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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 (RdRp), 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

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can also help viruses repair their genomes or generate defective interfering (DI) RNAs (Allison et al., 1990; Ayllon 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-

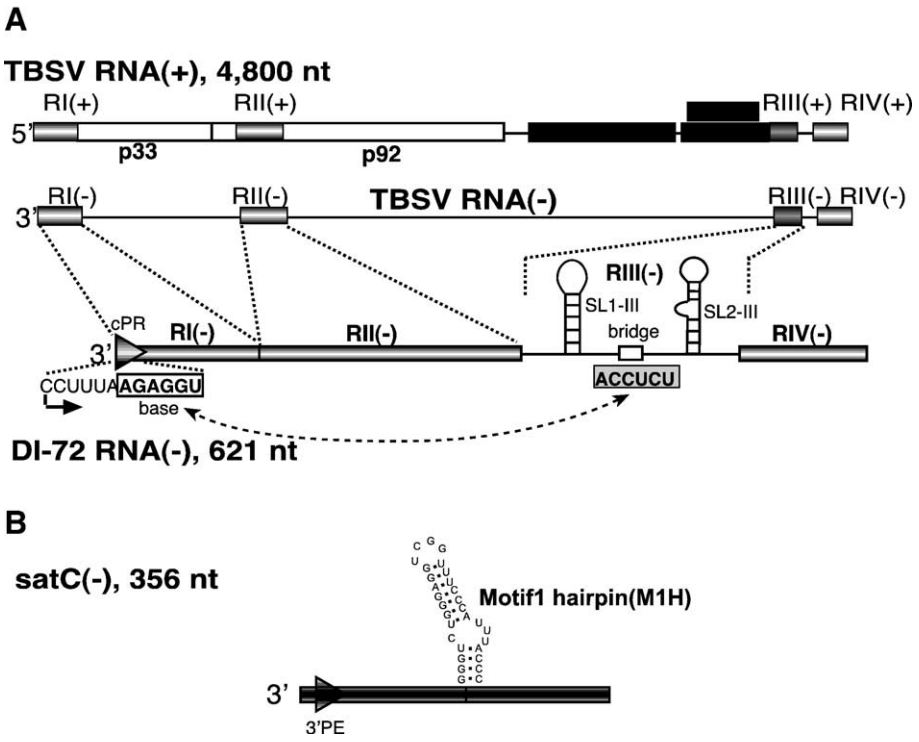


Fig. 1. (A) A schematic presentation of a typical tombusvirus genome [TBSV, top: plus-strand with five genes and middle: minus strand, shown in 3′-to-5′ that carries the RIII(−) REN] and a prototypical DI RNA (DI-72, minus-strand is shown). The four noncontiguous regions that are present in the DI-72 RNA are indicated with gray boxes. RIII(−) includes the replication enhancer, which consists of two stem-loops and a single-stranded bridge sequence. The bridge sequence is thought to interact with the base sequence in the minimal plus-strand initiation promoter (cPR), which contains the initiation site as indicated with a solid arrow. (B) Schematic presentation of satC RNA, which is associated with TCV. The location of the M1H replication enhancer and the 3′PE (that supports initiation in the in vitro RdRp assay) are shown in the minus-strand RNA.

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(−)] 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 of either TBSV or satC of TCV, termed cPR and 3′PE, respectively) 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(−), 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

