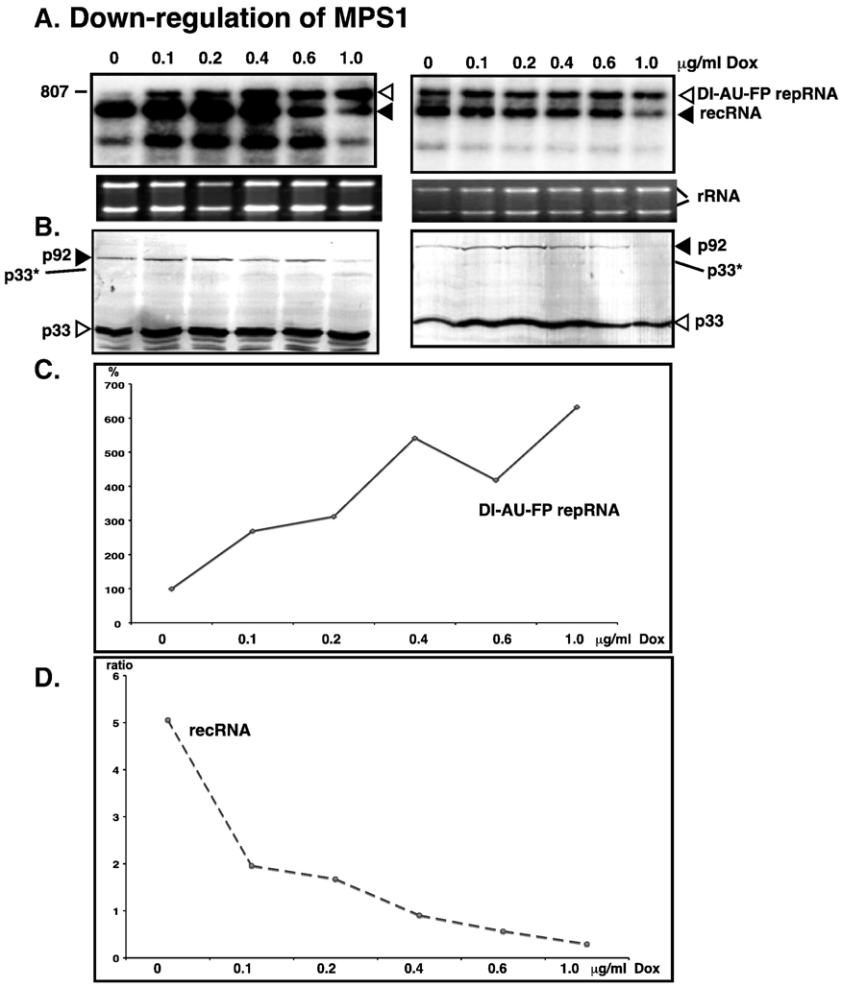


Fig. 8. The effect of down-regulation of Mps1p on the expression levels of p33 and $p92^{pol}$ and on TBSV repRNA accumulation and recombination in yeast. See further details in Fig. 6.

*Eco*RI and cloning into the *Xho*I/*Eco*RI site of the pYC/DI-AU-FP (Serviene et al., 2006).

Yeast transformation

Yeast was co-transformed with three plasmids by using the lithium acetate/ssDNA/polyethylene glycol method (Gietz and Woods, 2002), and transformants were selected by complementation of auxotrophic markers. The yTHC strains were co-transformed with pGAD-His92 and pGBK-His33/DI-AU-FP, expressing DI-AU-FP RNA under the control of the *GAL1* promoter and p33 under control of *ADH1* promoter (Serviene et al., 2006).

Yeast cultivation

In most experiments, yeast was pre-grown at 29 °C in SC-HUT^- with 2% glucose, 10 μg/ml doxycycline and without added $CuSO_4$ until reaching cell density of 1.0 (OD_{600}). Then yeast was diluted to 0.1 (OD_{600}) in SC-HUT^- medium containing 2% galactose and given concentration of $CuSO_4$ and doxycycline as indicated in the legends to figures. After 6 h, the yeast growth media were changed to SC-HUT^- with 2% glucose and given concentration of $CuSO_4$ and doxycycline, followed by additional culturing of yeast cells for 11 h at 29 °C.

For studying the effect of repRNA level on TBSV replication/recombination (Fig. 5), yeast was pre-grown at 29 °C in SC-UHL^- without $CuSO_4$ until reaching cell density of 1.0 (OD_{600}). Then yeast was diluted to 0.1 (OD_{600}) in SC-UHL^- medium with given concentration of $CuSO_4$ and cultured for 17 h.

For studying TBSV replication/recombination in the yTHC yeast strains (Figs. 6–8), yeast was pre-grown at 29 °C in SC-UHL^- with 2% glucose until reaching cell density of 1.0 (OD_{600}). Then yeast was diluted to 0.1 (OD_{600}) in SC-UHL^- medium containing 2% galactose and cultured for 6 h followed by changing the medium to SC-UHL^- with 2% glucose and additional culturing for 11 h.

RNA analysis

Total RNA isolation and Northern blot analysis were performed as described previously. The ^{32}P-labeled bands were imaged with Typhoon (GE Healthcare) and analyzed by the ImageQuant program.

Protein analysis

Total protein extraction and Western blot were performed as described previously (Panaviene et al., 2004, 2005). The primary antibody was anti-His6 (GE Healthcare), and the secondary antibody was HPR-linked anti-mouse IgG (BioRad). Immunoreactive bands were detected by ECL technique (GE) and imaged with Typhoon (GE Healthcare) and analyzed by the ImageQuant program.

CNV replicase assay

We obtained "membrane-enriched" (ME) CNV replicase preparations, which are suitable to test the replicase activity on the endogenous templates present within the CNV replicase preparation (Panaviene et al., 2004, 2005). Frozen yeast cells were resuspended in 150 μl extraction buffer (200 mM sorbitol, 50 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 10 mM KCl, 10 mM β-mercaptoethanol, yeast protease inhibitor mix, Sigma)/well in a 96 deep-well plate followed by addition of 250 μl volume of glass beads into each well of a 1 ml deep well plate. Then, the yeast cells were broken with a Genogrinder (2 min at 1500 rpm). We then added 600 μl pre-chilled extraction buffer/well. The samples were centrifuged at 100×*g* for 1.5 min at 4 °C. The supernatant was transferred to microcentrifuge tube followed by centrifugation at 21,000×*g* for 10 min at 4 °C. The pellet was resuspended in 700 μl extraction buffer. Because no template was added to the in vitro reaction, the replicase preparation could only use the co-purified RNA template. The ME replicase assay was performed for 120 min at 25 °C (Panaviene et al., 2004, 2005). The ^{32}P-labeled replicase products made on the endogenous template present within the ME fraction were phenol/chloroform extracted, precipitated with isopropanol/ammonium acetate and analyzed under denaturing conditions (i.e., 5% PAGE containing 8 M urea) (Nagy and Pogany, 2000). The amounts of 6xHis-p33 and 6xHis-p92 in the ME fractions were detected with monoclonal anti-His (GE Healthcare) and secondary alkaline phosphatase-conjugated anti-mouse antibody (Sigma) as described (Pogany et al., 2005). Western blots were developed using BCIP and NBT (Sigma).

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