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The RNA Replication Enhancer Element of Tombusviruses Contains Two Interchangeable Hairpins That Are Functional during Plus-Strand Synthesis†

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Replication of the RNA genomes of tombusviruses, which are small plus-sense RNA viruses of plants, may be regulated by *cis*-acting elements, including promoters and replication enhancers that are present in the RNA templates. Using a partially purified RNA-dependent RNA polymerase (RdRp) preparation (P. D. Nagy and J. Pogany, Virology 276:279-288, 2000), we demonstrate that the minus-strand templates of tombusviruses contain a replication enhancer, which can upregulate RNA synthesis initiating from the minimal plus-strand initiation promoter by 10- to 20-fold in an in vitro assay. Dissection of the sequence of the replication enhancer element revealed that the two stem-loop structures present within the ~80-nucleotide-long enhancer region have interchangeable roles in upregulating RNA synthesis. The single-stranded sequence located between the two stem-loops also plays an important role in stimulation of RNA synthesis. We also demonstrate that one of the two hairpins, both of which are similar to the hairpin of the minus-strand initiation promoter, can function as a promoter in vitro in the presence of short cytidylate-containing initiation sites. Overall, the in vitro data presented are consistent with previous in vivo results (D. Ray and K. A. White, Virology 256:162-171, 1999) and they firmly establish the presence of a replication enhancer on the minus-stranded RNA of tombusviruses.

The replication process of tombusviruses, which are single-component plus-strand RNA viruses of plants, is carried out by the virally coded RNA-dependent RNA polymerase (RdRp). First, a complementary (minus-strand) RNA is synthesized from the plus-strand templates, followed by plus-strand RNA synthesis on the minus-strand intermediates. The replication process is highly asymmetrical, leading to excess plus-strand progeny over the minus-strand intermediates (2, 3, 6, 22). The different steps in replication are regulated by *cis*-acting elements present in the plus- and minus-stranded RNA templates, including promoter (initiation) elements and a recently discovered replication enhancer element (8, 18, 20). In spite of their significance in tombusvirus RNA replication, the detailed roles of *cis*-acting elements are not completely understood.

Tombusvirus infections are frequently associated with small parasitic defective interfering (DI) RNAs, which are deletion derivatives of the tombusvirus genomic RNA (4, 5, 7, 10, 24). The typical DI RNAs contain three or four short conserved segments of the genomic RNA, each of which may harbor *cis*-acting elements (Fig. 1) (24). DI RNAs are excellent templates to study RNA replication in vivo and in vitro because they do not code for essential proteins and have small sizes (16, 24). Interestingly, several DI RNAs can interfere with the accumulation of the genomic RNA and reduce the intensity of symptoms in tombusvirus-infected plants (5, 11, 23). Overall, the DI RNAs must use the replication system of the helper tombusviruses, which is known to include two virally coded replicase proteins (p33 and p92) and probably host factors (11, 22), although no evidence for host factors has yet been published for tombusviruses.

Based on in vitro assays with a partially purified tombusvirus RdRp, the minimal promoter elements were defined to be short 3′-terminal sequences present on either the plus- or minus-stranded RNAs (16, 18). Namely, the core minus-strand initiation promoter is 19 nucleotides (termed the gPR promoter), while the core plus-strand initiation promoter (termed the cPR11 promoter) is 11 nucleotides in length (18). In spite of the differences in sequence and structure, the two promoter elements supported comparable levels of RNA synthesis that initiated de novo (without the need for a primer) from the 3′-terminal cytidylate (18).

The activity of the minimal promoters may be regulated by other *cis*-acting elements located in tombusvirus RNAs. In support of this model, a novel *cis*-acting replication sequence that is present internally within the 3′ untranslated region of tombusviruses (termed region III) (20) (Fig. 1) has been characterized recently. This *cis*-acting sequence is not absolutely required for DI RNA accumulation, but its deletion decreased RNA accumulation by 10-fold in protoplasts (20). Interestingly, the region III sequence stimulated RNA accumulation when present at different locations and even in the opposite orientation in the DI RNA. Based on these observations, Ray and White (20) postulated that region III has a replication enhancer-like function during tombusvirus replication. Interestingly, *Turnip crinkle virus*, a related carmovirus, and its associated *satC* RNA also contain replication enhancer elements, based on in vivo and in vitro studies (15, 17).

In this paper, using an in vitro assay with the partially purified tombusvirus RdRp, we demonstrate that one of the four segments, namely, region III, present in a prototypical DI RNA stimulated RNA synthesis most efficiently. Our in vitro

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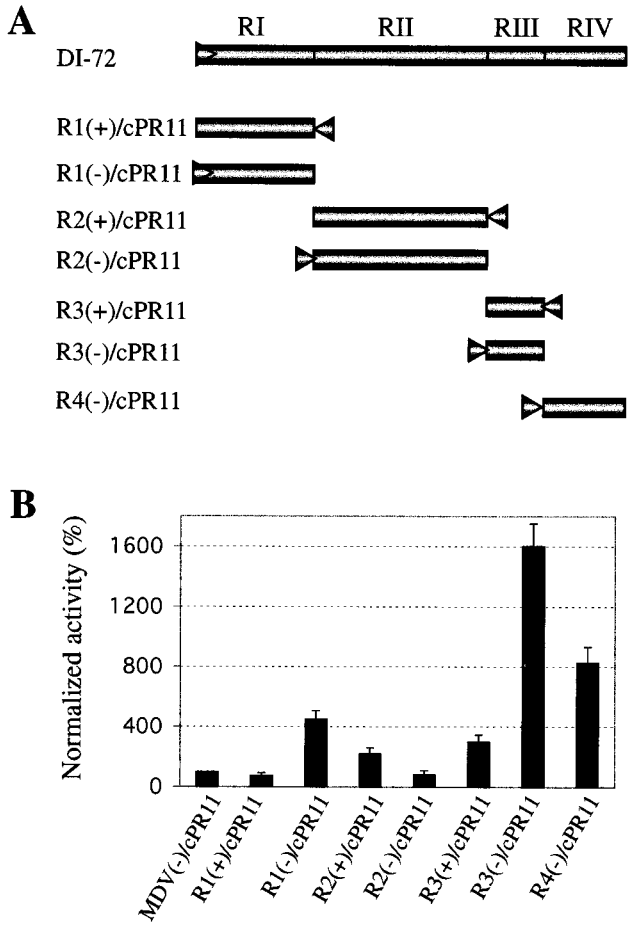

FIG. 1. Enhancement of template activity of tombusvirus RdRp by conserved regions of a TBSV-associated DI RNA. (A) Schematic representation of the RNA genome of a prototypical DI RNA (DI-72) and the RNA constructs tested. Region I (169 nucleotides) is derived from the 5′ nontranslated region of the genomic TBSV RNA. Region II is 239 nucleotides and originated from the coding region (the p92 ORF), while region III (82 nucleotides) represents the end of the p22 ORF plus part of the 3′ noncoding region. Region IV is 131 nucleotides long and is derived from the very 3′-terminal noncoding region of the TBSV RNA. The previously characterized 11-nucleotide-long cPR11 promoter (represented by a triangle) was fused to the 3′ end of each segment, both plus and minus stranded, to generate seven constructs. The triangles point leftward in the plus-stranded constructs and rightward in the minus-stranded constructs. Note that region I(−) originally contained cPR11 at its 3′ end, and therefore no additional cPR11 was fused to that construct [R1(−)/cPR11]. Also, region IV(+) was not tested because that segment already contains a promoter sequence (termed gPR). (B) Relative template activities of the above RNA constructs in an in vitro tombusvirus RdRp assay. The radiolabeled RdRp products, synthesized by in vitro transcription with CNV RdRp, were analyzed on denaturing gels, quantified with a phosphoimager and normalized to the number of templated urilydates ([^{32}P]UTP was used for labeling in the RdRp reaction). RNA templates were used in equal molar amounts. Half of the RdRp products were treated with single-strand-specific RNase to confirm the double-stranded nature of the RdRp products (not shown) (16). The template activity of the construct MDV(−)/cPR11 (Fig. 2A), which contained a 221-nucleotide-long minus-stranded satellite RNA sequence derived from the unrelated bacteriophage Qβ (1) fused to cPR11 at the 3′ end, was set at 100% in these studies. Each experiment was repeated two or three times.

studies show that the minus-stranded region III functions as a "strong" replication enhancer, since it can stimulate RNA synthesis by 10- to 20-fold compared to other viral or artificial sequences. Dissection of the sequence elements required for enhancement of RNA synthesis revealed that the two stem-loops present in minus-stranded region III have redundant functions. Moreover, the single-stranded region separating the two stem-loops can also affect the level of RNA synthesis. Overall, the data presented in this paper give new insights into the mechanism of regulation of RNA synthesis by tombusviruses.

