Mutations in the RNA-binding domains of tombusvirus replicase proteins affect RNA recombination in vivo

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Abstract

RNA recombination, which is thought to occur due to replicase errors during viral replication, is one of the major driving forces of virus evolution. In this article, we show evidence that the replicase proteins of Cucumber necrosis virus, a tombusvirus, are directly involved in RNA recombination in vivo. Mutations within the RNA-binding domains of the replicase proteins affected the frequency of recombination observed with a prototypical defective-interfering (DI) RNA, a model template for recombination studies. Five of the 17 replicase mutants tested showed delay in the formation of recombinants when compared to the wild-type helper virus. Interestingly, two replicase mutants accelerated recombinant formation and, in addition, these mutants also increased the level of subgenomic RNA synthesis (Virology 308 (2003), 191–205). A trans-complementation system was used to demonstrate that mutation in the p33 replicase protein resulted in altered recombination rate. Isolated recombinants were mostly imprecise (nonhomologous), with the recombination sites clustered around a replication enhancer region and a putative cis-acting element, respectively. These RNA elements might facilitate the proposed template switching events by the tombusvirus replicase. Together with data in the article cited above, results presented here firmly establish that the conserved RNA-binding motif of the replicase proteins is involved in RNA replication, subgenomic RNA synthesis, and RNA recombination.

Keywords: Replication; Recombination; RNA replication enhancer; Template switching; Subgenomic RNA; RNA binding; Replicase; Tomato bushy stunt virus; Cucumber necrosis virus; Defective-interfering RNA

Introduction

Errors have been proposed to occur during viral RNA replication often leading to RNA recombination, which is a process that joins RNA sequences that were either present on separate molecules or noncontiguous when present on the same molecule (Jarvis and Kirkegaard, 1991; Lai, 1992; Nagy and Simon, 1997). Depending on the sequences brought into proximity by RNA recombination, the changes in the viral genomes can be dramatic or minor. Due to the formation of viral genomes with novel traits, RNA recombination has been proposed to be a major driving force in viral evolution (Lai, 1992; Strauss and Strauss, 1988; Gibbs, 1997; Worobey and Holmes, 1999; Aranda et al., 1997; Fernandez-Cuartero et al., 1994). Accordingly, RNA recombination has been documented for a growing number of bacterial (Palasingam and Shaklee, 1992; Mindich et al., 1992), fungal (Shapira et al., 1991), plant (Allison et al., 1990; Ayllon et al., 1999; Borja et al., 1999; Cascone et al., 1993; Nagy and Bujarski, 1993; Rao and Hall, 1993; White and Morris, 1999), animal, and human viruses (Kirkegaard and Baltimore, 1986; Banner et al., 1990; Becher et al., 1999; Furuya et al., 1993; Hajjou et al., 1996; Keck et al., 1988; Khatchikian et al., 1989; King et al., 1982; Li and Ball, 1993; Molenkamp et al., 2001; Suzuki et al., 1998; Vezza et al., 1980; Walter et al., 2001; Weiss and Schlesinger, 1991; Worobey et al., 1999; Wu et al., 1999).

The most popular model of RNA recombination is that the viral replicase (due to yet incompletely understood reasons) makes significant errors during the replication pro-
cess, which could lead to template switching during complementary RNA synthesis (Jarvis and Kirkegaard, 1991; Lai, 1992; Nagy and Simon, 1997). After the switch from the donor to the acceptor RNA, the viral replicase can resume RNA synthesis using the nascent RNA (made on the donor template) as a primer on the acceptor RNA. This model assumes intricate relationship between viral replication and recombination processes in infected cells.

To understand the relationship between replication and recombination, we are using tombusviruses, which are single-component plus-stranded RNA viruses of plants. Two closely related tombusviruses, namely Tomato bushy stunt virus (TBSV) and Cucumber necrosis virus (CNV), are known to support RNA recombination at high frequencies (Borja et al., 1999; White and Morris, 1994a,b; 1995; Rochon, 1991). In addition to the genomic RNAs of tombusviruses, defective-interfering (DI) RNAs associated with these viruses are also frequently used templates for RNA recombination. The major advantages of using DI RNAs for both replication and recombination studies are that (i) DI RNAs are involved in RNA recombination with high frequencies (White and Morris, 1999); (ii) they do not contribute essential protein factors to replication, thus have higher genetic plasticity than the viral genomic RNA; and (iii) they use the same replication/recombination machinery as the genomic RNAs. The tombusvirus DI RNAs are mosaic types that are derived from the genomic RNA via two or three large sequence deletions (White and Morris, 1999; Fig. 1A). The sequence deletions during the DI RNA formation are thought to be the consequence of viral replicase jumping on the template and the deletions may occur in a stepwise manner (White and Morris, 1999).

The TBSV and CNV replicases contain two essential viral proteins (i.e., p33 and p92) and possibly host factors (Scholthof et al., 1995; Oster et al., 1998; Nagy and Pogany, 2000). The p92 protein contains the signature motifs of RNA-dependent RNA polymerases (RdRp), while the function of p33 is currently not known. Both proteins bind to single-stranded RNA, including the plus-stranded DI RNA (Rajendran and Nagy, 2003). Due to the overlapping expression strategy for these proteins, p33 and p92 contain the same RNA-binding region that includes an arginine/proline-rich sequence (termed the RPR motif; Rajendran and Nagy, 2003; Panaviene et al., 2003). Many mutations within the RPR motif of p33 and p92 were detrimental to virus and DI RNA replication. Several mutations in the RPR motif affected the function of p33 differently than that of p92, suggesting that the RPR motif plays somewhat different roles in these proteins (Panaviene et al., 2003). Interestingly, few mutations in the RPR motif of p33 could also affect the level of subgenomic RNA synthesis (Panaviene et al., 2003). These observations demonstrated that p33 and p92 and their RPR motifs play major roles in tombusvirus RNA replication. Therefore, due to the proposed interrelationship between replication and recombination, many of the mutations might also affect RNA recombination as well.

