Subgenomic RNA as a riboregulator: negative regulation of RNA replication by Barley yellow dwarf virus subgenomic RNA 2

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Abstract

Barley yellow dwarf virus (BYDV) generates three 3′-coterminal subgenomic RNAs (sgRNAs) in infected cells. Translation of BYDV genomic RNA (gRNA) and sgRNA1 is mediated by the BYDV cap-independent translation element (BTE) in the 3′ untranslated region. sgRNAs 2 and 3 are unlikely to be mRNAs. We proposed that accumulation of sgRNA2, which contains the BTE in its 5′UTR, regulates BYDV replication by trans-inhibiting translation of the viral polymerase from genomic RNA (gRNA). Here, we tested this hypothesis and found that: (i) co-inoculation of the BTE or sgRNA2 with BYDV RNA inhibits BYDV RNA accumulation in protoplasts; (ii) Brome mosaic virus (BMV), engineered to contain the BTE, trans-inhibits BYDV replication; and (iii) sgRNA2 generated during BYDV infection trans-inhibits both GFP expression from BMV RNA and translation of a non-viral reporter mRNA. We conclude that sgRNA2, via its BTE, functions as a riboregulator to inhibit translation of gRNA. This may make gRNA available as a replicase template and for encapsidation.

Thus, BYDV sgRNA2 joins a growing list of trans-acting regulatory RNAs.

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Keywords: sgRNA; Barley yellow dwarf virus; BTE

Introduction

Most RNA viruses replicate in the cytoplasm of their host cells. Thus, translation rather than transcription is often the major step at which viral gene expression is regulated. Many viral translational control strategies are conferred by RNA structures in cis (Gale et al., 2000; Macdonald, 2001; Mazumder et al., 2003). In recent years, diverse regulatory RNAs known as riboregulators have been discovered in prokaryotes and eukaryotes (Lease and Belfort, 2000; Rastinejad et al., 1993; Reinhart et al., 2000). Riboregulators function in trans, mainly post-transcriptionally. Only a few trans-regulatory RNAs from RNA viruses have been reported (Albarino et al., 2003; Eckerle and Ball, 2002; Sit et al., 1998). Here, we show that subgenomic RNA 2 (sgRNA2) of Barley yellow dwarf virus (BYDV) acts as a riboregulator to negatively regulate viral replication in trans.

BYDV is the type member of genus Luteovirus in the family Luteoviridae. BYDV RNA has a complex set of primary and secondary structures that regulate many non-canonical translation events (Miller et al., 2002). These include cap-independent translation (Guo et al., 2001; Wang et al., 1997, 1999), −1 ribosomal frameshifting (Barry and Miller, 2002; Paul et al., 2001), leaky scanning (Chay et al., 1996), and stop codon readthrough (Brown et al., 1996). BYDV has a positive sense RNA genome of 5677 nt that encodes six open reading frames (ORFs) (Miller et al., 1997, 2002). Its genomic RNA (gRNA) and subgenomic RNAs (sgRNAs) have no 5′-cap and no 3′-poly(A) tail (Allen et al., 1999), yet they are translated efficiently. The 105-nt cap-independent translation element (TE) in the 3′ untranslated region (UTR) of BYDV RNA facilitates efficient translation initiation at the 5′-proximal AUG (Guo et al., 2000). Similar structures are present in the 3′ UTRs of necrovi-
ruses (Meulewaeter et al., 2004; Shen and Miller, 2004) and dianthoviruses (Mizumoto et al., 2003), so we now refer to this element as a member of the BYDV-like class of TE, or BTE (Shen and Miller, 2004).

The BTE (i) binds translation factors (E. Allen and E. Pettit, personal communication) that presumably recruit the ribosome, (ii) is brought into proximity with the 5′ end by direct base pairing to the 5′ UTR (Guo et al., 2001), and (iii) functions both in the 5′ UTR and in the 3′ UTR (Guo et al., 2000). Most of the 869-nt 3′ UTR of BYDV gRNA is required for full cap-independent and poly(A) tail-independent translation in oat protoplasts (Guo et al., 2000; Wang et al., 1999). sgRNA2 corresponds to the 869-nt 3′ UTR of BYDV RNA and the BTE is at the 5′ end of sgRNA2 (Fig. 1). sgRNA2 encodes a small ORF (ORF 6) that varies from 4.3 to 7.2 kDa and is poorly conserved between isolates. It is absent in BYDV-related Soybean dwarf (Rathjen et al., 1994) and Bean leafroll (Domier et al., 2002) viruses. After much effort to detect the product of ORF 6 (P6) or translatability of sgRNA2, it appears that ORF 6 is not translated in vivo (A. Rakotondrafara, personal communication).