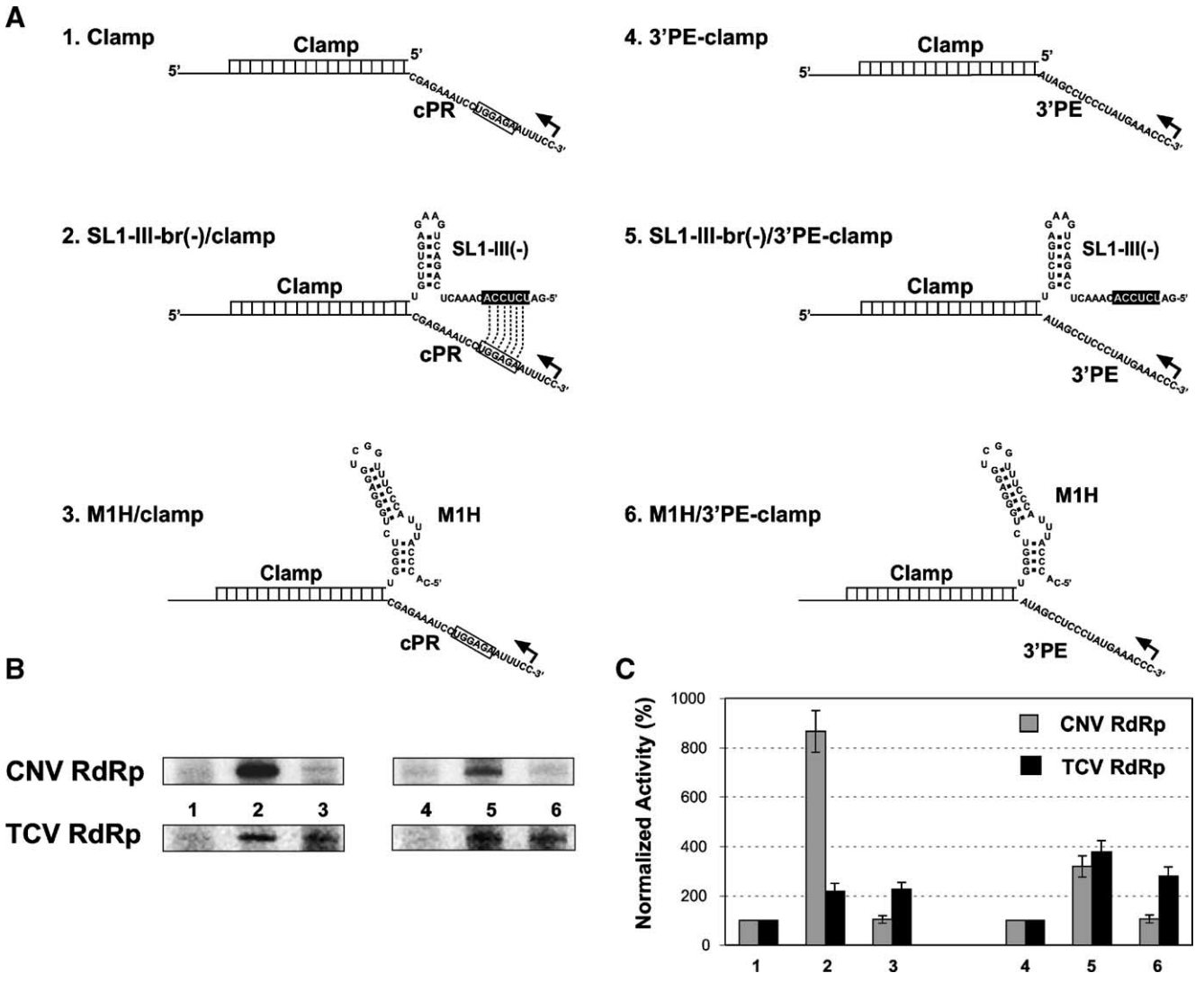


Fig. 2. Stimulation of complementary RNA synthesis by heterologous REN in *trans*. (A) Schematic representation of RNAs used in the in vitro *trans*-replication assay. Each of the shown templates consists of two RNA strands. The template strand contains either the cPR promoter (constructs 1–3) or the 3′PE (constructs 4–6) at the 3′ location and the clamp sequence. The nontemplate strand carries the complementary sequence of the clamp, which can base-pair with the template RNA (schematically shown by a ladder). Two of the constructs carry the minimal TBSV REN [SL1-III(−) with the bridge sequence], whereas additional two constructs contain M1H from satC of TCV. Note that the template strand and the nontemplate strand can form identical "clamp" structures. (B) Representative denaturing gel of ^{32}P-labeled RdRp products synthesized by in vitro transcription with either the CNV RdRp or the TCV RdRp, as shown. The gel-isolated annealed RNAs were used in equal molar amounts. (C) Relative stimulation of RNA synthesis by the REN elements. The amount of RNA products obtained (see panel B) was quantified using a phosphoimager. Each experiment was repeated two or three times.

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 *trans* by ~9-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 ~3-fold from the heterologous 3′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 *cis*-acting elements, albeit with different efficiency. We also observed ~2.5- 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 *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 3′PE 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 3′PE 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 3′PE 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 ^{32}P-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., RIII(−), 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

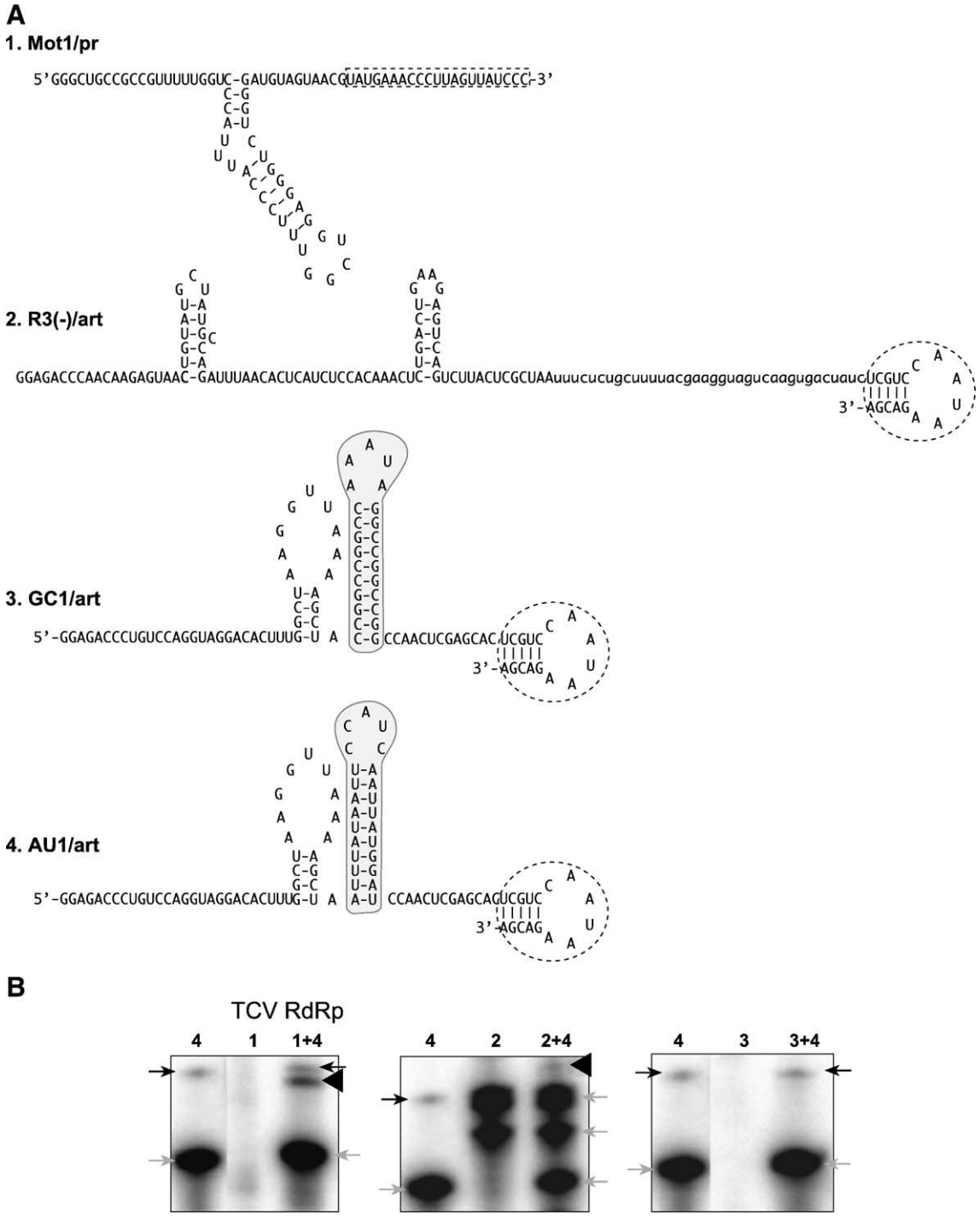


Fig. 3. Stimulation of template-switching by the heterologous REN in the TCV RdRp assay in vitro. (A) Sequences and structures of RNA templates used in the TCV RdRp reactions. Construct Mot1/pr contains the minus-stranded M1H replication enhancer (shown as the hairpin structure) and the 3′ proximal plus-strand 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 circuled). 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 the shown initiation (see panel A) and a cryptic initiation site.