This assumption was tested in this work using a single-cell (protoplasts) system, which supports efficient recombination of DI RNAs when the wild-type (wt) p33 and p92 proteins are present. We find that mutations in the RPR motif in p33/p92 proteins resulted in either “fast,” wt-like, or “slow” recombinant formation. The altered recombination efficiency in these RPR motif mutants was mapped to the p33 protein. We also find that wt or selected replicase mutants with altered recombination frequencies supported the relative accumulation of new recombinants versus the parental DI RNAs to similar extent; suggesting that postrecombinational selection is unlikely the reason for the observed differences among the mutants and the wt virus. Overall, we provide evidence that the replicase proteins of a tombusvirus, particularly the RNA-binding region, are involved in RNA recombination.

**Results**

**Rationale**

To study the role of replicase proteins in RNA recombination in tombusviruses, first we developed an efficient recombination system based on a prototypical DI RNA (i.e., DI-72 that is derived from TBSV via three deletions, Fig. 1A). Since we wanted to test the effect of the RNA-binding domains of the replicase proteins on recombination, we chose DI RNA sequences, namely, region II (RII; Shapka and Nagy, unpublished data) and region III (RIII; Panavas and Nagy, unpublished data), which, as minus-stranded sequences, are known to bind to the replicase proteins with high affinity in vitro. While the function of RII(−) is currently unknown, RIII(−) has recently been defined as a strong replication enhancer (Ray and White, 1999, 2003; Panavas and Nagy, 2003). It has also been demonstrated recently that RIII(−) promotes template switching by the CNV RdRp preparation in vitro (Cheng and Nagy, unpublished data). Interestingly, RIII(−) served as both donor and acceptor sites during the recombination events in vitro. Therefore, we reasoned that due to their binding to the viral replicase proteins, RIII(−) and possibly RII(−) sequences might constitute recombination hot spots in vivo as well. This hypothesis was tested in protoplasts below.

**Replication and recombination of TBSV DI RNAs carrying duplicated RII and RIII sequences**

To test the effect of RII and RIII sequences on the ability of DI RNA to replicate and to recombine, we made four constructs, as shown in Fig. 1A. Namely, DI-33 and DI-333 carried two and three copies of RII sequences, while constructs DI-2233 contained two copies of RII and RIII sequences. The fourth construct, DI-2323 carried a tandem repeat of RII and RIII (Fig. 1A).

Each of the gel-isolated TBSV DI RNA transcripts ob-
Fig. 1. Replication of DI RNAs carrying duplicated copies of known and putative cis-acting elements in protoplasts. (A) Schematic representation of TBSV genomic and DI RNAs used in these experiments. The genomic RNA is shown at the top with five genes (indicated with white and black boxes), which include the p33 and p92 replicate proteins (they overlap in sequence within the p33 gene). Note that CNV has similar replicate genes to those of TBSV. The prototypical DI RNA, DI-72, contains four conserved segments derived from the TBSV genomic RNA, termed RI to RIV, shown as gray boxes. The shown DI RNA constructs carry one or two copies of the 82-nt RII, a known replication enhancer. In addition, DI-333 and DI-2233 also contain two copies of the 239-nt RII sequence (as shown, framed boxes represent inserted copies of RII and/or RIII), which is a putative cis-acting replication element. Note that TBSV DI RNAs can only replicate in the presence of a helper virus, such as TBSV or the closely related CNV (not shown). (B) Accumulation of (+)-strands of DI-72 RNA and its derivatives in N. benthamiana protoplasts in the presence CNV helper RNA. DI RNAs (1 μg) were coc-electroporated with CNV RNA (2 μg) into protoplast cells. Then the total RNA from the first passage (after incubation for 48 h) was used for electroporation into yet another batch of protoplasts (termed second passage). Total RNA from the second samples contained at least two different-sized recombinant-like RNAs (ΔRIIΔRIII), Figs. 1B–C). These experiments detected only the parental (input)-sized (+) and (−) DI RNAs even after 48 h of incubation (termed zero passage), suggesting that (i) these DI RNAs can replicate in protoplasts, and (ii) they do not support recombinant formation/accumulation at a detectable extent (Fig. 1B). Interestingly, none of the DI RNAs with repeated RII or RIII sequences replicated as efficiently as the wt DI RNA (lowest and the highest accumulation was ~25 and 60% of wt for DI-333 and DI-33, respectively, Fig. 1B). Also, the amounts of plus-strands were decreased significantly more than the amounts of minus-strands for all four constructs tested (Figs. 1B–C, especially at the 24-h time point).

It is possible that the lack of recombinant accumulation with these mutants in the zero passage protoplasts is due to the limited time available for replication of the putative recombinants after their formation during protoplast incubation (viability of the protoplast cells sharply declined after 48 h under the test conditions, not shown). Therefore, to increase the chance for the formation/accumulation of recombinants, we made a passage with the total RNA obtained after 48 h of incubation in protoplasts via electroporation into a new batch of protoplasts (termed first passage). Then the total RNA from the first passage (after incubation for 48 h) was used for electroporation into yet another batch of protoplasts (termed second passage) and so on (Fig. 2). Northern blot analysis of the total RNA extracts obtained from the various protoplast samples after the first passage revealed the occurrence of novel, ~100–250 bp shorter than input, recombinant-like RNAs for constructs DI-33, DI-333, and DI-2233 in addition to the parental-sized DI RNAs (first passage, Fig. 2A). Interestingly, many of the samples contained at least two different-sized recombinant-like DI RNAs, suggesting that more than one recombination event took place in cells. Total RNA from the second passage (viability of the protoplast cells sharply declined after 48 h) was used for electroporation into yet another batch of protoplasts (termed second passage and so on (Fig. 2). Northern blot analysis of the total RNA extracts obtained from the various protoplast samples after the first passage revealed the occurrence of novel, ~100–250 bp shorter than input, recombinant-like RNAs for constructs DI-33, DI-333, and DI-2233 in addition to the parental-sized DI RNAs (first passage, Fig. 2A). Interestingly, many of the samples contained at least two different-sized recombinant-like DI RNAs, suggesting that more than one recombination event took place in cells. Total RNA from the second
passage showed a pattern similar to that from the first passage, except the input-sized DI RNAs became hardly detectable (Fig. 2A). Overall, recombinant-like DI RNAs were observed in 100% of the protoplast experiments (first and second passages) with constructs DI-33, DI-333, and DI-2233, based on Northern blot (Fig. 2A) and RT-PCR (Fig. 2B) analyses.

