Heterologous RNA replication enhancer stimulates in vitro RNA synthesis and template-switching by the carmovirus, but not by the tombusvirus, RNA-dependent RNA polymerase: Implication for modular evolution of RNA viruses

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Abstract

The viral RNA plays multiple roles during replication of RNA viruses, serving as a template for complementary RNA synthesis and facilitating the assembly of the viral replicase complex. These roles are coordinated by cis-acting regulatory elements, such as promoters and replication enhancers (REN). To test if these RNA elements can be used by related viral RNA-dependent RNA polymerases (RdRps), we compared the potential stimulatory effects of homologous and heterologous REN elements on complementary RNA synthesis and template-switching by the tombus- (Cucumber necrosis virus, CNV), carmovirus (Turnip crinkle virus, TCV) and hepatitis C virus (HCV) RdRps in vitro. The CNV RdRp selectively utilized its cognate REN, while discriminating against the heterologous TCV REN. On the contrary, RNA synthesis by the TCV RdRp was stimulated by the TCV REN and the heterologous tombusvirus REN with comparable efficiency. The heterologous REN elements also promoted in vitro template-switching by the TCV and HCV RdRps. Based on these observations, we propose that REN elements could facilitate intervirus recombination and postrecombinational amplification of new recombinant viruses.

Keywords: Replication; Interviral recombination; RNA promoter; RNA replication enhancer; In vitro RNA synthesis; Template-switching

Introduction

Distinct RNA elements play complex regulatory roles during replication of plus-strand RNA viruses (Ahlquist, 2002; Buck, 1996; Dreher, 1999; Kao et al., 2001; Lai, 1998; White and Nagy, 2004). These elements include the essential promoter (initiation) elements (Dreher, 1999; Kao et al., 2001) as well as regulatory RNA elements, such as template recruitment elements (RE) (Monkewich et al., 2005; Pogany et al., 2005; Sullivan and Ahlquist, 1999), RNA replication enhancers (REN) that up-regulate (Nagy et al., 1999; Panavas and Nagy, 2003a; Ray and White, 2003) and RNA replication silencer that down-regulate (Pogany et al., 2003; Zhang et al., 2004) RNA synthesis. The cis-acting RNA elements are thought to perform their functions by regulating the activity of the viral replicase, which contains the viral-coded RNA-dependent RNA polymerase (RdRp), viral auxiliary proteins and host proteins (Ahlquist et al., 2003). In spite of the significance of the cis-acting elements in virus replication, their roles have only been studied for a limited number of (+)-stranded RNA viruses (reviewed by Buck, 1996; Dreher, 1999; Kao et al., 2001).

An important feature of RNA viruses is their ability to recombine their genomes, which could lead to the generation of new viruses or strains (Aaziz and Tepfer, 1999b; Lai, 1992; Nagy and Simon, 1997; Simon and Bujarski, 1994; Worobey and Holmes, 1999). RNA recombination
can also help viruses repair their genomes or generate defective interfering (DI) RNAs (Allison et al., 1990; Aylon et al., 1999; Borja et al., 1999; Hillman et al., 1987; Lai, 1992; Nagy and Bujarski, 1992, 1995; White and Morris, 1994). Frequent RNA recombination is thought to occur due to replicase errors that include template-switching or jumping during RNA synthesis (Lai, 1992; Nagy and Simon, 1997). The replicase driven template-switching model is supported by numerous observations, including the effect of mutations within replication proteins on distribution of junction sites (Nagy et al., 1995) or on the frequency of RNA recombination (Figlerowicz et al., 1997; Panaviene and Nagy, 2003). Moreover, purified RdRp preparations of Qbeta phage, *Brome mosaic virus* (BMV), *Hepatitis C virus* (HCV), *Bovine viral diarrhea virus* (BVDV), *Cucumber necrosis virus* (CNV) and *Turnip crinkle virus* (TCV) have been shown to support template-switching in vitro (Biebricher and Luce, 1992; Cheng and Nagy, 2003; Kim and Kao, 2001). Nonreplicative RNA recombination has also been demonstrated for several (+)-stranded RNA viruses (Chetverin et al., 1997; Gmyl et al., 1999). Overall, RNA recombination is a major process that can lead to genome rearrangements and joining of previously separate sequences that likely facilitate rapid RNA virus evolution.