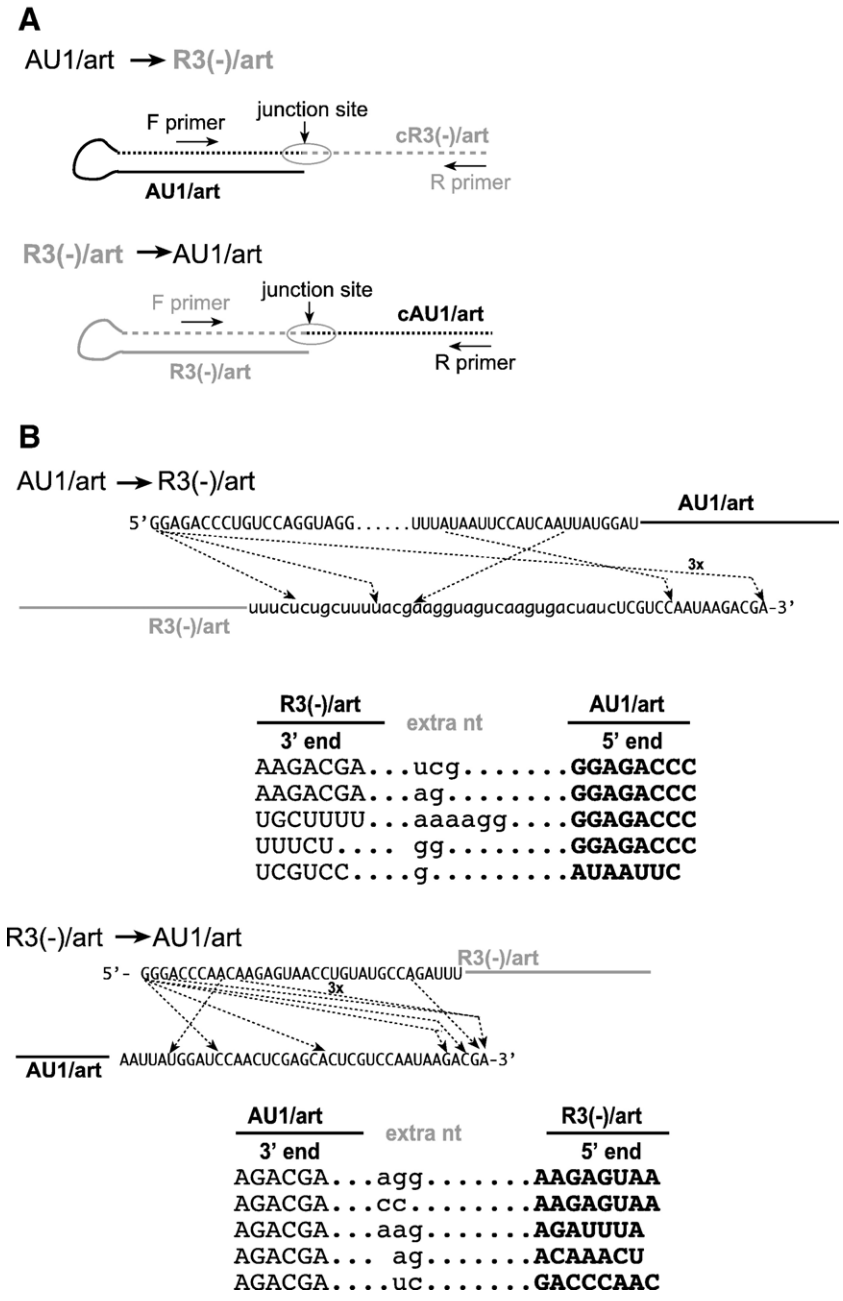


Fig. 4. (A) Schematic representation of the strategy used for the RT-PCR analysis of the hetero-recombinants obtained in the TCV RdRp assay. Two different sets of primers were used to detect the two types of hetero-recombinants formed (depending on which template was used as a donor during the recombination events). The dotted lines represent the newly synthesized RNA strands, which are complementary to the original templates (as indicated by letter "c" in front of the names of the RNAs). (B) Sequence analysis of the junction sites in the hetero-recombinants. After the RT-PCR analysis, the bands representing the hetero-recombinants were gel-isolated, cloned in *E. coli* and a representative number of clones were sequenced. Arrows indicate the template-switching by the recombinant TCV RdRp from the donor template (top) to the acceptor template (bottom). The frequencies of clones with identical sequences are indicated by numbers next to the arrows. Those recombinants that contained extra sequences (not derived from the template) at the junction sites are also shown at the bottom of each panel. The presence of extra nucleotides at the junction sites in recombinant templates is consistent with a TCV polymerase-mediated mechanism. The extra sequences in recombinants formed by end-to-end (but not in case of the internal positions) template-switches can also be the result of T7-polymerase-mediated nontemplate additions.