In contrast to the above three constructs, DI-2323 supported recombinant formation/accumulation less efficiently. The first recombinants were observed (at hardly detectable level) in 50% of the samples after the second passage (Fig. 2A). Half of the samples still did not contain recombinants after the third passage, suggesting that this construct could support recombinant formation/accumulation relatively inefficiently.

The observed novel DI RNAs in protoplasts appear to be true recombinants, since they can be detected in the total RNA extracts using either ethidium bromide stained gels (not shown), Northern blotting (Fig. 2A), or RT-PCR analysis (Fig. 2B). Control RT-PCR reactions performed on gel-isolated DI RNAs (the same preparations that were used for electroporation to protoplasts) did not detect recombinant-sized DI RNAs for DI-33 and DI-333 constructs (Fig. 2B). In contrast, recombinant-sized RT-PCR products were observed in the control RT-PCR reactions in the case of DI-2233 and DI-2323, because of the presence of the 239-nt RII repeat in these constructs. These were precise recombinants between the duplicated RII sequences (leading to deletion of one of the duplicated copies, not shown), and we did not count them in our analysis (Fig. 3). Nevertheless, DI-2233 and DI-2323 generated unique-sized recombinants that were not present in the control RT-PCR reactions. These recombinants were imprecise and were included in our analysis (see below).

Cloning and sequencing of the above putative recombinants demonstrated that (i) they were indeed recombinants, derived from the input DI RNAs via single deletions; and (ii) the recombination junction sites were varied in the recombinants (imprecise recombination) (Fig. 3). The fact that many of the recombinants obtained with DI-33 and DI-333 had unique and imprecise junction sites (Figs. 3A–B) shows the genome plasticity of the DI RNA. The occurrence of precise recombinants obtained with DI-33 and the lack of these recombinants for DI-333 indicate that
the same sequences might support different recombinants depending on the neighboring sequences. Many recombinants obtained with constructs DI-2323 and DI-2233 had the junction sites within the first copy of RII and the second copy of RIII, albeit other recombinants still carried portions of duplicated sequences (Figs. 3C–D). Note that we did not study precise recombination with constructs DI-2323 and DI-2233 due to the artifactual products in the control RT-PCR reactions (see above and Fig. 2B). Overall, we found that the majority of the recombinants lacked most of the inserted (duplicated) sequences, and they frequently contained further deletions in RII and RIII. The observed variability among the obtained DI RNA recombinants (Fig. 3) in junction site pattern is similar to previous findings with de novo generated recombinants in tombusvirus populations, which also contained unique deletion sites (White and Morris, 1999).

Testing DI RNA replication and recombination supported by CNV with mutations in the RPR motif of the replicase proteins

We have previously tested 19 RPR motif mutants that contained either deletions, insertions of extra arginines, or
substitutions (arginine/proline to alanine or lysine) in protoplasts (Panaviene et al., 2003). Among these mutants, 14 supported gCNV and DI-72 accumulation at detectable levels (Panaviene et al., 2003). To increase the number of viable mutants with possibly altered properties in replication/recombination, we generated a new set of RPR motif mutants (total of nine) that had arginine to histidine, lysine, glutamine, or alanine changes (single and double mutants, R637-K, R637-Q, R637-A/H643-H, R637-A/R643-H, R637-A/H646-H, R637-A/R649-H, Table 1). Additional mutants carried proline to glycine or tyrosine mutations (constructs P640-Y, P652-Y, P640-652-G, Table 1). Testing the ability of these mutants to support DI-333 RNA replication in protoplasts revealed that seven of them were 75–95% as efficient as the wt CNV (Fig. 4 and Table 1). Two double mutants, namely R637-A/H643-H and R637-A/H646-H, which carried arginine-to-histidine mutations, were able to support DI RNA replication only inefficiently (1–2% of wt level, Table 1).

To test the ability of the RPR motif mutants to support recombination in protoplast, we used a total of 17 mutants and the wt virus in a recombination assay with DI-333. When tested after the first passage, 10 of the RPR motif mutants supported the formation/accumulation of recombinant DI RNAs with comparable efficiency to the wt CNV (Fig. 5A). Five mutants, P640-A, P640-Y, P652-A, P652-Y, and P640-A/P652-A, however, supported the formation/accumulation of recombinant DI RNAs inefficiently (~15–40% of that of wt, Fig. 5B). We found that the low recombination efficiency for the above proline mutants was repeatable in three separate experiments (not shown). In those samples, which accumulated recombinant DI RNAs, the relative level of DI RNA accumulation (compared to the level of the parental DI-333 RNA) was low (~5%). These observations suggest that the recombinant DI RNAs are formed inefficiently or their formation is greatly delayed in the presence of the helper virus carrying the above proline mutations in the RPR motif ("slow mutants").

On the contrary, two RPR motif mutants (R646-A and R643+R) accumulated recombinant DI RNAs apparently faster than the wt helper did when tested with DI-333 RNA (not shown). To further test this observation, we used DI-2323, which can support recombination less efficiently than DI-333 in the presence of the wt helper (Fig. 2A). We found that in the presence of mutants R646-A or R643+R, DI-2323 RNA supported recombinant DI RNA accumulation in 82–100% of samples, while the corresponding value is ~18% in the presence of the wt helper (Fig. 5C) after the second passage. The relative amount of the recombinant DI RNAs (in comparison with the parental DI-2323 RNA) was also higher in samples obtained with R646-A and R643+R ("fast mutants") than with the wt. As an additional control, we also used mutant R643-A (which supported wt level of recombination with DI-333, Fig. 5A) in combination with DI-2323. Importantly, we found that R643-A supported low (~17%), wt level of recombination with DI-2323 after the second passage (Fig. 5C). These observations suggest that the recombinant DI RNAs might be formed more efficiently and/or earlier during infections in the presence of the helper

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**Table 1**
The level of accumulation of DI RNAs in the presence of gCNV carrying mutations in the RPR motif