In several RNA viruses, recombination events do not occur randomly, but instead, the recombination sites cluster in defined regions within the RNA genomes (Lai, 1992; Nagy and Simon, 1997; Simon and Bujarski, 1994). The recombination hot spot regions frequently contain AU-rich stretches (Nagy and Bujarski, 1996, 1997; Shapka and Nagy, 2004), form intra- or intermolecular secondary structures (stem-loop structures and heteroduplexes formed between complementary stretches present in separate RNAs) (Havelda et al., 1997; Nagy and Bujarski, 1993) or localized within cis-acting elements, such as RENs (Cheng and Nagy, 2003; Cheng et al., 2002; Miller et al., 1995; Nagy et al., 1999, 2001). These hot spot elements could play central roles in recombination, which could result in reshuffling of RNA domains around these hot spot sequences. Accordingly, sequence comparison of large number of viruses led to the model of “modular viral evolution”, which predicts that various RNA elements and/ or coding sequences are used as functional modules during RNA recombination events (Botstein, 1980; Dolja and Carrington, 1992; Gibbs, 1987). This is thought to speed up virus evolution by utilizing various combinations of “battle-tested” functional modules to gain new cis-acting elements or novel genes. Accordingly, a novel functional promoter element was created for TCV RdRp in vitro by joining of two functional modules, a short initiation sequence and a REN (Nagy et al., 1999). In addition, insertion of the BMV RdRp specificity element (termed CAM) into host tRNAs rendered the nontemplate tRNA compatible for complementary RNA synthesis in the in vitro BMV RdRp assay (Ranjith-Kumar et al., 2003).

To demonstrate the advantage of modular viral evolution, it would be useful to understand if an RNA virus could use the cis-acting elements of another virus. It is predicted that a “modular element” would be successful if it could promote RNA recombination events involving the heterologous viral RdRp, and/or if it could facilitate the efficient postrecombinational replication of the recombinant. Therefore, in this paper, we analyzed whether a tombusvirus (CNV) RdRp and a carmovirus (TCV) RdRp could recognize heterologous cis-acting elements in vitro for replication and recombination. CNV has a ~4.8 kb, single component (+)-stranded RNA genome that codes for two replicase proteins, named p33 and p92 (Fig. 1) (White and Nagy, 2004). Previous works with partially purified CNV and TBSV RdRps revealed that two separate short promoters, termed gPR and cPR (Fig. 1), are essential for minus- and plus-strand synthesis, respectively (Panavas et al., 2002a). Additional works led to the identification of a REN element, which stimulated (+)-stranded RNA synthesis by ~10-fold in vitro and in vivo (Fig. 1A; Panavas and Nagy, 2003b; Ray and White, 2003). The related TCV has a smaller genome (~4 kb) that codes for 5 proteins, including the p28 and p88 replication proteins (Fig. 1) (Simon, 1999). Purified, plant-derived active replicase preparations are available for both CNV and TCV, whereas *E. coli*-derived recombinant p88 RdRp is also functional in case of TCV and a recombinant CNV replicase preparation has been obtained from yeast (Nagy and Pogany, 2000; Panaviene et al., 2004; Rajendran et al., 2002; Song and Simon, 1994). In addition, TCV is known to be associated with small parasitic RNAs, termed satC and satD RNAs (Simon, 1999). Previous in vivo and in vitro works dissected functional promoter and REN elements in satC (Fig. 1B), facilitating the studies described below (Guan et al., 2000; Nagy et al., 1999).

To understand if heterologous REN elements could be used by the CNV replicase (for simplicity, the term RdRp will be used hereafter) and TCV RdRp preparations, in this report we analyzed complementary RNA synthesis and template-switching by these enzymes. The in vitro assays demonstrated that the TCV RdRp could use the tombusvirus REN efficiently during complementary RNA synthesis and for template-switching. On the contrary, the CNV RdRp used the cognate REN more efficiently than the heterologous TCV REN. Overall, our results suggest that the REN could be an important modular element to facilitate RNA recombination and subsequent template amplification of the recombinant viral RNA.