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

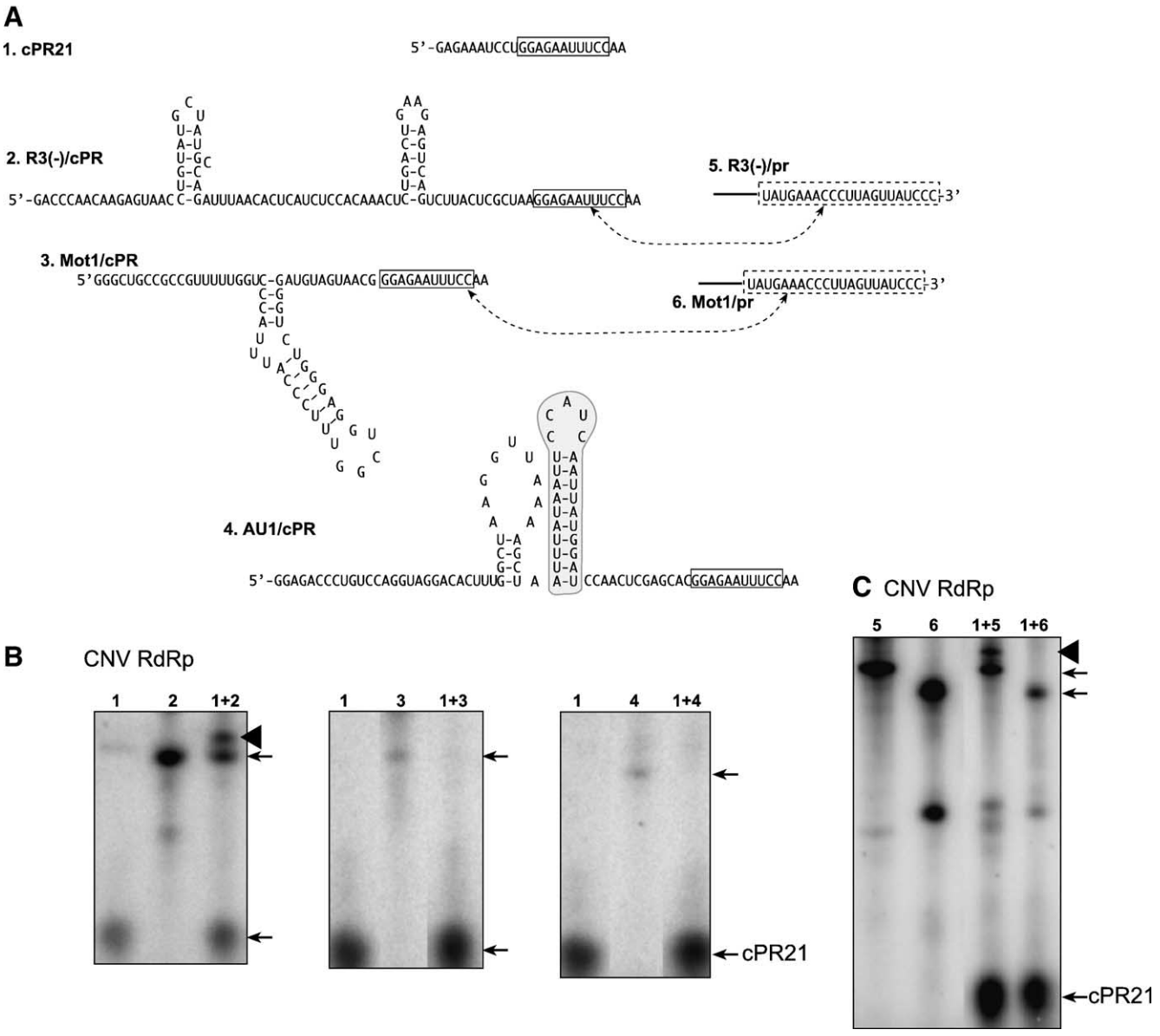


Fig. 5. Absence of stimulation of template-switching by the heterologous REN in the CNV RdRp assay in vitro. (A) Sequences and structures of RNA templates used in the CNV RdRp reactions. The minus-stranded 3′-terminal cPR promoter of the related TBSV is boxed, whereas the 3′PE of satC is boxed with dotted line. Note that only the altered 3′ ends are shown in constructs 5 and 6, whereas the remaining not shown sequences are the same as in constructs 2 and 3, respectively. See Fig. 3 for further details. (B–C) Denaturing gel analyses of the template-switching assays. The de novo RdRp products are depicted with arrows, whereas the hetero-recombinants formed between the two different templates are marked with black arrowhead. Note that CNV RdRp initiates de novo on each template, but with variable efficiency.

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′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 cPR21 and Mot1/pr (lane 1 + 6, Fig. 5C). This is in contrast with the efficient generation of hetero-recombinants between cPR21 and R3(−)/pr carrying the homologous TBSV REN (lane 1 + 5, 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*

(Luo et al., 2000; Zhang et al., 2005). We expected that the templates, which were also tested above in the CNV and TCV RdRp assays, might support complementary RNA synthesis and template-switching (to form homo-recombinants) in vitro.

Based on using four separate templates with the TBSV REN, the TCV REN, the AU1 and GC1 sequences, respectively, we found that the HCV RdRp was able to initiate RNA synthesis de novo and it also performed 3′-terminal extension (Fig. 6), similar to CNV and TCV RdRps (Guan et al., 2000; Nagy and Pogany, 2000; Panaviene et al., 2004; Rajendran et al., 2002). Template R3(−)/cPR carrying the TBSV RIII(−) REN supported the most efficient de novo initiation (100%) and 3′-terminal extension (35%) in the standard HCV RdRp assay (Fig. 6B, lane 1). Construct Mot1/pr carrying the M1H REN of TCV satC was almost as efficient in de novo initiation (81%) and 3′-terminal extension (33%) (Fig. 6B, lane 2), whereas constructs GC1/cPR and AU1/cPR carrying GC- and AU-rich sequences, respectively, supported inefficient de novo initiation (9% and 26%, respectively) and 3′-terminal extension (3% and 11%) (Fig. 6B, lanes 3 and 4). These data indicate that the complementary RNA synthesis by the HCV RdRp can be stimulated by the heterologous RENs more efficiently than by artificial sequences. In addition, we conclude that the TBSV and the TCV satC-derived minimal promoters are sufficient to support initiation of complementary RNA synthesis by the HCV RdRp in vitro.

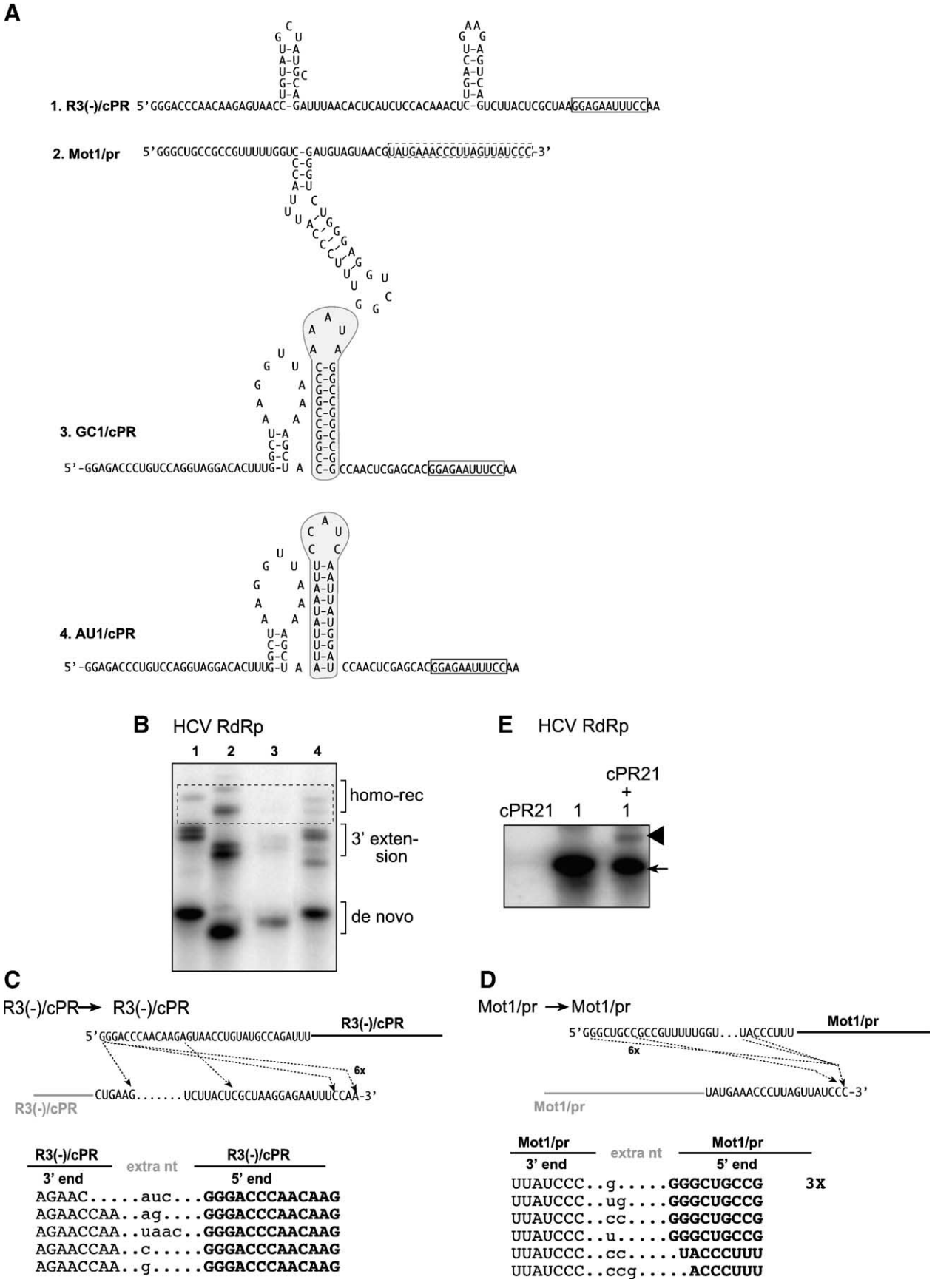
Interestingly, homo-recombinants were also observed when the template RNAs contained either RIII(−) or M1H RENs (constructs R3(−)/cPR and Mot1/pr, lanes 1 and 2 in Fig. 6B). The efficiency of template-switching by the HCV RdRp was 7% and 3% for R3(−)/cPR and Mot1/pr, respectively, when compared to de novo product observed with R3(−)/cPR (100%). In contrast, construct GC1/cPR did not support homo-recombinants at detectable levels, while construct AU1/cPR gave 0.5% homo-recombinants (Fig. 6B, lanes 3 and 4). RT-PCR amplification, cloning and sequencing of the homo-recombinants confirmed that they had similar junctions (Figs. 6C–D) to the homo-recombinants obtained with CNV and TCV RdRps (Cheng and Nagy, 2003). These data confirmed that the HCV RdRp could utilize the heterologous RENs for template-switching in vitro.