<table>
<thead>
<tr>
<th>Mutants</th>
<th>RPR motif</th>
<th>DI-RNA replication (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>WT</td>
<td>RPRRRP</td>
<td>100</td>
</tr>
<tr>
<td>R637-K</td>
<td>KPRRRP</td>
<td>73 ± 8</td>
</tr>
<tr>
<td>R637-H</td>
<td>HPRRRP</td>
<td>65 ± 7</td>
</tr>
<tr>
<td>R637-Q</td>
<td>QPRRRP</td>
<td>82 ± 12</td>
</tr>
<tr>
<td>P640-Y</td>
<td>RYRPRR</td>
<td>73 ± 7</td>
</tr>
<tr>
<td>P652-Y</td>
<td>PRPRRY</td>
<td>68 ± 5</td>
</tr>
<tr>
<td>P640-652-G</td>
<td>RGRRRG</td>
<td>72 ± 5</td>
</tr>
<tr>
<td>R637-A</td>
<td>P643-H</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>R637-A/P646-H</td>
<td>APFRRP</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>R637-A/P649-H</td>
<td>APFRRP</td>
<td>45 ± 7</td>
</tr>
<tr>
<td>P640-A</td>
<td>RARRRP</td>
<td>35 ± 7</td>
</tr>
<tr>
<td>P652-A</td>
<td>RPRERRA</td>
<td>62 ± 5</td>
</tr>
<tr>
<td>P640-652-A</td>
<td>RARRRA</td>
<td>49 ± 6</td>
</tr>
<tr>
<td>R637-A</td>
<td>APRRNP</td>
<td>42 ± 8</td>
</tr>
<tr>
<td>R643-A</td>
<td>RPARRP</td>
<td>32 ± 7</td>
</tr>
<tr>
<td>R643-K</td>
<td>RPRRPR</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>R646-A</td>
<td>RPRRKP</td>
<td>2 ± 5</td>
</tr>
<tr>
<td>R646-K</td>
<td>RPRRKP</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>R649-A</td>
<td>RPRRPP</td>
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</tr>
<tr>
<td>R649-K</td>
<td>RPRRRP</td>
<td>43 ± 7</td>
</tr>
<tr>
<td>R643+R</td>
<td>RPRRRP</td>
<td>2 ± 4</td>
</tr>
</tbody>
</table>

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*The top includes those RPR mutants that were analyzed in Fig. 4, while mutants shown at the bottom part of the table are from Panaviene et al. (2003).

*Abbreviations of amino acids: A, alanine; G, glycine; H, histidine; P, proline; Q, glutamine; R, arginine; K, lysine; Y, tyrosine. Boldfaced letters represent mutated amino acids.

The level of DI-333 RNA in the presence of wt gCNV was chosen as 100%. The experiment was repeated three times.

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![Fig. 4](image-url)
virus carrying the $R_{646}^{-}A$ and $R_{643}^{+}R$ mutations than in the presence of wt or $R_{643}^{-}A$.

The observed differences among the wt gCNV and several mutants in supporting the occurrence of novel recombinant DI RNAs might be due to (i) differences in the frequency of recombinant generation. In other words, it is possible that the mutated replicases might have reduced or increased capacity to generate recombinants (i.e., slow or fast mutants, Figs. 5B and C). (ii) It is also possible that there are differences in replication levels of the new recombinants and the parental DI RNAs (i.e., the difference is at the level of postrecombinational amplification). To test if those RPR motif mutations, which generated recombinants slower than wt (e.g., $P_{640}^{-}A$, $P_{640}^{-}Y$, $P_{652}^{-}A$, $P_{652}^{-}Y$, and $P_{640}^{-}A/P_{652}^{-}A$), might show reduced relative replication levels for the recombinant DI RNAs (compared to the parental DI RNAs), we performed template competition experiments between the parental DI-333 RNA and two different recombinant DI RNAs (derived from experiments shown in Figs. 2–3) in the presence of wt gCNV or the above mutants. One recombinant, namely no. 13, had the larger deletion that extended 51 nt into RII in DI-333 (Fig. 3B), while recombinant no. 6 had only 6 nt deleted from RII and contained a 5 nt stretch of the marker sequence (Fig. 3B). We found that after 48 h of incubation, both recombinant DI RNAs replicated 15- to 22-fold better than the parental DI-333 RNA in the template competition experiments. More importantly, these recombinant DI RNAs replicated at the same level in the presence of wt or mutant gCNV background (Figs. 6A–B). This data argue that, after their formation, the recombinant DI RNAs should be replicated by either the wt or the mutant gCNV to levels higher than that of the parental DI-333. Since we detected the parental DI RNAs readily,

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**Table 5.** Effect of RPR motif mutations on recombination frequency of DI RNAs. (A–B). Northern blot analysis of total RNA extracts (first passage) obtained from protoplasts that contained DI-333 and either wt gCNV or one of the RPR motif mutants. Protoplasts (zero passage) were coelectroporated with 1 $\mu$g of gCNV (carrying wt or mutated RPR motif) and 0.15 $\mu$g of gel-purified DI-333 transcript. For “passaging” the viral RNA, protoplasts were coelectroporated with the same amounts (5 $\mu$g each) of total RNA extracts obtained from zero passage protoplast 24 h after electroporation. The frequency of recombinant DI RNA accumulation (number of recombinants/total number of first passage protoplast samples) is shown on the bottom. Further details are as shown in the legend to Fig. 1. (C) RPR motif mutants that increased recombination frequency in the initial tests (performed with DI-333, not shown) were tested in the presence of DI-2323 that recombines inefficiently (Fig. 2). Protoplasts (zero passage) were coelectroporated with 0.4 $\mu$g of wt gCNV and 1 $\mu$g of gel-purified in vitro transcript of DI-2323 RNA or 5 $\mu$g of the given RPR motif mutant RNA and 2 $\mu$g of DI-2323 RNA, respectively. The higher amounts of RNA were used for the mutants because of the low replication level of DI RNAs when these mutants were used as helpers (Table 1, and Panaviene et al., 2003). For passaging the viral RNA, protoplasts were coelectroporated with the same amounts (5 $\mu$g each) of total RNA extracts obtained from zero passage protoplast 48 h after electroporation, plus additional 0.4 $\mu$g wt or 5 $\mu$g of the given RPR motif mutant gCNV transcripts. Total RNA extract after the second passage was analyzed by Northern blot as described in (A). The bottom panel includes an additional control, mutant $R_{643}^{-}A$, which supports level recombination with both DI-2323 and DI-333 (see B). Note that the amount and conditions for $R_{643}^{-}A$ was the same as for $R_{646}^{-}A$.
but not the recombinants (Fig. 5B), in the presence of slow mutants (i.e., P_{640}−A, P_{640}−Y, P_{652}−A, P_{652}−Y, and P_{640}−A/P_{652}−A), it is unlikely that the differences between the wt and the slow mutants are due to differences in supporting the accumulation of recombinant DI RNAs over the parental DI RNA. Instead, it is more likely that the differences are due to the altered efficiency of generation of recombinant DI RNAs by the wt and the selected mutants (see Discussion).