**Results**

**Rationale**

REN elements play major roles in replication of tombus- and carmoviruses (Nagy et al., 1999, 2001; Panavas and Nagy, 2003a; Ray and White, 2003) by enhancing plus-
strand RNA synthesis. In addition, RENs have also been demonstrated to serve as recombination hot spots in vivo (Cascone et al., 1993; Panaviene and Nagy, 2003) and to facilitate template-switching of the viral replicase in vitro (Cheng and Nagy, 2003; Cheng et al., 2002; Nagy et al., 1998). The stimulatory effect of RENs on RNA synthesis and recombination is likely the consequence of efficient binding of viral RdRps to REN sequences. Accordingly, binding of p33 and p92 replication proteins to the TBSV REN [i.e., RIII(C0)] was documented with purified recombinant TBSV proteins in vitro (Panavas and Nagy, 2005). CNV and TBSV p33 and p92 proteins bind to the TBSV REN with comparable efficiency (not shown), thus we consider CNV replication protein–TBSV REN interaction as a homologous type. Altogether, it is currently unknown if viral RdRp can utilize heterologous REN elements, which are dissimilar to the homologous (cognate) REN, during RNA synthesis and template-switching.

The TCV and the CNV RdRp preparations are capable of de novo initiation from the cognate promoter sequences or 3′-terminal extension on various added templates in vitro (Cheng and Nagy, 2003; Guan et al., 2000; Nagy et al., 1998, 1999; Panavas et al., 2002a, 2002b; Song and Simon, 1994). In addition, template-switching by the TCV and CNV RdRps has also been demonstrated in vitro (Cheng and Nagy, 2003). Therefore, in this paper, we tested the possible stimulatory effect of heterologous TBSV and TCV RENs on complementary RNA synthesis and template-switching using purified CNV and TCV RdRp preparations (see results below).

**Stimulation of TCV RdRp activity, but not the CNV RdRp activity, by a heterologous REN in vitro**

To test if a heterologous REN, when compared to the cognate REN, can stimulate template activity of CNV and TCV RdRps, we took advantage of a previously developed trans-replication assay (Panavas and Nagy, 2005). In this assay, the template RNA carried an initiation sequence (the minimal plus-strand initiation promoter (cPR), which contains the initiation site as indicated with a solid arrow) at the 3′ end and a “clamp” sequence. The template RNA was hybridized to a nontemplate RNA carrying a sequence complementary to the clamp sequence (Fig. 2A). Importantly, the 5′ end of the nontemplate RNA consisted of either the minimal tombusvirus REN [SL1-III-br(C0)], Fig. 2A, constructs SL1-III-br(clamp and SL1-III-br(3′PE-clamp)] or the carmovirus M1H REN (Fig. 2A, constructs M1H/clamp and M1H/3′PE-clamp) or lacked extra sequence (constructs Clamp and 3′PE-clamp, Fig. 2A). This arrangement allowed for (i) complementary RNA synthesis to take place on the template RNA sequences; and (ii) the tethering...
of the REN to the template strand helped positioning the REN in the close vicinity of the promoter present in the template RNA, which is necessary for stimulation of RNA synthesis (Panavas and Nagy, 2005). However, because the REN is present on the nontemplate RNA, it is not transcribed by the viral RdRp. As shown previously, the minimal TBSV REN stimulated RNA synthesis in \textit{trans} by \textasciitilde9-fold in the CNV RdRp assay (Figs. 2B–C, lane 2) (Panavas and Nagy, 2003a, 2005). Here, we show that the minimal TBSV REN also stimulated de novo RNA synthesis by \textasciitilde3-fold from the heterologous 3’V PE promoter in the CNV RdRp assay when compared to the RNA template lacking the enhancer sequence (Figs. 2B–C, compare lanes 4 and 5). Thus, the minimal TBSV REN stimulated complementary RNA synthesis by the CNV RdRp from both homologous and heterologous promoters, suggesting that CNV RdRp can recognize properly these \textit{cis}-acting elements, albeit with different efficiency. We also observed \textasciitilde2.5- and 3-fold stimulatory effect of the M1H REN on template activity of TCV RdRp when compared to the RNA template lacking the enhancer sequence (Figs. 2B–C, lanes 1 versus 3 and 4 versus 6), suggesting that, similar to the CNV RdRp, the TCV RdRp was stimulated by its cognate REN element in the \textit{trans}-replication assay.