MATERIALS AND METHODS

Tombusvirus RdRp preparation. *Cucumber necrosis virus* (CNV) RdRp preparations were obtained from systemically infected leaves of *Nicotiana benthamiana* plants as described by Nagy and Pogany (16). *N. benthamiana* plants were inoculated with CNV genomic RNA transcripts obtained by standard T7 RNA transcription with an *Sma*I-linearized clone of pK2/M5p20STOP for CNV (21).

Preparation of RNA templates. RNA templates were obtained by in vitro transcription reaction with T7 RNA polymerase with PCR-amplified DNA templates (13, 18). The templates and the primers for each PCR amplification are listed in Table 1, while the sequences of the primers are shown in Table 2. Several constructs were obtained by sequential PCR (18), where the product of the first PCR was gel purified and used for a second round of PCR, as shown in Table 1.

After T7 RNA transcription and phenol-chloroform extraction, unincorporated nucleotides were removed by repeated ammonium acetate-isopropanol precipitation (13, 16). The amounts and sizes of the obtained RNA transcripts were measured with a UV spectrophotometer and on a 5% polyacrylamide–8 M urea gel (denaturing polyacrylamide gel electrophoresis) analysis (13, 16).

Tombusvirus RdRp assay. CNV RdRp reactions were carried out as previously described (16). Briefly, each RdRp reaction mixture contained 100 nM template RNA, 50 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 10 mM dithiothreitol, 100 mM potassium glutamate, 1.0 mM each ATP, CTP, and GTP, 0.01 mM UTP (final concentration), and 0.5 μl of [^{32}P]UTP (from ICN) in a 50-μl total volume. The RdRp reaction was performed at 25°C for 60 min. After phenol-chloroform extraction and ammonium acetate-isopropanol precipitation, half of the RdRp products were treated with RNase I nuclease as recommended by the supplier (Ambion). Subsequently, the RdRp products were analyzed on a 20- or 32-cm-long denaturing 5% polyacrylamide–8 M urea gel, followed by analysis with a Phosphorimager as described elsewhere (16).

RESULTS

Mapping tombusvirus sequences that can upregulate RNA synthesis in vitro. The prototypical DI RNAs associated with CNV and the closely related *Tomato bushy stunt virus* (TBSV) contain four noncontiguous regions derived from the genomic RNA (9, 10, 24). Since DI RNAs replicate very efficiently in infected cells in the presence of the helper virus (both CNV and TBSV support DI RNA accumulation efficiently), it is likely that one or more of the conserved regions contain important *cis*-acting replication elements that may upregulate RNA synthesis.

To search for the presence of enhancers of RNA synthesis in DI RNAs, we first constructed seven different RNA templates that contained one of the plus- or minus-stranded segments of the prototypical DI RNA (DI-72, Fig. 1A) fused to the minimal plus-strand initiation promoter (termed cPR11), which represents the last 11 nucleotides of the 3′ end in the minus strand, 3′-CCUUUAAGAGG (18), at their 3′ ends, as shown in Fig. 1A. The plus-stranded region IV was not tested in these experiments because this sequence already contains a *cis*-acting element (the minus-strand initiation promoter, gPR, at the 3′ end [18]). The control construct was a heterologous tem-

TABLE 1. Primer sets used for PCR to generate constructs

Construct	Primers	Template
R1(+)/cPR11	31/226	DI-72
R1(−)/cPR11	20/15	DI-72
R2(+)/cPR11	17/227	DI-72
R2(−)/cPR11	232/14	DI-72
R3(+)/cPR11	18/490	DI-72
R3(−)/cPR11	253/23	DI-72
R4(−)/cPR11	235/22	DI-72
MDV(−)/cPR11	201/161/194[a]	MDV
MDV(−)/Hind	315/194	MDV
R3(−)/cPR11/Hind	253/316	DI-72
MDV(−)/R3(−)/cPR11	253/194	MDV(−)/Hind R3(−)/cPR11/Hind[b]
AU1/cPR11	248/251	AU1
AU3/cPR11	248/251	AU3
GC1/cPR11	248/251	GC1
GC2/cPR11	248/251	GC2
MDV/gPR	201/102/194[a]	MDV
R3(+)/gPR	18/680	DI-72
R3(−)/gPR	683/23	DI-72
Δ10	288/23	DI-72
Δ20	270/23	DI-72
Δ30	289/23	DI-72
Δ40	271/23	DI-72
Δ30/Δ13	289/291	DI-72
Δ30/Δ22	289/269	DI-72
Δ30/Δ30	289/268	DI-72
Δ30/Δ36	289/489	DI-72
Δ10/Δ13	481/291	DI-72
Δ10/Δ22	481/269	DI-72
Δ10/Δ30	481/268	DI-72
GGGU	723/757/739[a]	DI-72
L8	724/758/739[a]	DI-72
Lgcuu	725/759/739[a]	DI-72
2cm	727/760/739[a]	DI-72
S-rigleft	729/761/739[a]	DI-72
S-leftleft	730/762/739[a]	DI-72
S-intloop	731/763/739[a]	DI-72
S-AU	732/764/739[a]	DI-72
S-GC	733/765/739[a]	DI-72
S-4	484/766/739[a]	DI-72
S-8	487/767/739[a]	DI-72
R3(−)/cPR11Δ2	481/23	DI-72
R3(−)/cPR11Δ4	480/23	DI-72
R3(−)/cPR11Δ6	453/23	DI-72
R3(−)/cPR11Δ8	479/23	DI-72
R3(−)/cPR11Δ9	478/23	DI-72

[a] The first and last primers were used for the first round of PCR, followed by sequential PCR with the second and last primers.
[b] MDV(−)/Hind and R3(−)/cPR11/Hind were digested with *Hin*dIII followed by ligation in order to obtain the template for PCR.

plate, the 221-nucleotide MDV(−) satellite RNA associated with bacteriophage Qβ, which was fused to cPR11 (construct MDV[−]/cPR11, Fig. 2A) (1, 18).

As has been shown before, construct MDV(−)/cPR11 can support a basal level of RNA synthesis, with initiation starting from the 3′ end of the cPR11 sequence in the in vitro tombusvirus RdRp assay (18). Therefore, the level of RNA synthesis obtained with MDV(−)/cPR11 was set at 100% in this study (Fig. 1B).

We tested the template activities of each of the above seven constructs in addition to the control MDV(−)/cPR11 with our in vitro tombusvirus RdRp assay (Fig. 1B). The relative template activity of each construct was calculated after normalization of the level of RNA synthesis based on the number of templated uridylates incorporated in the RdRp reaction (due to the use of [^{32}P]UTP in the RdRp reaction). These experiments revealed that region III(−) [see construct R3(−)/cPR11, Fig. 1B] stimulated RNA synthesis that is initiated from the cPR11 promoter 16-fold more strongly than MDV(−)/cPR11 in vitro. This observation demonstrates that region III(−) can upregulate RNA synthesis, possibly due to its RNA replication enhancer function. Interestingly, minus-stranded regions I and IV as well as plus-stranded region II and region III also enhanced RNA synthesis by two- to eightfold (Fig. 1B). These data suggest that several segments of the prototypical DI RNA may be involved in regulation of RNA synthesis.

Since region III(−) was the strongest enhancer of RNA synthesis among the tombusvirus sequences tested, we continued our studies by focusing on this particular sequence.

It is possible that the 16-fold differences between the levels of RNA synthesis obtained with R3(−)/cPR11 and MDV(−)/cPR11 may be due not only to the stimulative effect of region III(−) sequences, but also to the putative inhibitory effect of MDV(−) sequences on RNA synthesis. To exclude this possibility, we constructed MDV(−)/R3(−)/cPR11, which contained both MDV(−) and region III(−) sequences in addition to the 3′-terminal cPR11 promoter (Fig. 2A). The normalized level of RNA synthesis obtained with MDV(−)/R3(−)/cPR11 was ninefold higher than that with MDV(−)/cPR11 (Fig. 2B). This confirms the stimulatory effect of region III(−) even when it is present on a heterologous RNA.

To further test the relative "strength" of the region III(−) replication enhancer, we compared the in vitro template activities of R3(−)/cPR11 to that of constructs carrying either AU-rich or GC-rich artificial sequences in addition to the 3′-terminal cPR11 (Fig. 3A). Construct R3(−)/cPR11 had 10- to 20-fold higher template activity than the constructs with artificial sequences (compare lane 1 with lanes 2 to 6, Fig. 3B and C). We conclude that the minus-stranded region III sequence functions as a "strong" replication enhancer (see also Fig. 1B and Discussion).

To examine whether the strong region III(−) replication enhancer can only function in the presence of the cPR11 promoter, we replaced the cPR11 sequence in construct R3(−)/cPR11 with the 19-nucleotide-long gPR promoter, which represents the minus-strand initiation promoter present at the very 3′ end of the plus-stranded DI and the tombusvirus genomic RNAs (Fig. 4A) (18). The control constructs were MDV(−)/gPR and R3(+)/gPR, which both contained the gPR promoter at the 3′ end and either the MDV(−) sequence or the region III(+) sequence (Fig. 4A).