In addition to conferring cap-independent translation in cis, the BTE inhibits translation in trans, in vitro (Wang et al., 1997, 1999). In wheat germ extract, either the BTE alone or full-length sgRNA2, which harbors the BTE at its 5′ end, trans-inhibits translation of BYDV genomic RNA (gRNA) and (to a much lesser extent) sgRNA1 (Wang et al., 1999). The inhibition does not require translation of ORF 6, but does require a functional BTE. Based on these in vitro data, we proposed that in later stages of the virus replication cycle, accumulation of sgRNA2 inhibits translation of RNA-dependent RNA polymerase (RdRp) from gRNA while allowing translation of structural proteins from sgRNA1. Thus, viral RNA replication would be inhibited by sgRNA2 introduced prematurely in the replication cycle. Here, we tested this hypothesis. We found that both replicating and nonreplicating RNAs containing the BTE inhibit BYDV RNA accumulation in trans. sgRNA2 generated during BYDV infection trans-inhibits translation of a reporter gene and gene expression from BMV RNA, which suggests that BYDV sgRNA2 inhibits viral replication via inhibition of translation. We also showed that the BTE in cis increases translation of capped RNA. Our data suggest that sgRNA2, via its BTE, functions as a riboregulator to negatively control translation of the viral RdRp, thus replication of BYDV RNA.

Results

Nonreplicating BTE and sgRNA2 RNAs trans-inhibit accumulation of BYDV RNAs in infected plant cells

In wheat germ extract, both the 105-nt BTE and sgRNA2, which harbors the BTE at its 5′ end, trans-inhibit the translation of BYDV genomic RNA (gRNA) and sgRNA1 (Wang et al., 1999). In natural infection, the molar ratio of sgRNA2 to sgRNA1 and gRNA is similar to the ratio that strongly inhibits translation of gRNA and weakly inhibits translation of sgRNA1 in vitro. Thus, we predict that addition of excess BTE or sgRNA2 during inoculation with BYDV RNA should inhibit BYDV replication via premature inhibition of translation of the RdRp from genomic RNA. To test this prediction, we co-inoculated oat protoplasts with the 105-nt BTE (TE105) or the 109-nt nonfunctional mutant BTE (TEBF) transcripts and wild-type infectious BYDV transcript, Pav6. TEBF contains a GAUC duplication in the BamHI4837 site that completely abolishes cap-independent translation mediated by the BTE in cis (Wang et al., 1997). The accumulated BYDV gRNA and sgRNA levels at 24 h post-inoculation (hpi) were detected by northern blot hybridization. When co-inoculated with Pav6 RNA into oat protoplasts, nonreplicating TE105 RNA trans-inhibited replication of BYDV RNA as predicted. However, we were surprised to find that when we increased the molar ratio of BTE/Pav6 to ≥20:1, replication of BYDV RNA was partially recovered (Fig. 2B, lanes 6 and 7). These results were highly reproducible in many experiments using different RNA and protoplast preparations.
We next examined the trans-effects of the BTE in a replicating context, but still isolated from other potential regulatory elements in BYDV RNA. To do this, we developed an expression system from an unrelated virus, Brome mosaic virus (BMV). BMV is a tripartite RNA virus in the Bromoviridae family with three genomic RNAs. Only RNAs 1 and 2 are required for viral RNA replication. RNA3 encodes two ORFs including the coat protein gene. The coat protein mRNA, subgenomic RNA 4, is generated from RNA3 during infection (Miller et al., 1985). All BMV RNAs are capped (Dasgupta et al., 1975), so BMV has no apparent need for a cap-independent translation element. To monitor gene expression and to avoid complications caused by encapsidation, the BMV coat protein ORF was replaced with that of GFP (Fig. 3). The BTE or TEBF sequence from BYDV was inserted into the intergenic region between the 3a and coat protein genes of BMV RNA 3, just upstream of the GFP ORF start codon (Fig. 3). This places the BTE in the 3′ UTR of the 3a gene on RNA 3 and in the 5′ UTR of the GFP-encoding subgenomic RNA 4. The resulting viruses were designated BMV.TEGFP and BMV.TEBFGFP (Fig. 3).

We inoculated oat protoplasts simultaneously with BYDV PAV6, and BMV RNAs 1 + 2 combined with various RNA 3 transcripts (Fig. 3) that harbor the BTE or its nonfunctional counterpart, TEBF. BMV RNAs 1, 2, and 3

Replicating BTE trans-inhibits accumulation of BYDV RNA

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were inoculated in a molar ratio of 1:1:2. To ensure that the effects were conferred specifically by the BTE, we also included tRNA and wild-type BMV.GFP RNA (no BTE) as controls. When co-inoculated with BYDV PAV6, BMV.TEGFP and BMV.TEBFGFP RNAs replicated similarly, as revealed in northern blot hybridization (Fig. 4A, lanes 3,4). The BYDV-specific probe detected the engineered BMV RNA3 accumulation due to the presence of the

![Fig. 4](image)

**Fig. 4.** Effects of BTE-containing BMV replication on BYDV RNA accumulation. Northern blot analyses were done as in Fig. 2. BYDV gRNA and sgRNAs are indicated. BMV RNAs 3 and 4 were also detected by the BYDV-derived probe because they contain the BTE. Below each blot, stained gels show relative loading of total RNA in each lane. Inoculum (A) in lane 1: no RNA; lane 2: PAV6; lane 3: PAV6 + 4 μg of BMV.TEGFP; lane 4: PAV6 + 4 μg of BMV.TEBFGFP; lane 5: PAV6 + 4 μg of tRNA; lane 6: PAV6 + 4 μg of BMV.GFP. (B) Effects of increasing BMV RNA inocula on BYDV RNA accumulation. Lane 1: PAV6; lanes 2–5: PAV6 + 1, 2, 4, and 8 μg of BMV.TEGFP, respectively.