To test if the viral RdRps can also be stimulated by the heterologous RENs, we performed CNV RdRp assays on RNAs that included the M1H REN of TCV satC (constructs 3 and 6, Fig. 2A). In comparison with the RNA lacking
REN sequences, the heterologous M1H REN did not stimulate RNA synthesis from the cPR or 3PE promoters by the CNV RdRp (lanes 1 versus 3 and 4 versus 6, Figs. 2B–C). In contrast, the minimal REN of TBSV enhanced the activity of the TCV RdRp by ~2- and 4-fold (lanes 2 and 5, Figs. 2B–C), depending on the presence of either the cPR or 3PE promoter. Surprisingly, this level of stimulation is comparable to that observed with the homologous M1H REN (lanes 3 and 6, Figs. 2B–C), suggesting that the TCV RdRp can recognize the minimal TBSV REN as efficiently as the M1H REN of TCV satC. Overall, these data suggested that, unlike the CNV RdRp, the TCV RdRp could be stimulated by a heterologous REN. On the contrary to REN, the heterologous minimal plus-strand initiation promoters (cPR of TBSV and 3PE of TCV satC) were utilized by both enzymes with comparable efficiencies (lanes 1 and 4, Fig. 2B).

Heterologous REN stimulates template-switching activity of the TCV RdRp in vitro

To test if the heterologous REN could stimulate template-switching by the TCV RdRp, we assayed the generation of recombinant RNAs in an in vitro template-switching assay. In the template-switching assay, the viral RdRp first has to initiate complementary RNA synthesis on the donor RNA, followed by strand elongation and pausing/termination. This process generates the primer RNA, which is subsequently used by the jumping RdRp to resume RNA synthesis on the acceptor RNA template. Because the template-switching assay leads to the labeling of the recombinant RNAs with 32P-UTP, they can be detected in denaturing PAGE gels (Cheng and Nagy, 2003).

We selected four RNA templates for the TCV RdRp-driven template-switching assay: the first template contained the cognate M1H REN of the TCV satC, which is known to support efficient template-switching in vitro (construct Mot1/pr, Fig. 3A) (Cheng and Nagy, 2003). The second template contained the heterologous TBSV REN [i.e., RI3(−), see construct R3(−)/art, Fig. 3A], whereas the third template had an artificial GC-rich sequence (GC1/art, Fig. 3A) (Cheng and Nagy, 2003; Nagy and Bujarski, 1998; Shapka and Nagy, 2004). The fourth template contained an artificial AU-rich sequence (AU1/art, Fig. 3A) (Cheng and Nagy, 2003; Nagy and Bujarski, 1997; Shapka and Nagy, 2004), which was used as a reference template in the template-switching assay. The control TCV RdRp reaction containing Mot1/pr (with homologous REN) and the reference template (AU1/art RNAs; Fig. 3B, lane 1 + 4) resulted in the efficient generation of recombinants between Mot1/pr and AU1/art sequences (Cheng and Nagy, 2003). These recombinants, termed hetero-recombinants (marked by an arrowhead in lane 1 + 4, Fig. 3B), represented 7.9% of the RdRp products when compared to the primer-extension product generated on the AU1/art reference template (100%, lane 1 + 4, marked with a gray arrow in Fig. 3B). RT-PCR amplification, cloning and sequencing of these hetero-recombinants demonstrated that template-switching occurred mostly from the 5′ end of one template to the 3′ end of the other template or end-to-internal positions (not shown) (Cheng and Nagy, 2003). The second type of recombinant is formed between two AU1/art templates and they are termed homo-recombinants (marked with black arrows in Fig. 3B).

The combination of R3(−)/art template carrying the heterologous TBSV REN and AU1/art reference template also supported the formation of hetero-recombinants with 6.4% efficiency in the TCV RdRp assay (lane 2 + 4, Fig. 3B). However, we had to use 5 times more R3(−)/art templates than Mot1/pr templates to detect hetero-recombinants in comparable TCV RdRp assays (Fig. 3B). Therefore, template-switching between R3(−)/art and AU1/art templates is estimated to occur at ~5-fold lower efficiency than between Mot1/pr and AU1/art templates in the TCV RdRp assay. In contrast, combination of AU1/art and GC1/art templates did not lead to detectable levels of hetero-recombinants when templates were used in the same amount as with the combination of R3(−)/art and AU1/art (Fig. 3B, lane 3 + 4 versus 2 + 4). These data indicate that the heterologous REN can promote template-switching by the TCV RdRp in vitro more efficiently than that observed with a control template (GC1/art).