Comparison of the in vitro template activities of these constructs revealed that construct R3(−)/gPR carrying the region III(−) was the most active among the templates tested, showing almost sevenfold higher activity than that of MDV(−)/gPR (compare lane 3 with lane 1, Fig. 4B and C). Construct R3(+)/gPR carrying region III(+) had threefold higher activity than MDV(−)/gPR, while its template activity was about half that observed with R3(−)/gPR (lanes 2 and 3, Fig. 4B and C). These experiments suggest that region III(−) can enhance RNA synthesis from both the cPR11 and gPR promoters, serving as a "general" replication enhancer. The level of stimulation of RNA synthesis, however, is more than twofold

TABLE 2. Primers used for construction of DNA templates for T7 transcription

Primer	Sequence[a]	Origin	Position
14	*GTAATACGACTCACTATA*GGGTTCTCTGCTTTTACGAAG	TBSV	c1507–1524[b]
15	*GTAATACGACTCACTATA*GGGCATGTCGCTTGTTTGTTGG	TBSV	c150–168
17	*GTAATACGACTCACTATA*GGAGAAACGGGAAGCTCGC	TBSV	1285–1301
18	*GTAATACGACTCACTATA*GGAGAAAGCGAGTAAGACAG	TBSV	4394–4411
20	**GGAAATTCTCCAGGATTTCTC**[c]	TBSV	1–20
22	*GTAATACGACTCACTATA*GGGCTGCATTTCTGCAATGTTCC	TBSV	c4754–4776
23	*GTAATACGACTCACTATA*GGGACCCAACAAGAGTAACCTG	TBSV	c4461–4480
31	*GTAATACGACTCACTATA***GGAAATTCTCCAGGATTTC**	TBSV	1–19
102	GGGCTGCATTTCTGCAATGAATTCCACAAGTGACACCT	TBSV/TBSV	c4754–4776/c4666–4682
161	**TTGGAAATTCTCC**TTCCACAAGTGACACCT	TBSV/TBSV	1–11/c4666–4682
194	GTAATACGACTCACTATAGGGGAACCCCCCTTC	MDV	c206–221
201	TTCCACAAGTGACACCTGGGGACCCCCCGGAA	TBSV/MDV	c4666–4682/1–15
226	TT**GGAAATTCTCC**TTGTCGC**T**TGTTTGTTGG	TBSV/TBSV	1–11/c150–165
227	T**TGGAAATTCTCC**TTCTGCT**T**TTACGAAGG	TBSV/TBSV	1–11/c1505–1519
232	TT**GGAAATTCTCC**TTAGAAACGGGAAGCTCGC	TBSV/TBSV	1–11/1287–1302
235	TT**GGAAATTCTCC**TTCCTGTTTACGAAAGT	TBSV/TBSV	1–11/4649–4665
248	*GTAATACGACTCACTATA*GGAGACCCTGTCCAGGTAG	ART	
251	TT**GGAAATTCTCC**TTGTGCTCGAGTTGGATCC	TBSV/ART	1–11/—
253	TT**GGAAATTCTCC**TTAGCGAGTAAGACAGACTC	TBSV/TBSV	1–11/4399–4416
268	*GTAATACGACTCACTATA*GGAGATTTACACTCATCTC	TBSV	c4433–4450
269	*GTAATACGACTCACTATA*GGAGCTATGCCAGATTTACA	TBSV	c4441–4462
270	TT**GGAAATTCTCC**TTCAGTCTGAGTTTGTGGA	TBSV/TBSV	1–11/4418–4435
271	TT**GGAAATTCTCC**TTGAGTGTAAATCTGGCAT	TBSV/TBSV	1–11/4438–4455
288	TT**GGAAATTCTCC**TTACAGACTCTTCAGTCTG	TBSV/TBSV	1–11/4408–4425
289	TT**GGAAATTCTCC**TTTGTGGAGATGAGTGTA	TBSV/TBSV	1–11/4428–4445
291	*GTAATACGACTCACTATA*GGAACCTGTATGCTATGCC	TBSV	c4451–4468
315	GGCATGCAAGCTTGGGGGGGACCCCCCGGAAGG	ART/MDV	—/1–20
316	GGCATGCAAGCTTAAGAGTAACCTGTATG	ART/TBSV	—/c4458–4473
453	TT**GGAA**TACAGACTCTTCAGTCTGAGT	TBSV/TBSV	1–4/4408–4425
478	TTGGCAGACTCTTCAGTCTGA	TBSV	4408–4425
479	TT**GGAA**CAGACTCTTCAGTCTGA	TBSV/TBSV	1–4/4408–4425
480	TT**GGAAATT**ACAGACTCTTCAGTCTGA	TBSV/TBSV	1–7/4408–4425
481	TT**GGAAATTCTA**CAGACTCTTCAGTCTGACTTTGTGGA	TBSV/TBSV	1–9/4408–4425
484	TT**GGAA**TACAGCCTTCGCTGAGTTTGTGGAGATGA	TBSV/TBSV	1–4/4423–4440
487	TT**GGAA**TACAGACTGACTTCTCAGTCTGAGTTTGTGGA	TBSV/TBSV	1–4/4423–4440
489	TAATACGACTCACTATAGGACACTCATCTCCACAAACT	TBSV/TBSV	1–4/4407–4435
490	**GGAAATTCTCC**ACCCAACAAGAGTAACCT	TBSV/TBSV	1–11/c4463–4480
680	GGGCTGCATTTCTGCAATGACCCAACAAGAGTAACCT	TBSV/TBSV	c1–19/c4463–4480
683	GGGCTGCATTTCTGCAATGAGCGAGTAAGACAGACTC	TBSV/TBSV	c1–19/4399–4416
723	TT**GGAAATTCTCC**TTACCCACCCACCCACCCAAGTTTGTG	TBSV/ART/TBSV	1–11/—/4426–4433
724	TT**GGAAATTCTCC**TTACAGACTAGAAGAAGAGTCTGAGTTTGTG	TBSV/ART/TBSV	1–11/—/4426–4433
725	TT**GGAAATTCTCC**TTACAGACTCGAAAGTCTGAGTTTGTG	TBSV/ART/TBSV	1–11/—/4426–4433
727	TT**GGAAATTCTCC**TTAGCGCCTCTTCAGACGCAGTTTGTG	TBSV/ART/TBSV	1–11/—/4426–4433
729	TT**GGAAATTCTCC**TTAGTCTGACTTCTCAGACAGTTTGTG	TBSV/ART/TBSV	1–11/—/4426–4433
730	TT**GGAAATTCTCC**TTACAGACTCTTCTCAGACAGTTTGTG	TBSV/ART/TBSV	1–11/—/4426–4433
731	TT**GGAAATTCTCC**TTACAGACTCTTCAGAGTGAGTTTGTG	TBSV/ART/TBSV	1–11/—/4426–4433
732	TT**GGAAATTCTCC**TTATATATACTTCTATATAAGTTTGTG	TBSV/ART/TBSV	1–11/—/4426–4433
733	TT**GGAAATTCTCC**TTACCGGCCCTTCGGCCGGAGTTTGTG	TBSV/ART/TBSV	1–11/—/4426–4433
739	*TAATACGACTCACTATA*GGAGATTTACACTCATCTCCACAAACT	TBSV	c4426–4450
759	TT**GGAAATTCT**ACAGACTCGA	TBSV/ART	1–9/—
760	TT**GGAAATTCT**AGCGCCTCT	TBSV/ART	1–9/—
761	TT**GGAAATTCT**AGTCTGACT	TBSV/ART	1–9/—
762	TT**GGAAATTCT**ACAGACTCT	TBSV/ART	1–9/—
763	TT**GGAAATTCT**ACAGACTCT	TBSV/ART	1–9/—
764	TT**GGAAATTCT**ATATATACTTCT	TBSV/ART	1–9/—
765	TT**GGAAATTCT**ACCGGCCCT	TBSV/ART	1–9/—
766	TT**GGAAATTCT**ACAGCCTTCGCTGAGT	TBSV/ART	1–9/—
767	TT**GGAAATTCT**ACAGACTGACTTCTCAGT	TBSV/ART	1–9/—

[a] T7 promoter sequence is in italics.
[b] Position on the TBSV genomic RNA (c before the position stands for complementary strand) or MDV. The artificial (ART) sequences are shown with a dash.
[c] cPR11 and its derivatives are shown in bold.

higher in combination with the cPR11 promoter than with the gPR promoter.