We also tested the relative accumulation levels of recombinant DI RNAs in a competition assay with DI-2323 parental RNA for the fast mutants and the wt (Fig. 6C). Similar to the above observations, the two fast mutants tested (R_{646}−A and R_{646}+R, Fig. 6C) as well as wt gCNV supported the recombinant DI RNAs at comparable relative levels in the competition assay after 48 h of incubation (Fig. 6C). Based on this data, we conclude that the fast mutants do not support the recombinant DI RNAs over the parental DI RNA better than the wt gCNV. This observation further supports the finding that the observed differences in frequency of recombinant DI RNA occurrence are unlikely due to differences between the wt and the mutants in post-recombinational selection of recombinant DI RNAs over the parental DI RNAs (see Discussion).

To test if the mutations in the replicase genes of the tested mutants were stably maintained during infection, we RT-PCR amplified, cloned, and sequenced the replicase genes from total RNA extracts derived from either the first (for constructs P_{640}−A, P_{652}−A, R_{637}−A, R_{649}−A, and R_{649}−K) or the second passage (R_{643}+R, R_{646}−A, R_{643}−A, and R_{643}−K) as described earlier (Panaviene et al., 2003). We found that the site-specific mutations were stably maintained in the replicate genes of all these mutants (not shown). This indicates that the mutated replicase proteins were present in protoplasts at the end of the experiments.

**Mutations within the RPR motif of p33 affect RNA recombination**

Due to the overlapping expression strategy of p33/p92 genes in tombusviruses, the RPR motif is present in both replicase proteins. Therefore, it is possible that the observed differences among the RPR motif mutants in supporting formation of DI RNAs were influenced by mutations present in both replicase proteins. To test the involvement of these proteins in RNA recombination separately, we have developed a three-component, complementation-based system based on expression of the p33 protein from a DI RNA (DI-p33, Fig. 7A; Oster et al., 1998; Panaviene et al., 2003) and the p92 from the mutated gCNV RNA that carries a tyrosine mutation eliminating the p33 stop codon (construct gCNV-Y, Fig. 7A; Panaviene et al., 2003). The third component was DI-333, which can be replicated in trans by p33 and p92 expressed separately from DI-p33 and gCNV-Y (not shown).

To test the role of the RPR motif mutations in p33 on RNA recombination, we constructed four DI-p33 mutants, namely P_{640}−A, P_{640}−Y, P_{652}−A, and P_{652}−Y, which can express the p33 protein with the given RPR motif mutation. We coelectroporated N. benthamiana protoplasts with one of the DI-p33 mutants, the unmodified gCNV-Y and DI-333, followed by passaging the total RNA extracts obtained after 24 h incubation to new batch of protoplasts as described under Materials and methods. Total RNA extracts from the samples of the first passage were analyzed by Northern blotting to detect putative DI-333-derived recombinants (Fig. 7B). The control three-component system that included the wt p33 (expressed from wt DI-p33, Fig. 7B) supported formation of DI RNA recombinants efficiently (85% of samples contained recombinants), demonstrating that the three-component system is suitable for recombination studies. Similar experiments, which included one of the mutated DI-p33, instead of the wt DI-p33, in the three-component system, gave a lower frequency level of DI recombinant formation: 45% for P_{640}−A, 31% for P_{640}−Y, 47% for P_{652}−A, and 29% for P_{652}−Y (Fig. 7B). Moreover,
The RPR motif in the p33 replicase protein is involved in RNA recombination

The most popular models of viral RNA recombination predict a close relationship between replication and recombination. This is because recombination is thought to be an error made by the viral replicase during the replication process. Therefore, it is possible that replication and recombination might share the same viral replicase complex and the viral RNA templates, although the final RNA products generated by replication and recombination could be different. Indeed, there is a unique template switching step performed by the viral replicase during recombination that is not needed (based on current models) during replication. The template switching is proposed to occur from the donor to the acceptor RNAs during complementary RNA synthesis on the donor template (or from the donor site to the acceptor sites if they are on the same RNA molecule). Accordingly, in vitro experiments with the Qβ phage (Biebricher and Luce, 1992), poliovirus (Arnold and Cameron, 1999; Tang et al., 1997), Brome mosaic virus (BMV) (Dzianott et al., 2001; Kim and Kao, 2001), Bovine viral diarrhea virus (Kim and Kao, 2001), Turnip crinkle virus (TCV) (Nagy et al., 1998), and CNV RdRps (Cheng and Nagy, unpublished data) demonstrated that template switching by these enzymes is possible and occurs at a high frequency. If template switching also takes place in vivo, then mutations in the viral replicase proteins should affect not only replication, but recombination as well in vivo experiments. Indeed, the data obtained in this work demonstrate that several mutations within the RPR motif of p33/p92 replicate proteins affected RNA recombination. For example, a group of p33/p92 mutants of CNV that carried alterations of either or both prolines in the RPR motif (e.g., P_{640} → A, P_{640} → Y, P_{652} → A, P_{652} → Y, and P_{640} → A/P_{652} → A) supported recombination at reduced efficiency (frequency) when compared to wt (Fig. 5B). On the contrary, two of the arginine mutants (e.g., R_{646} → A and R_{643} → R; Fig. 5C) supported recombiant formation more efficiently than wt did. The observed differences among the RPR motif mutants and the relative levels of DI recombinants were significantly lower than those observed in the case of the wt p33 (Fig. 7B). This data suggest that mutations within the RPR motif in p33 can affect RNA recombination (see Discussion).