To test if the HCV RdRp could also support the formation of hetero-recombinants, we performed template-switching assays with the combination of cPR21 and R3(−)/cPR templates. These experiments revealed the formation of hetero-recombinants in the HCV RdRp assay (lane cPR21 + 1 in Fig. 6E) that were 17% of the de novo product (marked with an arrow in Fig. 6E). Thus, the efficiency of formation of hetero-recombinants was ~2.5-fold less in the HCV RdRp assay than in the CNV RdRp assay (Fig. 4B, lane 1 + 2). Nevertheless, the data are consistent with the model that HCV RdRp utilized the heterologous REN during template-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 REN 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).


A
1. R3(-)/cPR 5'GGGACCCAACAAGAGUAACC-GAUUUAACACUCAUCUCCACAAACUC-GUCUUACUCGCUAAGGAGAAUUUCCAA
2. Mot1/pr 5'GGGCUGCCGCCGUUUUUGGUC-GAUGUAGUAACGUAUGAAACCCUUAGUUAUCCC-3'
3. GC1/cPR
5'-GGAGACCCUGUCCAGGUAGGACACUUUG-U A C-G CCAACUCGAGCACGGAGAAUUUCCAA
4. AU1/cPR
5'-GGAGACCCUGUCCAGGUAGGACACUUUG-U A A-U CCAACUCGAGCACGGAGAAUUUCCAA
B HCV RdRp
1 2 3 4
homo-rec
3' exten-
sion
de novo
E HCV RdRp
cPR21
cPR21 1 +
1
C
R3(-)/cPR→ R3(-)/cPR
5'GGGACCCAACAAGAGUAACCUGUAUGCCAGAUUU
R3(-)/cPR
6x
CUGAAG.....UCUUACUCGCUAAGGAGAAUUUCCAA-3'
R3(-)/cPR
R3(-)/cPR extra nt R3(-)/cPR
3' end 5' end
AGAAC.....auc...GGGACCCAACAAG
AGAACCAA..ag....GGGACCCAACAAG
AGAACCAA..uaac..GGGACCCAACAAG
AGAACCAA..c.....GGGACCCAACAAG
AGAACCAA..g.....GGGACCCAACAAG
D
Mot1/pr →Mot1/pr
5'GGGCUGCCGCCGUUUUUGGU...UACCCUUU
Mot1/pr
6x
UAUGAAACCCUUAGUUAUCCC-3'
Mot1/pr
Mot1/pr extra nt Mot1/pr
3' end 5' end
UUAUCCC..g.....GGGCUGCCG 3X
UUAUCCC..ug....GGGCUGCCG
UUAUCCC..cc....GGGCUGCCG
UUAUCCC..u.....GGGCUGCCG
UUAUCCC..cc.....UACCCUUU
UUAUCCC..ccg.....ACCCUUU

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 could 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/M5p20STOP for CNV (Rochon, 1991). CNV RdRp preparations were

Fig. 6. Stimulation of template-switching by the heterologous REN in the HCV RdRp assay in vitro. (A) Sequences and structures of RNA templates used in the HCV RdRp reactions. See further details in Fig. 3. (B) Denaturing gel analysis of the template-switching assay. The de novo, 3′ extension and homo-recombinants (boxed) products are bracketed. (C–D) Sequence analyses of the junction sites in the homo-recombinants. After the RT-PCR analysis, the bands representing the homo-recombinants were gel-isolated, cloned in *E. coli* and a representative number of clones was sequenced. Arrows indicate the template-switching by the recombinant HCV RdRp from the donor template (top) to the acceptor template (bottom). See further details in the legend in Fig. 4. (E) Detection of hetero-recombinants in the HCV RdRp assay. See further details in the legend in Fig. 5. The presence of extra nucleotides at the junction sites in recombinant templates is consistent with a HCV polymerase-mediated mechanism. Indeed, the ability to add extra nucleotides to the end of RNA templates by the HCV RdRp has been shown previously (Ranjith-Kumar et al., 2001).