Defining the minimal replication enhancer element in vitro. To define the minimal region III(−) sequences that are capable of stimulation of RNA synthesis from the cPR11 promoter sequence, we made a 10-, 20-, 30-, and 40-nucleotide deletion series that started from the 3′ end (called the 3′ junction) of region III(−) in construct R3(−)/cPR11 (Fig. 5A). Testing the in vitro template activities of the resulting constructs revealed that deletions of up to 30 nucleotides from the 3′ junction of

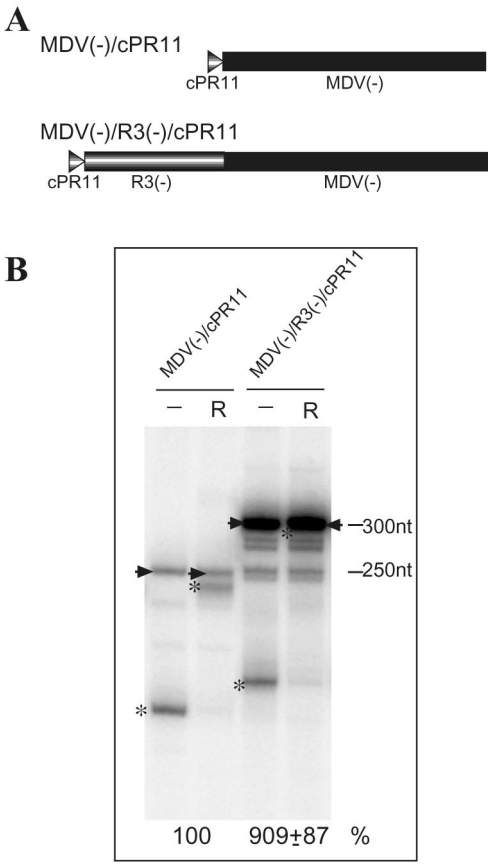

FIG. 2. Region III(−) enhances RNA synthesis by the CNV RdRp when present on a heterologous template. (A) Schematic representation of the constructs tested in in vitro CNV RdRp assays. cPR11 is shown with a triangle, while the minus-stranded MDV RNA is represented with a black box. The 82-nucleotide-long region III(−) sequence is shown with a gray box. The sequences are shown in the 3′ to 5′ orientation because they represent minus-stranded sequences. (B) Representative denaturing gel analyses of radiolabeled RNA products synthesized by in vitro transcription with CNV RdRp. Arrows point to the RdRp products generated by de novo initiation from the 3′ terminus, while asterisks depict products that were generated by self-priming from the 3′ end. Note that the de novo products are RNase insensitive (R, RNase-treated; —, untreated samples), while the self-primed products are partially RNase sensitive. The self-primed products move faster in the untreated samples due to their highly stable secondary structure (hairpin structures [16]). The positions of the molecular size markers are shown on the right (in nucleotides). The relative efficiency of template activities (only the de novo products that are pointed at by the arrows were measured) is shown at the bottom. Each experiment was repeated three times.

region III(−) did not decrease the level of RNA synthesis from cPR11 (see constructs Δ10, Δ20, and Δ30, lanes 2 to 4, Fig. 5B and C). In contrast, deletion of 40 nucleotides did result in a fivefold drop in template activity (construct Δ40, lane 5, Fig. 5A). These data suggest that the 3′ 30 nucleotides of region III(−) are dispensable for stimulation of RNA synthesis in vitro, while the sequence located between 30 and 40 nucleotides from the 3′ junction is important for RNA synthesis.

Secondary-structure analysis of the region III(−) sequence with the M-fold program (12) predicts the existence of two hairpins, termed SL1-III(−) and SL2-III(−), which are separated by a single-stranded region (Fig. 5A). The existence of SL1-III(−) and SL2-III(−) is further supported by solution structure analysis (20a). Deletion of 10 to 30 nucleotides from the 3′ junction of region III(−) is predicted to remove part of or the entire SL1-III(−) hairpin (Fig. 5A). Deletion of a further 10 nucleotides (construct Δ40), however, removes part of the single-stranded region in region III(−). Overall, these data indicate that while SL1-III(−) is dispensable for enhancement of RNA synthesis in vitro, the single-stranded portion of region III(−) may contain a sequence important for the functional enhancer.

To further define the role of sequences in region III(−) that are involved in the enhancement of RNA synthesis, we made a series of four sequence deletions from the 5′ junctions in construct Δ30 (Fig. 5A), which lacked the SL1-III(−) hairpin and showed a high level of template activity in the above deletion experiments (lanes 4 and 6, Fig. 5B and C). Testing the template activities of the resulting constructs revealed that removal of 13 nucleotides starting from the 5′ junction in region III(−) (construct Δ30/Δ13, Fig. 5A) was not detrimental to simulation of RNA synthesis (Fig. 5B and C, lane 7). In contrast, deletion of 22, 30, and 36 nucleotides, which also started from the 5′ junction, did reduce the level of RNA synthesis by two- to fourfold (constructs Δ30/Δ22, Δ30/Δ30, and Δ30/Δ36, Fig. 5, lanes 8, 9, and 10). Since the latter deletion mutants lacked significant portions of the SL2-III(−) hairpin, we propose that the SL2-III(−) hairpin is required for enhancement of RNA synthesis in vitro in the absence of the SL1-III(−) hairpin.

Enhancement of RNA synthesis by SL1-III(−) hairpin in vitro. The experiments shown in Fig. 5 demonstrated that while SL2-III(−) is required, the SL1-III(−) hairpin is dispensable for stimulation of RNA synthesis in the presence of the SL2-III(−) hairpin. The alternative explanation of the above observation, however, is that the two hairpins, SL1-III(−) and SL2-III(−), may play redundant roles during stimulation of RNA synthesis. To test whether SL1-III(−) can enhance RNA synthesis in the absence of SL2-III(−), we made a series of 5′ deletions in SL2-III(−) in construct Δ10 (Fig. 5A), which lacks a 10-nucleotide sequence from the 3′ junction (Fig. 6A) that is not part of SL1-III(−). This construct [R3(−) Δ10/cPR; Fig. 6A] contains both stem-loops and the internal single-stranded sequence of region III(−), in addition to a truncated version of the cPR11 promoter, which lacks two G's at its 5′ end. We found that this truncated version of cPR11 was more active than complete cPR11 in the presence of the region III(−) enhancer (see below).

Deletion of 13, 22, and 30 nucleotides starting from the 5′ junction of region III(−) removed parts of or the entire SL2-III(−) sequence, yet these deletions only slightly affected the level of RNA synthesis compared to that observed with the region III(−) sequence (constructs Δ10/Δ13, Δ10/Δ22, and Δ10/Δ30, Fig. 6B and C, lanes 2 to 4). Overall, these data demonstrate that the SL2-III(−) hairpin is redundant in the presence of the SL1-III(−) hairpin. Therefore, the results of the experiments shown in Fig. 5 and 6 suggest that the SL1-III(−) hairpin can serve as a strong replication enhancer in the absence of the SL2-III(−) hairpin.