To confirm that the detected hetero-recombinants obtained with the combination of R3(−)/art and AU1/art templates are true recombinants, the gel-isolated recombinants were amplified by RT-PCR, cloned and sequenced using the strategy illustrated in Fig. 4A. Comparison of the junction sites revealed that template-switching occurred from either the AU1/art template or from the R3(−)/art template (Fig. 4B). Interestingly, both end-to-end and internal recombination sites were found (Fig. 4B). Overall, these data suggest that the heterologous TBSV REN can be utilized by the TCV RdRp for template-switching. However, the efficiency of template-switching supported by the heterologous REN is ~25-fold lower than that supported by the homologous REN in vitro (based on calculations after adjustment of templates used in the in vitro RdRp reactions). Yet, the recombinants are readily detectable, unlike between GC1/art and AU1/art templates.

Heterologous REN does not stimulate template-switching activity of the CNV RdRp in vitro

To test if the heterologous REN from satC of TCV could stimulate template-switching by the CNV RdRp, we performed in vitro assays similar to those described above for the TCV RdRp. The difference in this assay is that the inefficient AU-rich template was replaced by the more efficient cPR21 template (containing the extended promoter) (Panavas et al., 2002a), which supports robust enough recombination for visualization in gels (Fig. 5). In
Fig. 3. Stimulation of template-switching by the heterologous REN in the TCV RdRp assay in vitro. (A) Sequences and structures of RNA templates used in the TCV RdRp reactions. Construct Mot1/pr contains the minus-stranded M1H replication enhancer (shown as the hairpin structure) and the 3’ proximal plus-strand initiation sequence (boxed with dotted line) of TCV satC. Construct R3(-)/art contains the RIII(-) replication enhancer and an artificial self-priming sequence (termed “art”, the sequence circled). In addition, there is a 36-nt RII(-)-derived sequence in R3(-)/art, shown with small letters, to facilitate the separation of homo- versus hetero-recombinants in these assays. Constructs GC1/art and AU1/art carry the art sequence and artificial GC-rich and AU-rich sequences (shaded), respectively (Cheng and Nagy, 2003). Note that all four templates are used for primer extension by the TCV RdRp in vitro. (B) Denaturing gel analysis of the template-switching assay. The RNA templates were used in equal amounts (15 pmol/reaction), except Mot1/pr (3 pmol/reaction), under the conditions described in the Materials and methods. The 3’ extension products are depicted with gray arrows, whereas the recombinants formed between identical templates (homo-recombinants) are marked with black arrows. Recombinants formed between the two different templates (hetero-recombinants) are marked with a black arrowhead. Note that R3(-)/art produced two different primer extension products due to the shown initiation (see panel A) and a cryptic initiation site.
the control assay, the combination of cPR21 and R3(-)/cPR, carrying the homologous TBSV REN, gave rise to readily detectable recombinants (control lane 1 + 2, Fig. 5B; 46.9% hetero-recombinants when compared to the de novo initiated product). In contrast, the combination of cPR21 and Mot1/cPR carrying the heterologous M1H REN did not result in detectable accumulation of hetero-recombinants (Fig. 5B, lane 1 + 3). Similarly, no template-switching products were observed in the control CNV RdRp assay that included cPR21 and AU1/cPR (Fig. 5B, lane 1 + 4). To test
if the lack of recombinant accumulation with the heterologous REN is due to inefficient initiation of CNV RdRp on the Mot1/cPR template, we replaced the heterologous cPR promoter sequence with the homologous 3\'-V\_PE promoter sequence (see construct Mot1/pr, Fig. 5A). This resulted in efficient initiation of RNA synthesis on Mot1/pr template (lane 6, Fig. 5C), but we still did not detect formation of hetero-recombinants between cPR\_21 and Mot1/pr (lane 1 + 6, Fig. 5C). Altogether, these data established that the CNV RdRp did not utilize the heterologous M1H REN efficiently in the in vitro template-switching assay.