**Mutations within the RPR motif of p92 have no major effect on RNA recombination**

To test the role of the RPR motif mutations in p92 on RNA recombination, we constructed four gCNV-Y mutants, namely P_{640} → A, P_{640} → Y, P_{652} → A, and P_{652} → Y, which can express the p92Y protein with the given RPR motif mutation, and used them in the three-component RNA recombination assay described above (the other components were wt DI-p33 and DI-333). Interestingly, all four gCNV-Y mutants supported DI recombination as efficiently as the wt gCNV-Y did in protoplasts when tested after the first passage (Fig. 7C). The relative amounts of recombinant versus parental DI-333 were also comparable for these gCNV-Y mutants and the wt gCNV-Y (Fig. 7B). Based on these results, we propose that the RPR motif in p92 (at least the particular mutations tested) might have a lesser effect on RNA recombination than the corresponding mutations in p33.

**Discussion**

The most popular models of viral RNA recombination predict a close relationship between replication and recombination. This is because recombination is thought to be an error made by the viral replicase during the replication process. Therefore, it is possible that replication and recombination might share the same viral replicase complex and the viral RNA templates, although the final RNA products generated by replication and recombination could be different. Indeed, there is a unique template switching step performed by the viral replicase during recombination that is not needed (based on current models) during replication. The template switching is proposed to occur from the donor to the acceptor RNAs during complementary RNA synthesis on the donor template (or from the donor site to the acceptor sites if they are on the same RNA molecule). Accordingly, in vitro experiments with the Qβ phage (Biebricher and Luce, 1992), poliovirus (Arnold and Cameron, 1999; Tang et al., 1997), Brome mosaic virus (BMV) (Dzianott et al., 2001; Kim and Kao, 2001), Bovine viral diarrhea virus (Kim and Kao, 2001), Turnip crinkle virus (TCV) (Nagy et al., 1998), and CNV RdRps (Cheng and Nagy, unpublished data) demonstrated that template switching by these enzymes is possible and occurs at a high frequency. If template switching also takes place in vivo, then mutations in the viral replicase proteins should affect not only replication, but recombination as well in vivo experiments. Indeed, the data obtained in this work demonstrate that several mutations within the RPR motif of p33/p92 replicate proteins affected RNA recombination. For example, a group of p33/p92 mutants of CNV that carried alterations of either or both prolines in the RPR motif (e.g., P_{640} → A, P_{640} → Y, P_{652} → A, P_{652} → Y, and P_{640} → A/P_{652} → A) supported recombination at reduced efficiency (frequency) when compared to wt (Fig. 5B). On the contrary, two of the arginine mutants (e.g., R_{646} → A and R_{643} → R; Fig. 5C) supported recombiant formation more efficiently than wt did. The observed differences among the RPR motif mutants and
wt are likely due to altered efficiency in recombinant formation (frequency of recombination) driven by the mutated replicase proteins. This is because these RPR motif mutants supported replication of selected recombinants at relative levels (when compared to the level of replication of the parental DI RNA) similar to that observed with wt CNV helper virus in a template competition experiment in protoplasts (Fig. 6). In other words, the above template competition experiments suggest that, after the RNA recombination step, a particular recombinant (such as the recombinants shown in Fig. 6) is predicted to have a comparable chance to outcompete the parental DI RNA in both the mutant and the wt p33/p92 background in protoplasts. Therefore, we suggest that the p33/p92 replicase proteins of CNV are directly involved in recombination. The direct involvement of the replicase proteins in recombination is also supported by our previous in vitro data that demonstrated the ability of a partially purified CNV RdRp preparation to support recombinant formation (Cheng and Nagy, unpublished data).

Interestingly, previous works with the BMV (which belongs to a different supergroup than Tombusviruses) 1a and 2a proteins also suggested the role of the viral replicase proteins in recombination (Nagy et al., 1995; Figlerowicz et al., 1997, 1998). Also, the polymerase acid protein subunit, a possible elongation factor, of the RdRp of influenza A virus has been proposed to affect DI RNA formation (Fodor et al., 2003). These findings are in agreement with the template switching based recombination models.

Development of a three-component complementation-based recombination system that contained tombusvirus RNAs expressing p33 and p92 proteins separately allowed us to study independently the role of the RPR motif mutations in p33 and p92. We found that selected RPR motif mutations, which affected recombination frequency in the two-component system where both p33 and p92 were simultaneously mutated, were capable of affecting RNA recombination when present in p33 alone. On the contrary, their effects were less obvious on DI RNA recombination when present in p92. This data indicate that the RPR motif in p33 is involved in recombinant formation. This further supports the model that RNA replication and RNA recombination might depend on similar factors and elements (also see below the discussion on the role of the RNA template). The actual function of p33 in tombusvirus replication/recombination will need further study.

Is there a relationship between recombination, replication, or subgenomic RNA synthesis?

Comparison of the abilities of the RPR motif mutants to support replication and recombination revealed the lack of correlation between the extent of replication and the efficiency of recombination. For example, mutants R_{646}−A and R_{643}+R supported replication poorly (Table 1), but they showed accelerated rate of recombination (Fig. 5C). On the other hand, mutants P_{640}−A, P_{640}−Y, P_{652}−A, and P_{652}−Y, which supported recombination inefficiently (Fig. 5B), were capable of supporting replication at levels (58–87%) similar to that obtained with mutants R_{637}−K, R_{637}−Q, P_{640}−652−G, and R_{637}−A/ R_{645}−H, etc. (Table 1), which could support ~wt-level of recombinants. The difference in the effect of a particular mutant on the efficiency of replication and recombination suggests that the RPR motif in p33 (and possibly in p92) plays different functions during replication and template switching. A similar observation was also made for BMV 2a mutants that affected the levels of replication and recombination differently (Figlerowicz et al., 1997, 1998). These observations are not surprising in the sense that protein factors and RNA elements (see below), which are involved in replication and recombination, are likely utilized somewhat differently by the replicase proteins.