Defining the sequence-structure requirement for a minimal

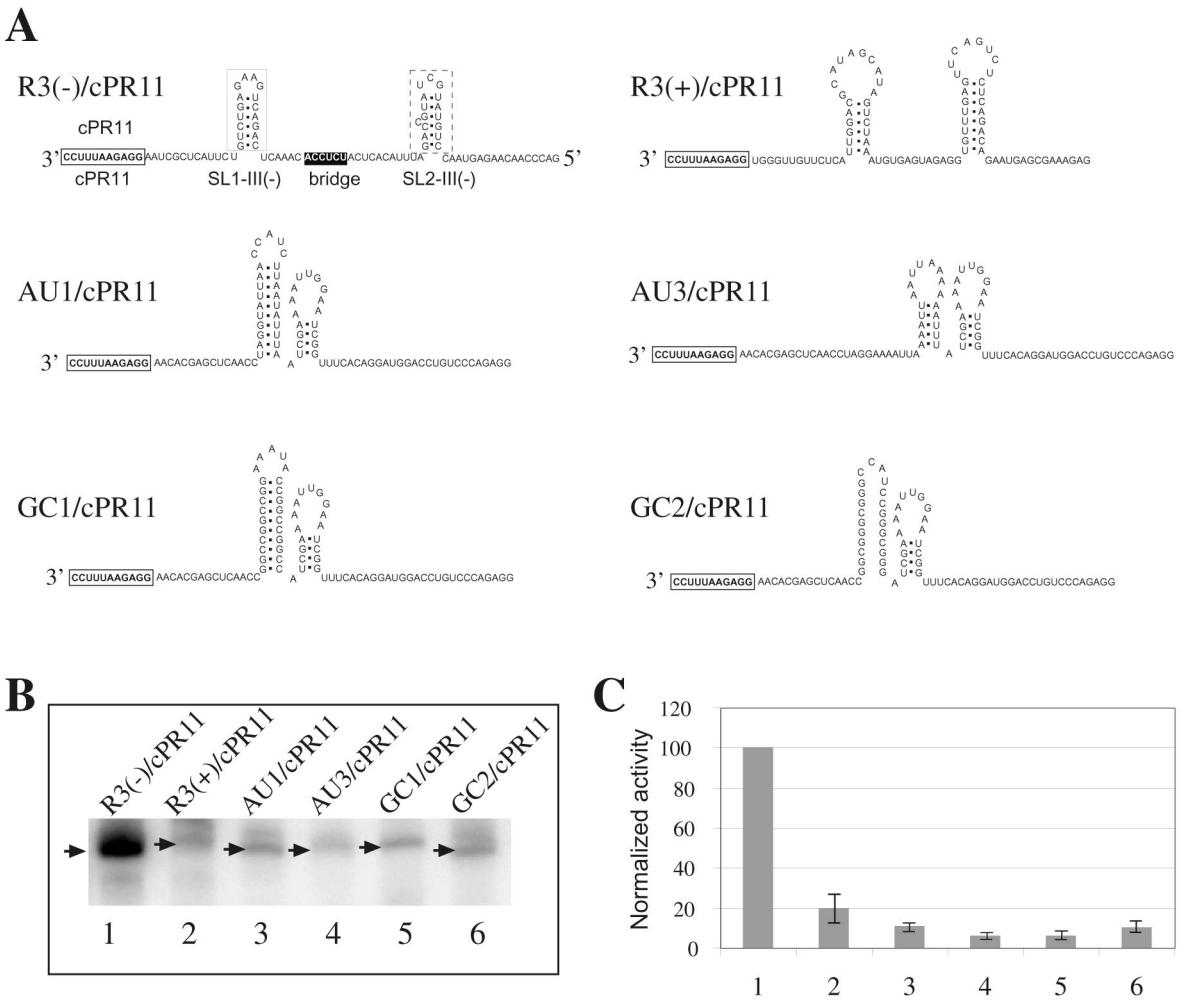

FIG. 3. Replacing region III(−) sequences with artificial sequences reduces template activities. (A) The actual sequences of the constructs tested are shown in the 3′ to 5′ orientation. The predicted secondary structures, based on the M-fold program (12), are shown: the two stem-loop structures, SL1-III(−) and SL2-III(−), and the cPR11 promoter are boxed. A 6-nucleotide-long region, termed the bridge, within the single-stranded portion of region III(−) that may interact with the cPR11 promoter is shown in a black box. (B) Representative denaturing gel analyses of radiolabeled RNA products synthesized by in vitro transcription with CNV RdRp. Arrows point to the RdRp products generated by initiation from the 3′ terminus. Note that the RdRp products obtained with the GC-rich templates migrate slightly aberrantly under the condition used, possibly due to their unusual structure. (C) Relative template activities of the above RNA constructs in an in vitro tombusvirus RdRp assay. The results were normalized as described in the legend to Fig. 1.

replication enhancer in vitro. The above experiments demonstrated that the SL1-III(−) and SL2-III(−) hairpins play interchangeable roles in enhancement of RNA synthesis. Although the overall sizes and structures of these hairpins are somewhat similar, their primary sequences are different. This suggests that the structures and/or the sizes of these hairpins are important during RNA synthesis. To test this model, we replaced the SL1-III(−) hairpin in construct Δ10/Δ30 (Fig. 6A), which supports RNA synthesis efficiently, with 11 different sequences, as shown in Fig. 7A. Replacing SL1-III(−) with an artificial single-stranded G/U-rich sequence (construct GGGU in Fig. 7A), which changed both the sequence and structure compared to SL1-III(−), inhibited RNA synthesis by 25-fold (lane 2 in Fig. 7B and C).

This experiment further supported our observation that SL1-III(−) might play a role in RNA synthesis. In contrast to the dramatic change in construct GGGU, replacing the 4-nucleotide-long loop region of SL1-III(−) with an 8-nucleotide-long loop sequence or with a different tetraloop sequence (constructs L8 and Lgcuu, Fig. 7A) inhibited RNA synthesis by only 5% and 10%, respectively (lanes 3 and 4, Fig. 7B and C). These data suggest that the loop region in SL1-III(−) does not play a major role in RNA synthesis. Introducing 6- and 16-nucleotide substitutions into the stem portion of SL1-III(−) (constructs 2cm and S-rigleft, Fig. 7A), which maintained the overall stability of the hairpin, reduced RNA synthesis by 20 to 30% (lanes 5 and 6 of Fig. 7B and C). This suggests that the primary sequence of SL1-III(−) plays a moderate role in RNA synthesis.

Therefore, we further tested the role of the stem by making four additional constructs that contained sequences that cannot form stable base pairs (construct S-leftleft, Fig. 7A) or can form secondary structures with a four-nucleotide internal loop region inside the stem, a weak AU-rich region, and an unusu-

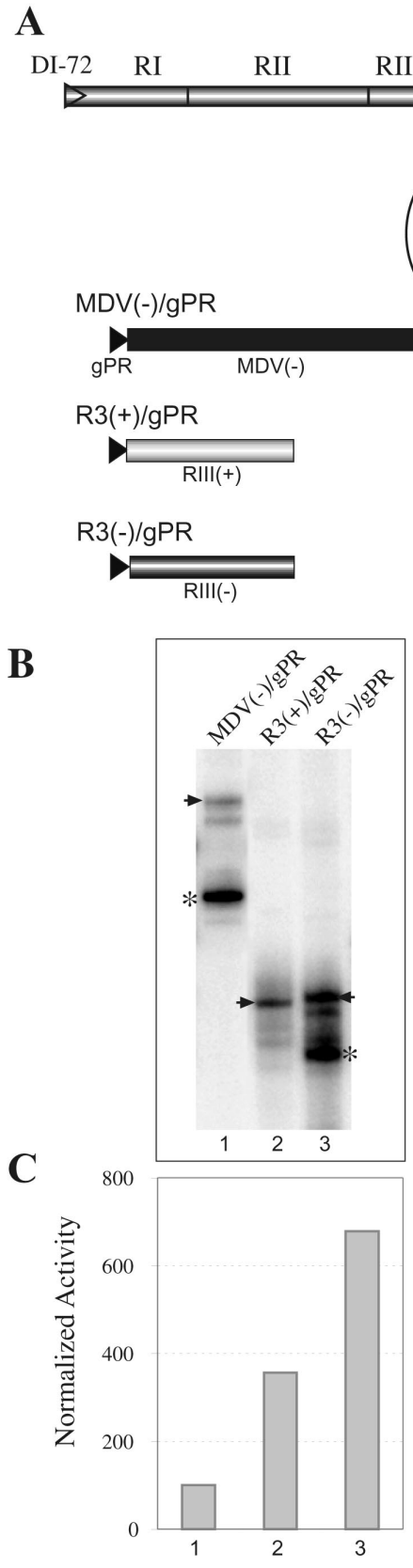

FIG. 4. Region III replication enhancer can enhance RNA synthesis from the minus-strand initiation promoter in vitro. (A) Schematic representation of the constructs tested in the in vitro CNV RdRp assays. The 19-nucleotide-long core minus-strand initiation promoter, gPR, is shown with a black triangle, while the minus-stranded MDV sequence (see Fig. 2) is indicated with a black box. Sequences representing region III(+) and region III(−) are shown with light and dark gray boxes, respectively. The constructs are drawn in the 3′ to 5′ orientation. (B) Representative denaturing gel analyses of radiolabeled RNA products synthesized by in vitro transcription with CNV RdRp. Arrows point to the RdRp products generated by de novo initiation from the 3′ terminus, while asterisks depict products that were generated by self-priming from the 3′ end. See the legend to Fig. 2 for details. (C) Relative template activities of the constructs shown in panel A (only the de novo products that were pointed at by the arrows were measured). See the legend to Fig. 1 for further details.

ally stable GC-rich stem region (constructs S-intloop, S-AU, and S-GC, respectively; Fig. 7A). We found that construct S-leftleft, similar to construct GGGU, with no well-defined stem structures supported RNA synthesis at a ~20-fold reduced level (lane 7 in Fig. 7B and C). The template activities of constructs S-intloop, S-AU, and S-GC (lanes 8 to 10 in Fig. 7B and C) were below 50%, suggesting that these structures are less efficient than the structure of SL1-III(−) in supporting RNA synthesis.

To test the role of the length of the stem in SL1-III(−) in RNA synthesis, we made two constructs, S-4 and S-8 (Fig. 7A), which contained stems that were either 2 bp shorter or longer than SL1-III(−). While construct S-4 (lane 11, Fig. 7B and C) with the shorter stem supported RNA synthesis at a level comparable to that of SL1-III(−), construct S-8 supported cRNA synthesis at a ~30% reduced level (lane 12, Fig. 7B and C). These data reveal that the length of the stem of SL1-III(−) can also affect the level of RNA synthesis.

SL1-III(−) hairpin functions as a promoter in the presence of initiation sequences. Interestingly, the structures of the SL1-III(−) and SL2-III(−) hairpins are similar to the structures of the core minus-strand initiation promoter gPR (18) and an internal initiation site present in the minus-stranded DI-72 (19). However, a significant difference between these promoters and the replication enhancers [SL1-III(−) and SL2-III(−) hairpins] may be the presence of two cytidylates 3′ to the hairpins for the promoters. These cytidylates may facilitate de novo RNA initiation by the tombusvirus RdRp.