**Heterologous REN stimulates template-switching activity of the recombinant HCV RdRp in vitro**

To further test if heterologous REN could support efficient template-switching, we also tested the HCV RdRp, which is distantly related to the CNV and TCV RdRps (O’Reilly and Kao, 1998). To make the HCV RdRp assays simpler, we used only single templates in these assays with the purified recombinant HCV RdRp obtained from *E. coli*
confirmed that they had similar junctions (Figs. 6C–D) to the cloning and sequencing of the homo-recombinants contaminants (Fig. 6B, lanes 3 and 4). RT-PCR amplification, GC1/cPR did not support homo-recombinants at detectable switching events. Overall, the obtained data in this work support previous findings that the HCV RdRp can use RNA templates efficiently for template-switching in vitro (Ranjith-Kumar et al., 2004).

Discussion

In contrast to the intra-virus recombination (occurring between strains of the same virus), our understanding of intervirus recombination, which occurs between two different viruses, is poor. This is mainly due to the infrequent isolation of intervirus recombinants (Aaziz and Tepfer, 1999a; Chapman et al., 1998; Goldbach, 1992; Masuta et al., 1998; Rao and Grantham, 1994). Accordingly, intervirus recombination faces many obstacles. First, different viruses must infect the same host cells and their RNAs must be available for recombination (i.e., they must be co-localized to the same replication complex). Second, template-switching by the viral replicase must occur between the different RNAs to form interviral recombinants. Third, the generated intervirus recombinants must be viable (replication-competent and infectious) in order to reach detectable levels. However, the abundance of virus-infected hosts, including double virus infections, favors the emergence of new intervirus recombinants from time to time. Indeed, the popular theory of modular virus evolution (Botstein, 1980; Dolja and Carrington, 1992; Gibbs, 1987) predicts that functional modules present in viral genomes could be reshuffled or recombined into other, even unrelated viruses. Comparison of large number of sequenced viral genomes strongly supports this model (Dolja and Carrington, 1992; Gibbs, 1987; Holmes et al., 1999; Worobey and Holmes, 1999). How do these functional modules participate in recombination? Are these events accidental (random) or nonrandom?

We tested the above model in vitro by using purified RdRp preparations of the related (but distinct) CNV and TCV and the more distantly related HCV. In addition, we used well-defined TBSV and TCV RdRps elements that have been shown to facilitate RNA recombination in vivo and in vitro (Cascone et al., 1993; Cheng and Nagy, 2003; Cheng et al., 2002; Nagy et al., 1998, 2001; Simon, 1999; Simon and Bujarski, 1994; White and Nagy, 2004). In contrast to the previous works, here we tested these elements in heterologous combinations (i.e., the TBSV REN with the TCV RdRp and the TCV REN with the CNV RdRp). Our in vitro assays revealed that the TCV RdRp was able to utilize the TBSV REN for template-switching, albeit less efficiently than it used its cognate REN (Fig. 3). Interestingly, the more distantly related HCV RdRp also utilized the TBSV REN as well as the TCV REN for template-switching in vitro (Fig. 6), arguing that RENs of various viruses could possibly promote intervirus recombination. However, this phenomenon does not seem to be universal, because the CNV RdRp did not utilize the heterologous M1H REN efficiently in vitro (Fig. 5).
The newly made intervirus recombinants are predicted to face rigorous selection pressure in their hosts. Therefore, it is unlikely that most intervirus recombination events would lead to the emergence of viable recombinants. However, heterologous REN elements could facilitate the postrecombinational amplification of the new recombinants if the viral RdRp can utilize the heterologous REN. Our data presented above (Fig. 2) support this model, because the TBSV REN enhanced RNA synthesis by ~3-fold in a trans-replication assay with the TCV RdRp. The same TBSV REN also stimulated RNA synthesis by the HCV RdRp by ~4- to 10-fold when compared to AU- and GC-rich templates (Fig. 6). Also, the M1H REN of TCV satC enhanced RNA synthesis by 3- to 8-fold in an HCV RdRp assay (Fig. 6). However, similar to template-switching, the stimulation of RNA synthesis by a heterologous REN does not seem to be universal, because the M1H REN of TCV satC did not enhance RNA synthesis in a trans-replication assay with the CNV RdRp (Fig. 2). It is currently unknown what is the reason for the difference in template utilization between the CNV RdRp and the TCV RdRp. The difference between these RdRps seems to be genuine because TCV RdRp prepared from plants and the recombinant TCV RdRp from E. coli show similar characteristics in in vitro assays when tested with several TCV- and TBSV-derived templates (Rajendran et al., 2002). Overall, these observations suggest that REN elements could be “functional modules” that facilitate intra- and interviral RNA recombination and possibly, postrecombinational amplification of the new recombinants.