One of the surprising observations of this work is that the two RPR motif mutants, namely R_{646}−A and R_{643}+R (Fig. 5C), which accelerated RNA recombination, also increased the relative amount of subgenomic RNAs (by ~2 to 20-fold when the levels of subgenomic RNAs were compared to the level of genomic RNA) in protoplast (Panaviene et al., 2003). This observation suggests that there might be a common step in RNA recombination and subgenomic RNA synthesis (such as termination of RNA synthesis?), which is stimulated by these RPR motif mutants. Future experiments will address this possibility.

The role of cis-acting elements in tombusvirus recombination

Based on in vitro studies with the partially purified CNV RdRp, we predicted that the RII sequence, which has been shown to be a strong replication enhancer in vitro (Panavas and Nagy, 2003) and in vitro (Ray and White, 1999, 2003), should be active in promoting RNA recombination. Accordingly, we found that DI RNAs carrying two or three copies of RIII recombed rapidly in protoplasts. Although the recombination junctions were mainly imprecise, they clustered within the RIII sequence, suggesting that RIII is a recombination hot-spot sequence. We show similar data for RII (construct DI-2233 and DI-2323 in Figs. 3C–D), which is a putative cis-acting element (albeit its function is currently unknown; Shapka and Nagy, unpublished data) that can bind to the replicase proteins in vitro. We have shown previously that insertions of short sequences (~110–150 nt) derived from GFP or barstar genes into DI RNA did not promote recombination (Shapka and Nagy, unpublished data) under the same conditions used in this work. The detailed roles of these elements in RNA recombination will be published elsewhere.

Interestingly, multiple copies of regions II and III elements have not increased the replication potential of the prototypical DI RNA. Instead, we observed (i) a moderate level of interference with replication (Fig. 1); and (ii) re-
duced competition against the recombinant DI RNAs (Fig. 6). The interference might take place during plus-strand synthesis since the amounts of plus-strands for DI RNAs with multiple copies of regions II and III decreased significantly more (at the 24 h time point) than the amount of minus-strands for all four constructs. Note that, similar to our observation, Ray and White (1999) also observed the lack of additional stimulation of DI RNA replication due to the duplicated RIII replication enhancer sequence in the presence of the TBSV helper virus. Also, a duplicated copy of the motif1 replication enhancer of satC, a satellite (chimeric) RNA associated with TCV infections (Nagy et al., 1999, 2001), did not further enhance the replication of satC when compared to satC carrying a single motif1 replication enhancer. It is possible that one copy of the replication enhancer is optimal for replication and additional copies might only disturb the replication process, thus leading to replicase errors, such as RNA recombination.

In summary, this is the first report on the role of a tombusvirus replicase protein (namely p33) in RNA recombination. This and a previous work (Panaviene et al., 2003) firmly establish that the RNA-binding domains in the replicase proteins are involved in RNA replication, subgenomic RNA synthesis, and RNA recombination. Additional structure/function studies will be helpful in understanding the mechanism of these processes that are vital for tombusvirus infection and evolution.

Materials and methods

Plasmid construction

For construction of cDNA clones of DI-33 and DI-333 DI RNA, we PCR-amplified RIII of DI-72 using primers 355 (5′-GGACGGTGCCGAGCTTGGCTGAGACAG-3′) and 356 (5′-GAACCATTCGAGACGCCTGGGGCATCTCCAGAAACAAC-3′) and template DI-72XPN (Shapka and Nagy, unpublished data). DI-72XPN, which contains XhoI and NcoI sites between RII and RIV, is a derivative of DI-72SXP obtained from Andy White (White and Morris, 1994a). The PCR product was digested with PstI, gel purified, and cloned into DI-72XPN linearized with PstI. We selected the appropriate DI-33 and DI-333 clones, which contained either one RII copy or two RIII copies, respectively, in 5′-3′ orientation by sequencing.

For construction of DI-2233 clone, RII of DI-72SXP was amplified by PCR using primers 386 (5′-GGACGGGGGCCCATCGATAGAAAAACGGGAAGCATCG-3′) and 387 (5′-GGACGGGGCCGATATCTCTGTTTTACGAAGGTAG-3′) and DI-72SXP template. The PCR product was digested with Apal, gel purified, and ligated into DI-33 cDNA treated with Apal. An additional copy of RII was inserted between the original RII and RIII in 5′-3′ orientation.

To make DI-2323 clone, RII of DI-72 was PCR-amplified using primers 388 (5′-GGACGAGATCTGGGTGGATCGATAAAAACGGGAAGCTTCGCC-3′) and 389 (5′-GGACGAGCATGGGGATACATCGTCTTTACGAAGGTAG-3′), and template DI-72SXP RNA. The PCR fragment was digested with BglII and MluI, followed by gel purification and ligation into DI-33 treated with BglII and MluI.

Construction of RPR motif mutants

Site-directed mutagenesis within the RPR motif of gCNV (Panaviene et al., 2003; Rajendran and Nagy, unpublished data) was performed with Quick Change XL Site-Directed Mutagenesis Kit (Stratagene). The primer pairs used for each mutant are shown in the Table 2, while the template was pK2MS (Rochon and Johnston, 1991), encoding a full-length cDNA clone for CNV. Before transformation into Escherichia coli (DH5α), the PCR products were digested with DpnI. The clones for each mutant were confirmed by sequencing using primer 27 (5′-GTATTTCAACCAAGGGAC-3′).

We made derivatives of gCNV-Y (expressing protein p92-Y) and DI-p33, carrying selected mutations in the RPR motif (Table 1) as described in Panaviene et al. (2003). To confirm the presence of the RPR mutation and that the termination codon was replaced with tyrosine codon in derivatives of gCNV-Y, each clone was sequenced using primer 631 (5′-GGAGGAATTCAGGTAATTGCCTGCAC-3′).

Cloning recombinant DI RNAs

To obtain cDNA clones for a representative number of recombinant DI RNAs, we performed reverse transcriptase (RT) reaction with primer 41 (5′-GGACGAATTCGGGCTGCATTTCTGCAATGTTCC-3′) and 40 (5′-GGACGAATTCGGGCTGCATTTCTGCAATGTTCC-3′) and template DI-72SXP RNA. The PCR fragment was digested with XbaI and EcoRI, gel isolated, and ligated into similarly treated pUC19 vector. The clones for sequencing were selected after restriction digestion with XbaI and EcoRI. The sequencing was done using primer 157 (5′-GGGCTGATTTCTGCAATGTTCC-3′). The RT-PCR products were digested with XbaI and EcoRI, gel isolated, and ligated into similarly treated pUC19 vector. The clones for sequencing were selected after restriction digestion with XbaI and EcoRI. The sequencing was done using primer 157 (5′-GGGCTGATTTCTGCAATGTTCC-3′). Note that cDNA clones with identical recombination junction sites were counted only once if they were derived from the same protoplast samples.