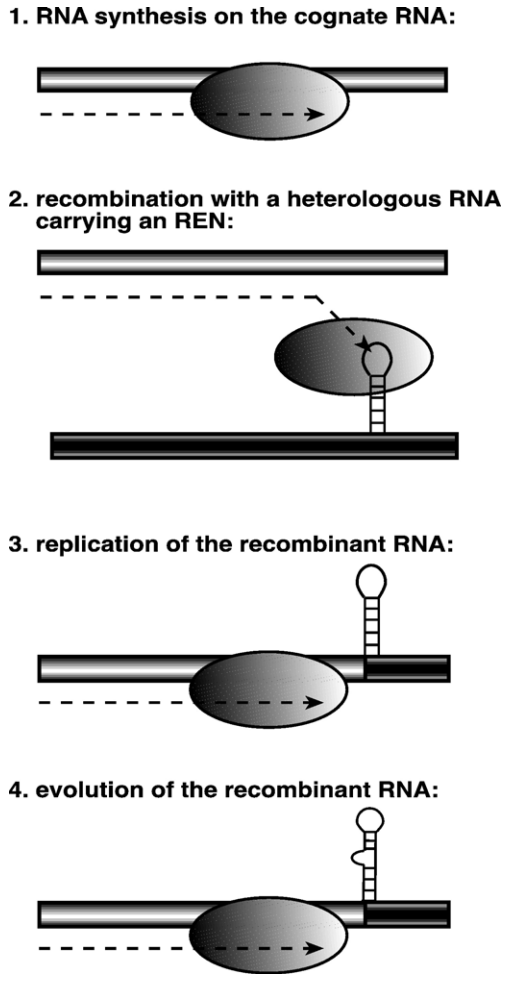


Fig. 7. Model on the role of REN in intra-viral recombination. Template-switching by the viral RdRp to a heterologous template carrying an REN element (indicated by a stem-loop structure) can lead to the formation of a recombinant viral RNA. The REN is predicted to be actively involved in guiding the "jumping" viral RdRp during recombination events and also the postrecombinational amplification of the new recombinant RNA. Subsequent replication and evolution of the recombinant viral RNA, via either additional recombination event(s) or point mutations, are predicted to give rise to a better-adapted virus.

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(+)/3′PE using primer pairs of #526 (5′-TAATACGACTCACTATAGGAC-TTGGGTATGATGGGTTTC-3′) and #515 (TTGG-AAATTCTCCAGGATTTCTCGTTACTCGCTCTGCAGT) as well as #526 and #627 (AAGGGTTTCATAGGGAGGC-TATTTACTCGCTCTGCAGT), respectively; (ii) to generate the partially complementary strand, we made clamp(−) and SL1-III-br/clamp(−) and using primer pairs: #523 (ATAT-CACACCTGTCTCCGAGAGGGA) and #514 (TAATAC-GACTCACTATAGGCTTACTCGCTCTGCAGT), as well as #523 and #535 (TAATACGACTCACTATAGATCTCCA-CAAACTCAGACTGA); (iii) to generate the partially complementary strand for M1H/clamp(−), we performed two-step PCR, first using primer pair of #523 and #628 (CCCATTTACCCTTTGGCTGGAGGGTCTGGGTCT-TACTCGCTCTGCAGT), followed by using primer pair of #523 and #629 (TAATACGACTCACTATAGGACCCATT-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 ^{32}P-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 (GTAATACGACTCACTATAGGGACCCAACAAGAGTAACCTG), which anneals to the 3′ end of complementary R3(−)/art, while the PCR reaction also contained the forward primer #251 (TTGGAAATTCTCCTTGTGCTCGAGTTGGATCC) 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 (GTAATACGACTCACTATAGGAGACCCTGTCCAGGTAG), which anneals to the 3′ end of the complementary AU1/art sequence for RT and the forward primer #18 (GTAATACGACTCACTATAGGAGAAAGCGAGTAAGACAG) 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

Aaziz, R., Tepfer, M., 1999a. Recombination between genomic RNAs of two cucumoviruses under conditions of minimal selection pressure. Virology 263 (2), 282–289.

Aaziz, R., Tepfer, M., 1999b. Recombination in RNA viruses and in virus-resistant transgenic plants. J. Gen. Virol. 80 (Pt. 6), 1339–1346.

Ahlquist, P., 2002. RNA-dependent RNA polymerases, viruses, and RNA silencing. Science 296 (5571), 1270–1273.

Ahlquist, P., Noueiry, A.O., Lee, W.M., Kushner, D.B., Dye, B.T., 2003. Host factors in positive-strand RNA virus genome replication. J. Virol. 77 (15), 8181–8186.

Allison, R., Thompson, C., Ahlquist, P., 1990. Regeneration of a functional RNA virus genome by recombination between deletion mutants and requirement for cowpea chlorotic mottle virus 3a and coat genes for systemic infection. Proc. Natl. Acad. Sci. U.S.A. 87 (5), 1820–1824.

Ayllon, M.A., Lopez, C., Navas-Castillo, J., Mawassi, M., Dawson, W.O., Guerri, J., Flores, R., Moreno, P., 1999. New defective RNAs from citrus tristeza virus: evidence for a replicase-driven template switching mechanism in their generation. J. Gen. Virol. 80 (Pt. 3), 817–821.

Biebricher, C.K., Luce, R., 1992. In vitro recombination and terminal elongation of RNA by Q beta replicase. EMBO J. 11 (13), 5129–5135.

Borja, M., Rubio, T., Scholthof, H.B., Jackson, A.O., 1999. Restoration of wild-type virus by double recombination of tombusvirus mutants with a host transgene. Mol. Plant-Microbe Interact. 12 (2), 153–162.

Botstein, D., 1980. A theory of modular evolution for bacteriophages. Ann. N. Y. Acad. Sci. 354, 484–490.

Buck, K.W., 1996. Comparison of the replication of positive-stranded RNA viruses of plants and animals. Adv. Virus Res. 47, 159–251.

Cascone, P.J., Haydar, T.F., Simon, A.E., 1993. Sequences and structures required for recombination between virus-associated RNAs. Science 260 (5109), 801–805.

Chapman, M.R., Rao, A.L., Kao, C.C., 1998. Sequences 5′ of the conserved tRNA-like promoter modulate the initiation of minus-strand synthesis by the brome mosaic virus RNA-dependent RNA polymerase. Virology 252 (2), 458–467.

Cheng, C.P., Nagy, P.D., 2003. Mechanism of RNA recombination in carmo- and tombusviruses: evidence for template switching by the RNA-dependent RNA polymerase in vitro. J. Virol. 77 (22), 12033–12047.

Cheng, C.P., Pogany, J., Nagy, P.D., 2002. Mechanism of DI RNA formation in tombusviruses: dissecting the requirement for primer extension by the tombusvirus RNA dependent RNA polymerase in vitro. Virology 304 (2), 460–473.

Chetverin, A.B., Chetverina, H.V., Demidenko, A.A., Ugarov, V.I., 1997. Nonhomologous RNA recombination in a cell-free system: evidence for a transesterification mechanism guided by secondary structure. Cell 88 (4), 503–513.