Additional findings of this work are that the minimal promoter elements of TBSV and TCV are functional in the CNV, TCV and HCV RdRp assays. Also, all three RdRps showed preference for using the AU-rich template over the GC-rich template for RNA synthesis and template-switching (Figs. 3, 5 and 6) and Cheng and Nagy, 2003; Cheng et al., 2002). This observation supports previous models that AU-rich sequences can constitute recombination hot spots for a number of RNA viruses, including retroviruses and plus-stranded RNA viruses (DeStefano et al., 1994; Nagy and Bujarski, 1997, 1998; Pilipenko et al., 1995; Shapka and Nagy, 2004; Wu et al., 1995).

**Model on the dual function of REN in intervirus recombination**

The theory of modular evolution predicts that viruses should not only be able to recombine with other distinct viruses, but also some of the newly formed interviral recombinants should be viable/replication-competent under certain conditions. Our testing of the effect of heterologous REN on complementary RNA synthesis (Fig. 2) is compatible with this assumption. Therefore, we propose that the same REN element that promotes RNA recombination, also can stimulate the amplification of the newly generated recombinants (Fig. 7). This dual function of REN in recombination and postrecombinational replication is likely the result of binding of the REN to the viral RdRp as shown earlier (Nagy et al., 1999; Panavas and Nagy, 2005). The binding of the RdRp to the REN is proposed to increase the local concentration of the RdRp on the template RNAs (Panavas and Nagy, 2005). This, in turn, is expected to increase the ability of the recombinant RNA to compete with the parental or other viruses, at least under certain conditions.

It is unlikely that the newly emerged interviral recombinant carrying the heterologous REN is highly adapted to its environment. However, further evolution of the recombinant RNA via point mutations or additional recombination events could lead to the emergence of highly adapted recombinants (Fig. 7). The proposed further evolution of REN-containing modules could make it difficult to identify these modules via sequence comparison among viruses. Interestingly, the emergence of satC RNA might have occurred via the above mechanism, because it was formed by multiple recombination events and accumulation of mutations (Simon, 1999). Moreover, the M1H REN present within the satC genome contains sequence stretches similar to promoter-like sequences of TCV.

Altogether, the data presented in this paper are consistent with the model that REN elements might actively promote intervirus recombination and increase the replication potential of new recombinants; thus facilitating modular evolution of RNA viruses. Acquiring of heterologous REN or other cis-acting sequences via intervirus recombination could be beneficial for viruses to speed up their adaptation to their hosts or “jumping” to new hosts.

**Materials and methods**

**Plant inoculation and CNV RdRp preparation**

*Nicotiana benthamiana* plants were inoculated with CNV genomic RNA transcripts obtained by standard T7 RNA transcription using *Sma*I cut clone of pK2/Msp20STOP for CNV (Rochon, 1991). CNV RdRp preparations were
obtained from systemically infected leaves as described (Nagy and Pogany, 2000).

Recombinant TCV and HCV RdRp preparations

The N-terminally truncated, highly active TCV RdRp (termed p88C) fused to the maltose binding protein was affinity-purified from *E. coli* as described (Rajendran et al., 2002). The highly active, C-terminally truncated recombinant NS5B HCV RdRp, expressed in *E. coli*, was purified to near homogeneity using Nickel-column chromatography as described (Luo et al., 2000; Zhang et al., 2005).