To generate full-length cDNA clones for selected recombinant DI RNAs (see control experiment in Fig. 6), selected partial cDNA clones, which were used for sequencing, were digested with XbaI and XhoI. This is followed by gel purification of the appropriate fragments and cloning into similarly treated DI-2323 (Fig. 1A).
Table 2
List of primers used for site-directed mutagenesis of CNV (pK2M5)

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primer pair</th>
<th>Sequencea</th>
<th>Positionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>R637–K</td>
<td>832</td>
<td>ATTGGTGTCACAGGAGACGCTGCGAGAAGACCT</td>
<td>622–654</td>
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<tr>
<td>R637–H</td>
<td>833</td>
<td>AGGTCTCTGGAGGCAGGTGGTGGGAGGCTACAT</td>
<td>654–622</td>
</tr>
<tr>
<td>R637–Q</td>
<td>834</td>
<td>AGGTCTCTGGAGGCAGGTGGTGGGAGGCTACAT</td>
<td>622–654</td>
</tr>
<tr>
<td>R637–Y</td>
<td>835</td>
<td>AGGTCTCTGGAGGCAGGTGGTGGGAGGCTACAT</td>
<td>654–622</td>
</tr>
<tr>
<td>P640–Y</td>
<td>840</td>
<td>GGCCTACAGAAAGATATTATGCAGCTAAGATTG</td>
<td>625–658</td>
</tr>
<tr>
<td>P652–Y</td>
<td>841</td>
<td>CAGGTCTCAGAAGAGATATTATGCAGCTAAGATTG</td>
<td>637–668</td>
</tr>
<tr>
<td>P660–G</td>
<td>842</td>
<td>CGCCCTCGCAGAGAATATTATGCAGCTAAGATTG</td>
<td>668–637</td>
</tr>
<tr>
<td>R637–A/R645–H</td>
<td>843</td>
<td>CAGGTCTCAGAAGAGATATTATGCAGCTAAGATTG</td>
<td>668–637</td>
</tr>
<tr>
<td>R637–A/R645–H</td>
<td>844</td>
<td>CCAGGTCTCAGAAGAGATATTATGCAGCTAAGATTG</td>
<td>626–760</td>
</tr>
<tr>
<td>R637–A/R646–H</td>
<td>845</td>
<td>CAGGTCTCAGAAGAGATATTATGCAGCTAAGATTG</td>
<td>628–660</td>
</tr>
<tr>
<td>R637–A/R646–H</td>
<td>846</td>
<td>CAGGTCTCAGAAGAGATATTATGCAGCTAAGATTG</td>
<td>631–663</td>
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<tr>
<td>R637–A/R649–H</td>
<td>847</td>
<td>CAGGTCTCAGAAGAGATATTATGCAGCTAAGATTG</td>
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<td>R637–A/R649–H</td>
<td>848</td>
<td>CAGGTCTCAGAAGAGATATTATGCAGCTAAGATTG</td>
<td>666–634</td>
</tr>
</tbody>
</table>

a The mutated sequences are underlined.

b The nucleotide positions within the p33/p92 ORF of CNV are indicated (Rochon and Johnston, 1991).

Preparation of RNA transcripts

To generate RNA transcripts, all pK2M5 and DI-72XPXN-derived plasmids were digested with Smal and were used in an in vitro transcription reaction with T7 RNA polymerase (Nagy et al., 1999). The obtained DI RNA transcripts used in recombination studies were gel purified from 1% agarose gel, followed by phenol/chloroform extraction, precipitation in 95% ethanol, and three times repeated wash with 70% ethanol to remove residual salts. For the rest of the in vitro RNA transcripts, DNA templates were removed by Dnase I treatment, followed by phenol/chloroform extraction, precipitation, and washing as described above. The RNA transcripts were electrophorized on 1% agarose gels and quantified by UV spectrophotometer (Beckman).

Preparation and electroporation of protoplasts

*N. benthamiana* protoplasts were prepared as described (Panaviene et al., 2003). At the end of the procedure, we resuspended 5 × 10^5 protoplasts in the electroporation buffer (10 mM HEPES, 10 mM NaCl, 120 mM KCl, 4 mM CaCl_2, 200 mM mannitol). For electroporation, we used the following amounts of RNA transcripts: 2 μg gCNV RNA and 1 μg gel-purified DI RNAs (unless specified otherwise in the figure legends). After electroporation, the samples were incubated in 2 ml of protoplast culture medium (Kong et al., 1997) in the dark for 24–48 h at 22°C.

Total RNA extraction from protoplasts and RNA analysis

Total RNA was extracted from protoplast using a standard phenol/chloroform method (Kong et al., 1997; Nagy et al., 2001). Aliquots of total RNA were analyzed on 1 or 1.2% agarose or 4% polyacrylamide/8 M urea gels. RNA samples were treated with formamide at 85°C right before loading on the gels. For Northern blot analysis, the RNA was transferred from the gels to Hybond XL membrane (Amersham-Pharmacia) by electrotransfer and hybridized with DI-72- (+) or DI-72- (−) specific probes (Panaviene et al., 2003). Hybridization was done in ULTRAhyb hybridization buffer at 68°C using supplier (Ambion) recommended conditions. The probes were made in an in vitro transcription reaction with T7 RNA polymerase in the presence of a [α-32P]UTP and DNA templates representing the 169-bp RI (Fig. 1A). These templates were generated by PCR using primers 15 (5’-GTAATACGACTCACTATACTC-3’) and 20 (5’-GGGACTCTTCTGAGAATTCTCC-3’) for amplification of (+) strands, and primers 31 (5’-GTAATACGACTCACTATACTC-3’) and 678 (5’-GGGACTCTTCTGAGAATTCTCC-3’) for (−) strand detection.

Acknowledgments

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