To examine if a replication enhancer can be converted into a promoter, we made SL1-III(−) hairpin-containing constructs that also contained cytidylates at 3′ positions. This was done by deleting 2, 4, 6, 8, and 9 nucleotides from the core cPR11 sequence in construct R3(−)Δ10 (Fig. 8A). These deletions were designed to leave the 3′-terminal cytidylates unmodified, since they are required for de novo initiation (18). The in vitro tombusvirus RdRp assay revealed that deletions of 2 to 8 nucleotides in cPR11 did not interfere with de novo RNA synthesis (constructs Δ2, Δ4, Δ6, and Δ8 in Fig. 8A and lanes 2 to 5 in Fig. 8B and C). Deletion of 9 nucleotides from cPR11, however, reduced RNA synthesis by fourfold (construct Δ9; lanes 6 in Fig. 8B and C).

The observation that construct Δ8 can support RNA synthesis efficiently suggests that the SL1-III(−) hairpin can support RNA synthesis in the presence of an upstream initiation sequence. The fact that construct Δ9 can support RNA synthesis at a reduced level, however, indicates that the two cytidylates

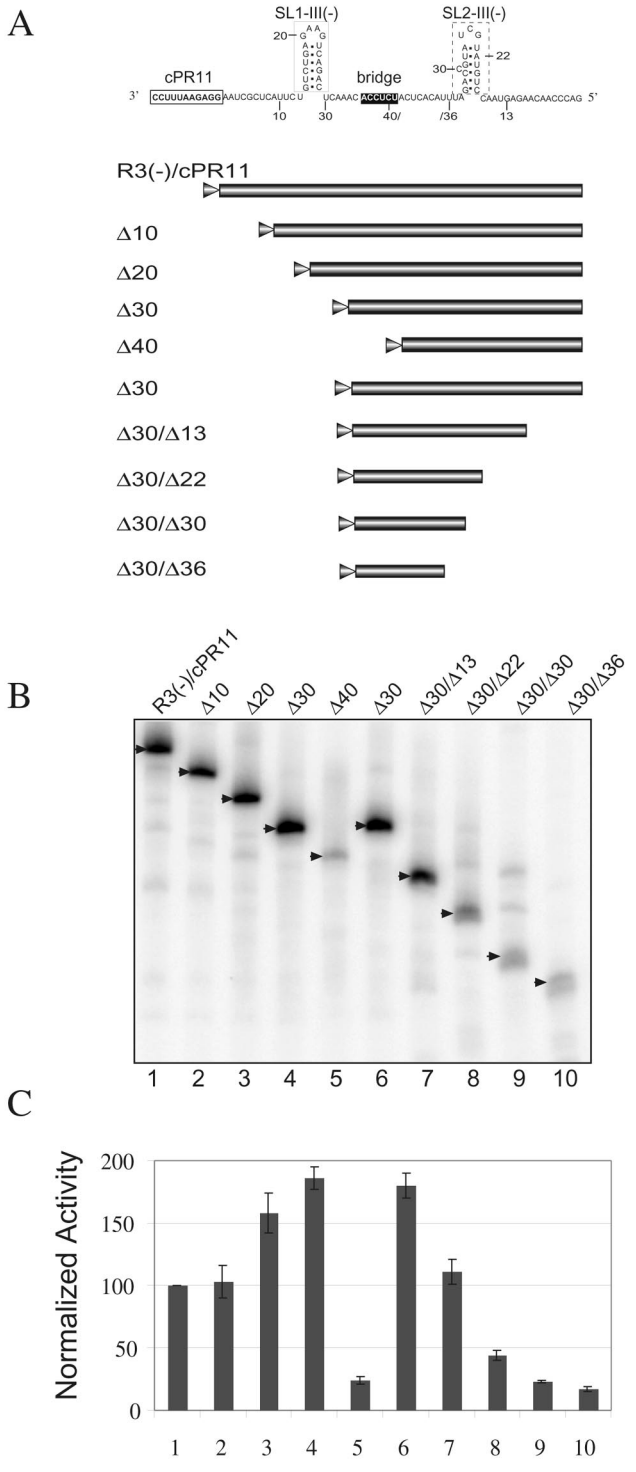

FIG. 5. SL2-III(−) hairpin functions as a replication enhancer in the in vitro CNV RdRp assay. (A) Schematic representation of the series of deletion constructs generated from R3(−)/cPR11 (Fig. 1). The actual sequence and predicted secondary structure of R3(−)/cPR11 are shown on the top (see Fig. 3 for detailed description of the individual sequence elements) in the 3′ to 5′ orientation. Sequences present in the constructs are indicated with gray bars. The gray triangle represents the cPR11 promoter sequence. The names of the constructs indicate the lengths of deletions. (B) Representative denaturing gel analyses of radiolabeled RNA products synthesized by in vitro transcription with CNV RdRp. Arrows point to the RdRp products generated by initiation from the 3′ terminus. (C) Relative template activities of the above RNA constructs in the in vitro tombusvirus RdRp assay. The results were normalized as described in the legend to Fig. 1.

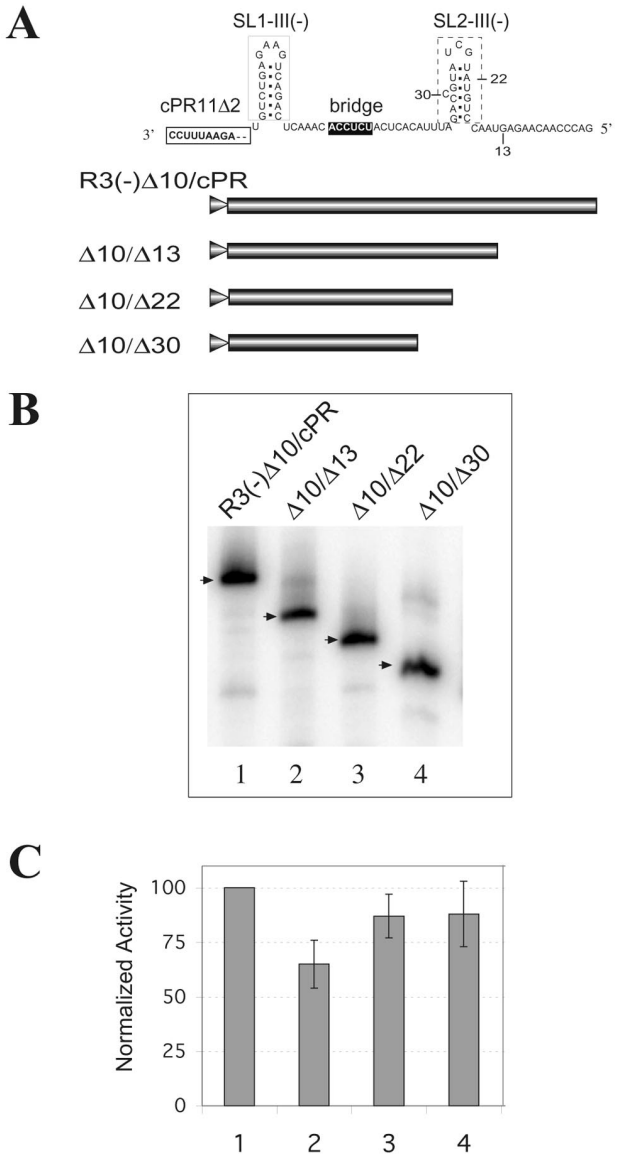

FIG. 6. SL1-III(−) hairpin functions as a replication enhancer in the in vitro CNV RdRp assay. (A) Schematic representation of the series of deletion constructs generated from construct R3(−)Δ10/cPR, which was derived from construct Δ10 (Fig. 5A) by a 2-nucleotide deletion in the cPR11 promoter sequence (termed cPR11Δ2; boxed). Note that cPR11Δ2 is as active as cPR11 in the presence of SL1-III(−) (see Fig. 8). The actual sequence and predicted secondary structure of construct R3(−)Δ10/cPR are shown on the top (see Fig. 3 for detailed description of the individual sequence elements) in the 3′ to 5′ orientation. Sequences present in the constructs are indicated with gray bars. The names of the constructs indicate the lengths of deletions. (B) Representative denaturing gel analyses of radiolabeled RNA products synthesized by in vitro transcription with CNV RdRp. Arrows point to the RdRp products generated by initiation from the 3′ terminus. (C) Relative template activities of the above RNA constructs in the in vitro tombusvirus RdRp assay. The results are normalized as described in the legend to Fig. 1.

(the initiation site) should be positioned properly in the vicinity of the SL1-III(−) hairpin for initiation to occur efficiently. Overall, these data illustrate that the entire promoter sequence (except the initiator sequence) is not essential for efficient RNA synthesis when a replication enhancer sequence is present in the vicinity of the initiation site.