Production of RNA templates

For the in vitro experiments, single-stranded RNA templates were obtained by in vitro transcription reaction with T7 RNA polymerase using PCR amplified DNA templates (Nagy and Pogany, 2000; Nagy et al., 1997). The templates used in template-switching assays were described previously (Cheng and Nagy, 2003). The DNA templates for the trans-replication assay were produced using PCR as follows: (i) to generate the template strand, we made clamp(+)cPR and clamp(+)3PE using primer pairs of 

#526 (5'-TAATACGACTCACTATAGG-3') and #515 (TGG-GTTATGATGGГTTTCT-3') as well as #526 and #627 (AAGGGTTTCAATAGGAGGCT-TATTTACTCGCTCTGCAGT), respectively; (ii) to generate the partially complementary strand, we made clamp(−) and SL1-III-br clamp(−) and using primer pairs: #523 (ATATCACACCTGTCTCGAGAGGA) and #514 (TAATACGACTCACTATAGAGTTACTCGCTCTGCAGT), as well as #523 and #535 (TAATACGACTCACTATAGATCTCAAATCTGAGCTGA); (iii) to generate the partially complementary strand for M1H/clamp(−), we performed two-step PCR, first using primer pair of #523 and #628 (CCCATTACCCCTTTTGGCTGGGTCTGGTCTCTC- TACTCGCTCTGCAGT), followed by using primer pair of #523 and #629 (TAATACGACTCACTATAGGACCATT- TACCCTTTGGCT). To make the partly double-stranded constructs for the trans-replication assay, two partially complementary, heat denatured single-stranded RNA transcripts (94 °C for 2 min) were annealed in STE buffer (10 mM TRIS, pH 8.0, 1 mM EDTA and 100 mM NaCl), followed by slow cooling (in 30 min) to 25 °C. The annealed RNAs were loaded onto 5% nondenaturing 15 cm long polyacrylamide gel. After electrophoresis, the gels were stained with ethidium bromide and the annealed RNA bands were excised. The annealed RNAs were eluted into 0.6 M ammonium acetate, followed by phenol/chloroform extraction and ethanol precipitation. RdRp reactions (50 μl) were carried out as previously described (Nagy and Pogany, 2000).

RdRp assays

The CNV and TCV RdRp assays were performed using the same buffer as described (Cheng and Nagy, 2003; Nagy and Pogany, 2000; Rajendran et al., 2002). The HCV RdRp assay was performed in Mn-containing buffer as described previously (Luo et al., 2000). Each RdRp reaction contained 0.5 μg of template RNA (Cheng and Nagy, 2003). After phenol/chloroform extraction, the RdRp products were analyzed under complete denaturing conditions (5% PAGE gels containing 40% formamide and 8 M urea, with the electrophoresis performed at 70 °C in a Bio-Rad DCode apparatus), followed by phosphorimager analysis as described (Cheng and Nagy, 2003; Nagy and Pogany, 2000). The quantified data were normalized based on the estimated number of 32P-UTP incorporated to each product (Cheng and Nagy, 2003; Panavas et al., 2002b).
RT-PCR and sequencing analysis

To detect recombinants in the RdRp assays, the recombinant RNA-containing bands were cut from denaturing gels and the RNAs were isolated as described earlier (Cheng and Nagy, 2003). The use of the recombinant-sized, gel-isolated RdRp products was expected to eliminate the possibility of recombination during the RT-PCR analysis. The RT reaction included the reverse primer #23 (GTAAACGACTCACTATA-
AGGGACCCCAAAAGAAGTACCTG), which anneals to the 3’ end of complementary R3(−)/art, while the PCR reaction also contained the forward primer #251 (TTGGA-
AAATTTCTCTTGCTGAGTGGATCC) to detect template-switching from AU1/art to R3(−)/art. To detect template-switching from R3(−)/art to AU1/art, we used reverse primer #248 (GTAAACGACTCACTATA-
GGACCCCTGCAAGTACCTG), which anneals to the 3’ end of the complementary AU1/art sequence for RT and the forward primer #18 (GTAAACGACTCACTATA-
GGAGAAAGCCTGCAAGTACCTG) for PCR. The obtained RT-PCR products were either gel-isolated or directly cloned into pGEM-T Easy vector (Promega). A representative number of independent clones were sequenced using the M13/pUC19 reverse primer (Gibco BRL).

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References