DeStefano, J.J., Bambara, R.A., Fay, P.J., 1994. The mechanism of human immunodeficiency virus reverse transcriptase-catalyzed strand transfer from internal regions of heteropolymeric RNA templates. J. Biol. Chem. 269 (1), 161–168.

Dolja, V.V., Carrington, J.C., 1992. Evolution of positive-strand RNA viruses. Semin. Virol. 3, 315–326.

Dreher, T.W., 1999. Functions of the 3′-untranslated regions of positive strand Rna viral genomes. Annu. Rev. Phytopathol. 37, 151–174.

Figlerowicz, M., Nagy, P.D., Bujarski, J.J., 1997. A mutation in the putative RNA polymerase gene inhibits nonhomologous, but not homologous, genetic recombination in an RNA virus. Proc. Natl. Acad. Sci. U.S.A. 94 (5), 2073–2078.

Gibbs, A., 1987. Molecular evolution of viruses; 'trees', 'clocks' and 'modules'. J. Cell Sci., Suppl. 7, 319–337.

Gmyl, A.P., Belousov, E.V., Maslova, S.V., Khitrina, E.V., Chetverin, A.B., Agol, V.I., 1999. Nonreplicative RNA recombination in poliovirus. J. Virol. 73 (11), 8958–8965.

Goldbach, R., 1992. The recombinative nature of potyviruses: implications for setting up true phylogenetic taxonomy. Arch. Virol. Suppl 5, 299–304.

Guan, H., Carpenter, C.D., Simon, A.E., 2000. Analysis of *cis*-acting sequences involved in plus-strand synthesis of a turnip crinkle virus-associated satellite RNA identifies a new carmovirus replication element. Virology 268 (2), 345–354.

Havelda, Z., Dalmay, T., Burgyan, J., 1997. Secondary structure-dependent evolution of Cymbidium ringspot virus defective interfering RNA. J. Gen. Virol. 78 (Pt. 6), 1227–1234.

Hillman, B.I., Carrington, J.C., Morris, T.J., 1987. A defective interfering RNA that contains a mosaic of a plant virus genome. Cell 51 (3), 427–433.

Holmes, E.C., Worobey, M., Rambaut, A., 1999. Phylogenetic evidence for recombination in dengue virus. Mol. Biol. Evol. 16 (3), 405–409.

Kao, C.C., Singh, P., Ecker, D.J., 2001. De novo initiation of viral RNA-dependent RNA synthesis. Virology 287 (2), 251–260.

Kim, M.J., Kao, C., 2001. Factors regulating template switch in vitro by viral RNA-dependent RNA polymerases: implications for RNA–RNA recombination. Proc. Natl. Acad. Sci. U.S.A. 98 (9), 4972–4977.

Lai, M.M., 1992. RNA recombination in animal and plant viruses. Microbiol. Rev. 56 (1), 61–79.

Lai, M.M., 1998. Cellular factors in the transcription and replication of viral RNA genomes: a parallel to DNA-dependent RNA transcription. Virology 244 (1), 1–12.

Luo, G., Hamatake, R.K., Mathis, D.M., Racela, J., Rigat, K.L., Lemm, J., Colonno, R.J., 2000. De novo initiation of RNA synthesis by the RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. J. Virol. 74 (2), 851–863.

Masuta, C., Ueda, S., Suzuki, M., Uyeda, I., 1998. Evolution of a quadripartite hybrid virus by interspecific exchange and recombination between replicase components of two related tripartite RNA viruses. Proc. Natl. Acad. Sci. U.S.A. 95 (18), 10487–10492.

Miller, W.A., Dinesh-Kumar, S.P., Paul, C.P., 1995. Luteovirus gene expression. Crit. Rev. Plant Sci. 14, 179–211.

Monkewich, S., Lin, H.X., Fabian, M.R., Xu, W., Na, H., Ray, D., Chernysheva, O.A., Nagy, P.D., White, K.A., 2005. The p92 polymerase coding region contains an internal RNA element required at an early step in tombusvirus genome replication. J. Virol. 79 (8), 4848–4858.

Nagy, P.D., Bujarski, J.J., 1992. Genetic recombination in brome mosaic virus: effect of sequence and replication of RNA on accumulation of recombinants. J. Virol. 66 (11), 6824–6828.

Nagy, P.D., Bujarski, J.J., 1993. Targeting the site of RNA–RNA recombination in brome mosaic virus with antisense sequences. Proc. Natl. Acad. Sci. U.S.A. 90 (14), 6390–6394.

Nagy, P.D., Bujarski, J.J., 1995. Efficient system of homologous RNA recombination in brome mosaic virus: sequence and structure requirements and accuracy of crossovers. J. Virol. 69 (1), 131–140.

Nagy, P.D., Bujarski, J.J., 1996. Homologous RNA recombination in brome mosaic virus: AU-rich sequences decrease the accuracy of crossovers. J. Virol. 70 (1), 415–426.

Nagy, P.D., Bujarski, J.J., 1997. Engineering of homologous recombination hotspots with AU-rich sequences in brome mosaic virus. J. Virol. 71 (5), 3799–3810.

Nagy, P.D., Bujarski, J.J., 1998. Silencing homologous RNA recombination hot spots with GC-rich sequences in brome mosaic virus. J. Virol. 72 (2), 1122–1130.

Nagy, P.D., Pogany, J., 2000. Partial purification and characterization of Cucumber necrosis virus and Tomato bushy stunt virus RNA-dependent RNA polymerases: similarities and differences in template usage between tombusvirus and carmovirus RNA-dependent RNA polymerases. Virology 276 (2), 279–288.

Nagy, P.D., Simon, A.E., 1997. New insights into the mechanisms of RNA recombination. Virology 235 (1), 1–9.

Nagy, P.D., Dzianott, A., Ahlquist, P., Bujarski, J.J., 1995. Mutations in the helicase-like domain of protein 1a alter the sites of RNA–RNA recombination in brome mosaic virus. J. Virol. 69 (4), 2547–2556.

Nagy, P.D., Carpenter, C.D., Simon, A.E., 1997. A novel 3′-end repair mechanism in an RNA virus. Proc. Natl. Acad. Sci. U.S.A. 94 (4), 1113–1118.

Nagy, P.D., Zhang, C., Simon, A.E., 1998. Dissecting RNA recombination in vitro: role of RNA sequences and the viral replicase. EMBO J. 17 (8), 2392–2403.

Nagy, P.D., Pogany, J., Simon, A.E., 1999. RNA elements required for RNA recombination function as replication enhancers in vitro and in vivo in a plus-strand RNA virus. EMBO J. 18 (20), 5653–5665.