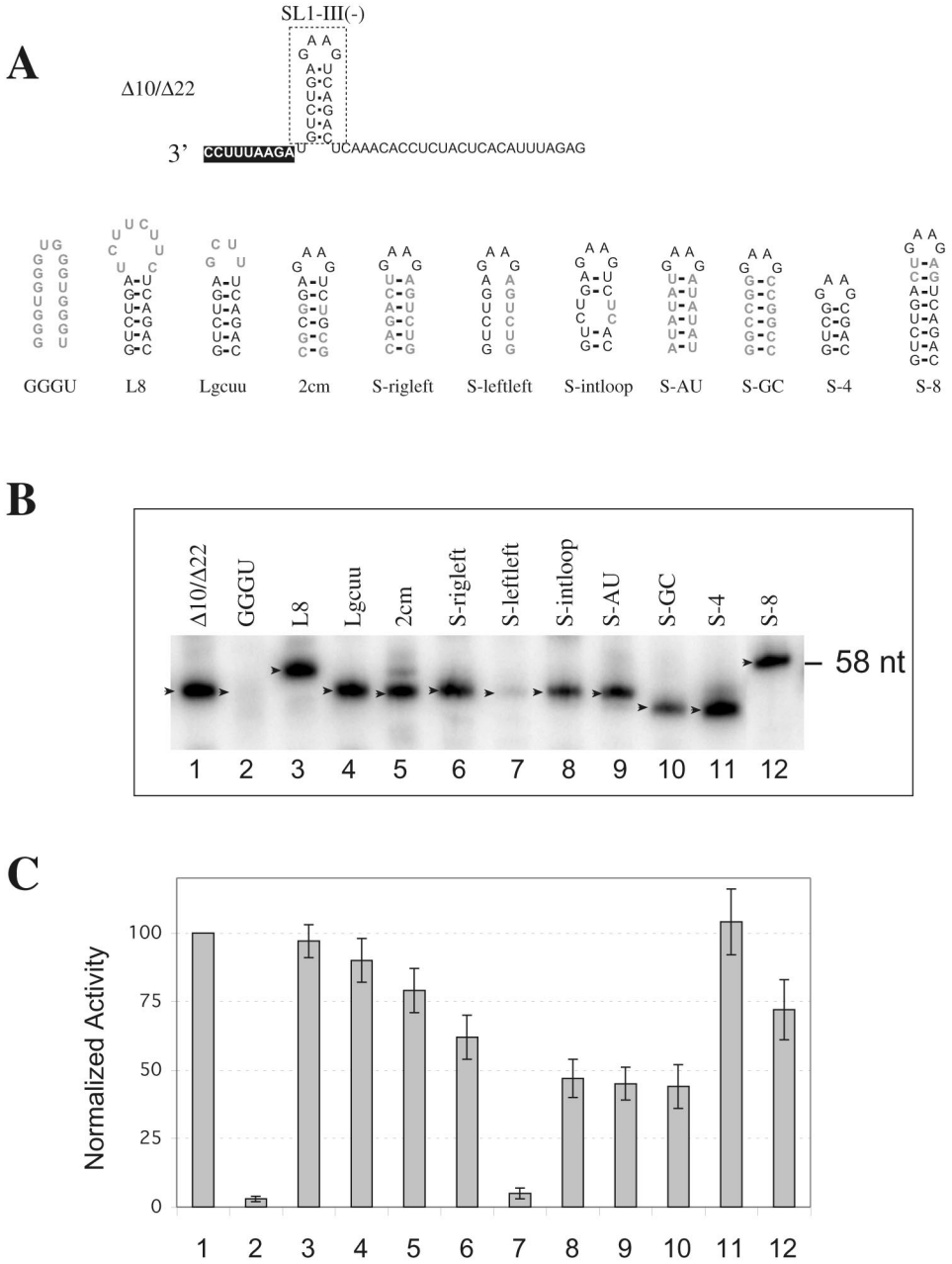

FIG. 7. Secondary structure of the SL1-III(−) hairpin plays a role in stimulation of RNA synthesis in the in vitro CNV RdRp assay. (A) The actual sequence and predicted secondary structure of construct Δ10/Δ22 (see Fig. 6) are shown on the top in the 3′ to 5′ orientation. Note that letters in a black box represents a 2-nucleotide deletion version of cPR11 (see Fig. 8), and the SL1-III(−) region is boxed with a dotted line. The 11 constructs are identical to Δ10/Δ22 except for the sequences shown, which replaced SL1-III(−) in each construct. Gray letters indicate mutated nucleotides, while black letters represent nucleotides present in SL1-III(−). (B) Representative denaturing gel analyses of radiolabeled RNA products synthesized by in vitro transcription with CNV RdRp. Arrows point to the RdRp products generated by initiation from the 3′ terminus. Note that a few RNAs move slightly aberrantly in the gels under the conditions used due to their unusual sequence or structure (for example, S-GC in lane 10). (C) Relative template activities of the above RNA constructs in the in vitro tombusvirus RdRp assay. The results were normalized as described in the legend to Fig. 1.

DISCUSSION

Replication of tombusviruses is being intensively studied with the help of DI RNAs that must contain *cis*-acting replication elements in order to compete efficiently with the helper virus for the viral RdRp during infection. Since the most competitive DI RNAs contain four noncontiguous segments derived from the parent virus, it is likely that these regions contain regulatory sequences. Indeed, deletion of either of the conserved segments debilitated the replication of DI RNAs in plants or in protoplasts (reviewed in references 22 and 24). Overall, two of the eight segments (i.e., plus and minus strands of the four conserved regions) are known to contain defined

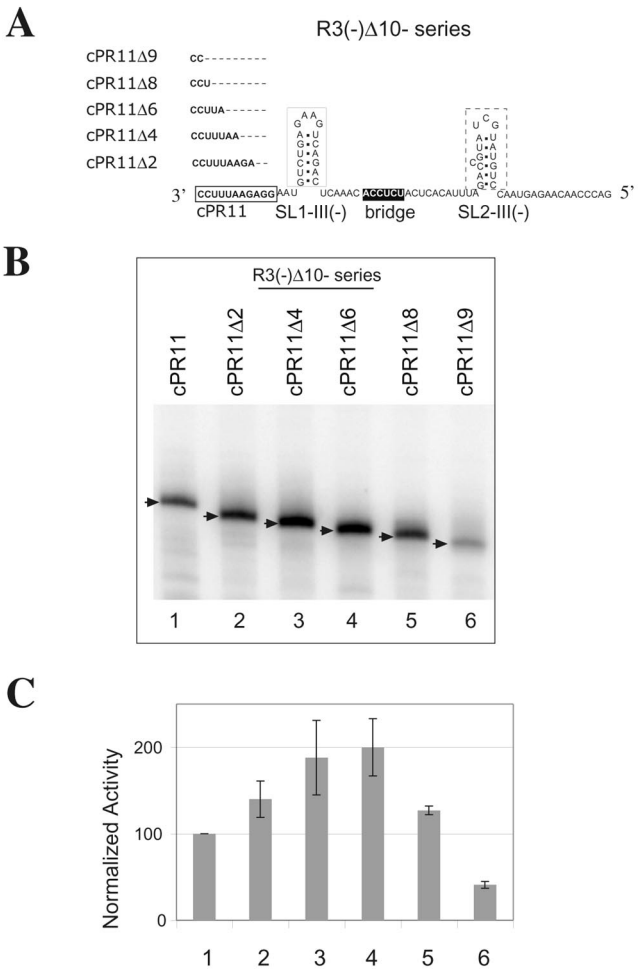

FIG. 8. Region III(−) replication enhancer facilitates RNA synthesis in the presence of an initiation sequence. (A) The actual sequence and predicted secondary structure of construct Δ10 [see Fig. 5A; indicated here as R3(−)Δ10] is shown in the 3′ to 5′ orientation. The individual sequence elements are shown as in Fig. 3. The five constructs are identical to construct R3(−)Δ10 except for the deletions within the cPR11 region (the deleted sequences are indicated by dashes). (B) Representative denaturing gel analyses of radiolabeled RNA products synthesized by in vitro transcription with CNV RdRp. Arrows point to the RdRp products generated by initiation from the 3′ terminus. (C) Relative template activities of the above RNA constructs in the in vitro tombusvirus RdRp assay. The results were normalized as described in the legend to Fig. 1.

cis-acting replication elements. These are region IV(+), which contains the minus-strand initiation promoter (e.g., gPR [18]), and region I(−), which carries the plus-strand initiation promoter (e.g., cPR11 [18]). It is possible that these and the other segments contain additional regulatory elements that potentially can up- or downregulate RNA synthesis initiated from the 3′-terminal promoters. Accordingly, Ray and White (20) obtained supporting data on the function of region III as a possible enhancer element in DI RNA replication in protoplasts. However, questions such as what strand of region III is involved in enhancement of replication and whether the effect is direct or indirect remain open.

By using a partially purified tombusvirus RdRp system, we tested the putative regulatory roles of the above segments in in vitro RNA synthesis. This was achieved by fusing the cPR11 promoter sequence separately to the 3′ end of each segment [except region IV(+), which contains the gPR sequence; thus, fusing cPR11 to its end would result in promoter duplication, which is outside the scope of this paper]. Interestingly, several segments, including region II(+), region III(+), region I(−), region III(−), and region IV(−), stimulated RNA synthesis from the cPR11 promoter in vitro by 2- to 16-fold. This suggests that RNA synthesis may be regulated by sequences present on both plus- and minus-stranded sequences.