Nagy, P.D., Pogany, J., Simon, A.E., 2001. In vivo and in vitro characterization of an RNA replication enhancer in a satellite RNA associated with turnip crinkle virus. Virology 288 (2), 315–324.

O'Reilly, E.K., Kao, C.C., 1998. Analysis of RNA-dependent RNA polymerase structure and function as guided by known polymerase structures and computer predictions of secondary structure. Virology 252 (2), 287–303.

Panavas, T., Nagy, P.D., 2003a. The RNA replication enhancer element of tombusviruses contains two interchangeable hairpins that are functional during plus-strand synthesis. J. Virol. 77 (1), 258–269.

Panavas, T., Nagy, P.D., 2003b. Yeast as a model host to study replication and recombination of defective interfering RNA of Tomato bushy stunt virus. Virology 314 (1), 315–325.

Panavas, T., Nagy, P.D., 2005. Mechanism of stimulation of plus-strand synthesis by an RNA replication enhancer in a tombusvirus. J. Virol. 79, 9777–9785.

Panavas, T., Pogany, J., Nagy, P.D., 2002a. Analysis of minimal promoter sequences for plus-strand synthesis by the Cucumber necrosis virus RNA-dependent RNA polymerase. Virology 296 (2), 263–274.

Panavas, T., Pogany, J., Nagy, P.D., 2002b. Internal initiation by the cucumber necrosis virus RNA-dependent RNA polymerase is facilitated by promoter-like sequences. Virology 296 (2), 275–287.

Panaviene, Z., Nagy, P.D., 2003. Mutations in the RNA-binding domains of tombusvirus replicase proteins affect RNA recombination in vivo. Virology 317 (2), 359–372.

Panaviene, Z., Panavas, T., Serva, S., Nagy, P.D., 2004. Purification of the cucumber necrosis virus replicase from yeast cells: role of coexpressed viral RNA in stimulation of replicase activity. J. Virol. 78 (15), 8254–8263.

Pilipenko, E.V., Gmyl, A.P., Agol, V.I., 1995. A model for rearrangements in RNA genomes. Nucleic Acids Res. 23 (11), 1870–1875.

Pogany, J., Fabian, M.R., White, K.A., Nagy, P.D., 2003. A replication silencer element in a plus-strand RNA virus. EMBO J. 22 (20), 5602–5611.

Pogany, J., White, K.A., Nagy, P.D., 2005. Specific binding of tombusvirus replication protein p33 to an internal replication element in the viral RNA is essential for replication. J. Virol. 79 (8), 4859–4869.

Rajendran, K.S., Pogany, J., Nagy, P.D., 2002. Comparison of turnip crinkle virus RNA-dependent RNA polymerase preparations expressed in *Escherichia coli* or derived from infected plants. J. Virol. 76 (4), 1707–1717.

Ranjith-Kumar, C.T., Gajewski, J., Gutshall, L., Maley, D., Sarisky, R.T., Kao, C.C., 2001. Terminal nucleotidyl transferase activity of recombinant Flaviviridae RNA-dependent RNA polymerases: implication for viral RNA synthesis. J. Virol. 75 (18), 8615–8623.

Ranjith-Kumar, C.T., Zhang, X., Kao, C.C., 2003. Enhancer-like activity of a brome mosaic virus RNA promoter. J. Virol. 77 (3), 1830–1839.

Ranjith-Kumar, C.T., Sarisky, R.T., Gutshall, L., Thomson, M., Kao, C.C., 2004. De novo initiation pocket mutations have multiple effects on hepatitis C virus RNA-dependent RNA polymerase activities. J. Virol. 78 (22), 12207–12217.

Rao, A.L., Grantham, G.L., 1994. Amplification in vivo of brome mosaic virus RNAs bearing 3' noncoding region from cucumber mosaic virus. Virology 204 (1), 478–481.

Ray, D., White, K.A., 2003. An internally located RNA hairpin enhances replication of Tomato bushy stunt virus RNAs. J. Virol. 77 (1), 245–257.

Rochon, D.M., 1991. Rapid de novo generation of defective interfering RNA by cucumber necrosis virus mutants that do not express the 20-kDa nonstructural protein. Proc. Natl. Acad. Sci. U.S.A. 88 (24), 11153–11157.

Shapka, N., Nagy, P.D., 2004. The AU-rich RNA recombination hot spot sequence of Brome mosaic virus is functional in tombusviruses: implications for the mechanism of RNA recombination. J. Virol. 78 (5), 2288–2300.

Simon, A.E., 1999. Replication, recombination, and symptom-modulation properties of the satellite RNAs of turnip crinkle virus. Curr. Top. Microbiol. Immunol. 239, 19–36.

Simon, A.E., Bujarski, J.J., 1994. RNA–RNA recombination and evolution in virus infected plants. Annu. Rev. Phythopathol. 32, 337–362.

Song, C., Simon, A.E., 1994. RNA-dependent RNA polymerase from plants infected with turnip crinkle virus can transcribe (+)- and (−)-strands of virus-associated RNAs. Proc. Natl. Acad. Sci. U.S.A. 91 (19), 8792–8796.

Sullivan, M.L., Ahlquist, P., 1999. A brome mosaic virus intergenic RNA3 replication signal functions with viral replication protein 1a to dramatically stabilize RNA in vivo. J. Virol. 73 (4), 2622–2632.

White, K.A., Morris, T.J., 1994. Nonhomologous RNA recombination in

tombusviruses: generation and evolution of defective interfering RNAs by stepwise deletions. J. Virol. 68 (1), 14–24.

White, K.A., Nagy, P.D., 2004. Advances in the molecular biology of tombusviruses: gene expression, genome replication, and recombination. Prog. Nucleic Acid Res. Mol. Biol. 78, 187–226.

Worobey, M., Holmes, E.C., 1999. Evolutionary aspects of recombination in RNA viruses. J. Gen. Virol. 80 (Pt. 10), 2535–2543.

Wu, W., Blumberg, B.M., Fay, P.J., Bambara, R.A., 1995. Strand transfer mediated by human immunodeficiency virus reverse transcriptase in vitro is promoted by pausing and results in misincorporation. J. Biol. Chem. 270 (1), 325–332.

Zhang, G., Zhang, J., Simon, A.E., 2004. Repression and derepression of minus-strand synthesis in a plus-strand RNA virus replicon. J. Virol. 78 (14), 7619–7633.

Zhang, C., Cai, Z., Kim, Y.C., Kumar, R., Yuan, F., Shi, P.Y., Kao, C., Luo, G., 2005. Stimulation of hepatitis C virus (HCV) nonstructural protein 3 (NS3) helicase activity by the NS3 protease domain by HCV RNA-dependent RNA polymerase. J. Virol. 79, 8687–8697.