Overall, region III(−) showed the highest enhancement in RNA synthesis, which was fivefold higher than that observed with region III(+). In contrast to the above virus-derived sequences, artificial sequences were unable to stimulate RNA synthesis from the cPR11 promoter (Fig. 3). We also demonstrated that region III(−) was capable of enhancement of RNA synthesis when present on a heterologous MDV template (Fig. 2). This suggests that stimulation of RNA synthesis by the region III(−) sequence is likely direct rather than indirect. Overall, we conclude that region III contains a replication enhancer that functions as a strong enhancer in the minus-stranded RNA and a weak enhancer in the plus strands of tombusviruses and DI RNAs. In agreement with our in vitro data, the results of protoplast experiments in the accompanying paper by Ray and White (20a) also support the role of the minus-stranded region III in enhancing tombusvirus replication.

Deletion analysis within region III(−) showed that the two stem-loop structures, SL-1-III(−) and SL2-III(−), play redundant and interchangeable roles in enhancement of RNA synthesis. Constructs carrying either hairpin supported RNA synthesis at a level comparable to that of the construct containing both hairpins [compare construct Δ30 with R3(−)/cPR1 in Fig. 5B and construct Δ10 with Δ10/Δ30 in Fig. 6B]. This suggests that there is no additive stimulatory effect coming from the combination of the two stem-loops. Functional redundancy in the region III sequence was also confirmed in protoplast experiments, which included mutants generated by scanning mutagenesis (20).

Interestingly, duplication of the region III sequences did not increase the level of DI RNA accumulation in protoplasts (20). Also, duplication of enhancer sequences, called the motif 1 hairpin, present in a *satC* gene associated with turnip crinkle virus had no additive effect on RNA accumulation in protoplasts (15). In contrast, enhancement with multiple enhancers was additive when inserted into a poorly replicating satellite RNA (15). Also, the replication enhancer present in the genomic turnip crinkle virus RNA showed an additive effect on RNA synthesis when fused with the motif 1 hairpin in the in vitro turnip crinkle virus RdRp assay (15). The reasons for these differences among known replication enhancers are currently unknown. It is possible that there is a limiting step in enhancement of RNA synthesis for some replication enhancers that cannot be eliminated by sequence duplication, while this limitation is not valid in the case of other enhancers. Further mechanistic experiments will address this question.

The deletion experiments with templates containing the region III(−) enhancer also revealed that a single-stranded region between the SL1-III(−) and SL2-III(−) hairpins is re-

quired for full enhancer activity (Fig. 5 and 6). The role of this region in enhancement of RNA synthesis will be presented in a separate paper. Briefly, we postulate that a 6-nucleotide-long segment (termed the bridge sequence in Fig. 3) within the single-stranded portion of region III(−) may base pair with the promoter sequence. This RNA-RNA interaction may serve to bring the promoter into proximity with the replication enhancer (Panavas and Nagy, unpublished data). Nevertheless, the single-stranded region alone, including the bridge sequence, cannot stimulate RNA synthesis as efficiently as in combination with either SL1-III(−) or SL2-III(−) (see constructs Δ30/Δ22, Δ30/Δ30, and Δ30/Δ36 in Fig. 5B and C).

To test whether the replication enhancer function of region III(−) is promoter specific, we compared the template activities of constructs containing either the cPR11 or the gPR promoter. Similar to region III(−), the minimal cPR11 sequence is also present in the minus-stranded tombusvirus RNA. In contrast, gPR is present in the plus-strand template. Region III(−) was capable of stimulating RNA synthesis from both the cPR11 and the gPR promoters, although the level of stimulation was more than twofold higher with cPR11 compared to the MDV(−)-containing control constructs (Fig. 1 and 4). The reason for the different efficiency of stimulation of RNA synthesis from the two different promoters is an interesting question, and it requires further experimentation.

The motif 1 hairpin replication enhancer of *satC* associated with turnip crinkle virus was also found to enhance RNA synthesis more efficiently from the minimal plus-strand initiation promoter than from the minus-strand initiation promoter in vitro (15, 17). However, unlike the region III(−) enhancer, the enhancement from the minus-strand initiation promoter by the motif 1 hairpin replication enhancer was rather small, suggesting that the motif 1 hairpin may function primarily in conjunction with the plus-strand initiation promoter during plus-strand RNA synthesis, while it may not have a significant role during minus-strand synthesis (17). In conclusion, our data suggest that region III(−) is a strong general replication enhancer that can stimulate RNA synthesis from different tombusvirus promoters.

From a mechanistic point of view, it is interesting how the two hairpins, SL1-III(−) and SL2-III(−), in spite of having rather dissimilar sequences, can stimulate RNA synthesis so efficiently. It is possible that the tombusvirus RdRp can recognize the two different hairpins based on their primary sequences. It is also possible that the secondary structures of these hairpins, which are somewhat similar, may facilitate recognition. The in vitro experiments favor the second model because mutagenesis of the SL1-III(−) hairpin followed by testing of the template activities of the resulting constructs revealed that the primary sequence of the stem and the loop has a smaller effect than the secondary structure on its function as a replication enhancer (Fig. 7). For example, swapping the right and the left side sequences of the stem that maintained base pairing but changed the primary sequence (see construct S-rigleft, Fig. 7) did reduce its template activity by over 30%. This effect, however, was much less than that of eliminating the secondary structure of the hairpin (see constructs GGGU and S-leftleft, Fig. 7), which reduced template activities by more than 20-fold. In addition to our in vitro data, the role of the secondary structure of SL-III(−) in tombusvirus replication was also supported by the in vivo results shown in the accompanying paper by Ray and White (20a).

Interestingly, templates with AU- and GC-rich stems and a weak stem that contained an internal loop (constructs S-AU, S-GC, and S-intloop, Fig. 7) had less than 50% activity in the RdRp assay. This suggests that the sequences or structures of these stems are not optimal for enhancing the activity of the tombusvirus RdRp. While lengthening of the 6-bp-long stem with 2 bp reduced template activity, shortening it by 2 bp did not reduce RNA synthesis, suggesting that 4 to 6 bp within the stem are optimal for RNA synthesis in vitro. It is possible that the stems of SL1-III(−) and SL2-III(−) can form similar surfaces (in either the minor or the major groove) that may facilitate RdRp binding better than the mutated or artificial stems tested in Fig. 7. Overall, the data are most consistent with the model that the secondary structures of the hairpins are important for recognition and/or binding by the tombusvirus RdRp.

The secondary structures of the SL1-III(−) and SL2-III(−) hairpins are similar to that of the gPR promoter, which is involved in initiation of minus-strand synthesis (Fig. 4A) (19). In addition, an internal promoter-like sequence located within region I(−) that can support internal initiation in vitro also has a similar stem-loop structure (19). Our current model predicts that all these elements are involved in binding to the tombusvirus RdRp. However, there is a major difference between the gPR and internal promoters and SL1-III(−) and SL2-III(−): the promoters can support cRNA synthesis, while SL1-III(−) and SL2-III(−) cannot do so in the absence of an upstream promoter [for example, the cPR11 promoter present in construct R3(−)/cPR11, Fig. 1]. It is possible, however, that this difference between the promoters and the enhancer hairpins might only be the result of the presence of initiation sequences for the promoters or the lack of it in the case of the enhancer hairpins. This was indeed supported by our observation that the three 3′-terminal nucleotides (3′-CCU in construct cPR11Δ8, Fig. 8) of the cPR11 promoter were sufficient to promote efficient de novo RNA synthesis in the presence of the SL1-III(−) replication enhancer (Fig. 8).

We propose that the putative binding of the RdRp to the replication enhancer may facilitate correct positioning of the RdRp over the 3′ end of the template, which includes the CCU initiation site that can lead to initiation. In the absence of the proper initiation site, the replication enhancer cannot support RNA synthesis. Therefore, this model states that the promoters and replication enhancers are similar in their abilities to bind to the RdRp, but they are different in their abilities to support initiation of RNA synthesis. This is likely due to the presence of an initiation sequence in the promoters, while replication enhancers lack these sequences. Similar observations were made for the motif 1 hairpin replication enhancer of the turnip crinkle virus-associated *satC* gene (14, 15). Although the motif 1 hairpin replication enhancer alone did not support de novo RNA synthesis, it was able to do so in the presence of initiator sequences in vitro (14, 15). The overall similarities in their secondary structures between some promoters and replication enhancers may be beneficial for these viruses, since this may reduce the complexity of the *cis*-acting elements that must be recognized by the viral RdRp.

In summary, with our in vitro tombusvirus RdRp system, we firmly established that region III of tombusviruses and DI

RNAs contains a replication enhancer that can directly facilitate de novo RNA synthesis from minimal promoters. Our in vitro findings on the role of region III as a replication enhancer are consistent with data obtained in protoplast experiments by Ray and White (20, 20a). Importantly, we demonstrate that minus-stranded region III is a stronger replication enhancer than plus-stranded region III. These results will open the way for future studies on the mechanistic details of stimulation of RNA synthesis by the tombusvirus replication enhancer.

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