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![Fig. 5](image)

**Fig. 5.** (A) Effects of the BTE in cis on expression of GFP from BMV.GFP fluorescence intensities were measured by using flow cytometry. Mock: oat protoplasts were electroporated without RNA; hpi, hours post-inoculation. Vertical bars indicate standard deviation. Each value is a mean of at least three replicates. (B) Accumulation of recombinant BMV RNAs 3 and 4. Total RNA was extracted from oat protoplasts 24 h after inoculation with BMV.GFP, BMV.TEGFP, or BMV.TEBFGFP, and used for northern blot hybridization. A 32P-labeled probe complementary to the full-length GFP gene sequence was used to detect recombinant BMV RNAs 3 and 4. The bottom panel shows the RNA loading control.

BTE or TEBF sequences. Replicating BMV.TEGFP inhibited accumulation of BYDV RNA (Fig. 4A, lane 3), whereas BMV.TEBFGFP (Fig. 4A, lane 4), tRNA (lane 5), and BMV.GFP (lane 6) did not inhibit BYDV RNA accumulation as much, if at all. The inhibitory effects conferred by the BTE from replicating BMV.TEGFP were dose-dependent (Fig. 4B). When the co-inoculated BMV.TEGFP RNA was increased from 1 to 4 μg, the amounts of BYDV gRNA and sgRNAs decreased (Fig. 4B, lanes 2–4). However, when co-inoculated with 8 μg of BMV.TEGFP, BYDV RNA accumulation was inhibited less (Fig. 4B, lane 5).

**Subgenomic RNA 2 produced during BYDV infection trans-inhibits gene expression of BMV RNA containing or lacking the BTE**

Having established that the BTE and sgRNA2 trans-inhibit BYDV RNA replication in vivo, we set out to test the
mechanism of the inhibition. Based on our in vitro data (Wang et al., 1999), we proposed that BTE or sgRNA2, when co-inoculated with BYDV RNA, attenuate replication via premature inhibition of translation of the RdRp from genomic RNA. Because of the difficulty of detecting RdRp, we used BMV.GFP and BMV.TEGFP as sensors to test whether sgRNA2 could trans-inhibit translation in vivo. Unexpectedly, we found that the BTE enhanced BMV gene expression in cis (Figs. 5A, 6C). By using flow cytometry and UV microscopy, we found that GFP expression levels from BMV.TEGFP were higher than those from BMV.GFP (Figs. 5A, 6C). The TEBF leader, which differs from the BTE by only four bases, reduced GFP expression to near background levels (Fig. 5A). This may be caused by the secondary structure in the TEBF impeding ribosome scanning to the start codon. BMV.TEGFP-infected cells fluoresced more brightly than BMV.GFP-infected cells (Fig. 6C), and the BTE increased the number of cells expressing detectable levels of GFP. The percentage of oat protoplasts with green fluorescence was 6.5% (±3.3%) in BMV.TEGFP-inoculated cells, 2.2% (±0.7%) in the BMV.GFP-inoculated group, and 0.25% (±0.12%) in the BMV.TEBFGFP-inoculated group. Standard deviations from at least three independent experiments are included in parentheses. For each independent experiment, the GFP expression levels from BMV.TEGFP were 2- to 5-fold higher than those from BMV.GFP.

The insertions of BTE and TEBF sequences had little effect on BMV.GFP RNA replication and synthesis of BMV RNA 4 (Fig. 5B). This result agrees with previous reports that insertion of a foreign gene within 17 bases downstream of the RNA 4 start site did not greatly affect subgenomic RNA synthesis (French et al., 1986). Because presence of the BTE had little effect on BMV RNA accumulation (Fig. 5A), the BTE enhanced BMV gene expression in cis (Figs. 5A, 6C).
5B), but increased GFP expression, these data indicate that the insertion of the BTE in the 5' UTR of non-BYDV (i.e. BMV) mRNA increased translation. Thus, in addition to providing cap-independent translation, the BTE also increases the translation of capped mRNAs.

To test whether sgRNA2 can trans-inhibit translation in vivo, we co-inoculated BMV.GFP RNA with wild-type BYDV infectious transcript PAV6, or with a mutant, PAV6ΔSG2 RNA. PAV6ΔSG2 contains a point mutation (G4810C) that knocks out sgRNA2 synthesis but has little effect on accumulation of the other BYDV RNAs (Koev and Miller, 2000). When co-inoculated with BMV.GFP RNA, wild-type PAV6 reduced GFP expression from BMV.GFP by 2- to 6-fold (Figs. 6A, C). PAV6ΔSG2 was less inhibitory (Figs. 6A, C). The degrees of inhibition of GFP expression by PAV6 were similar at different time points (Fig. 6A). The reason for the reduced inhibition by PAV6ΔSG2 was not due to reduced replication of PAV6ΔSG2 relative to PAV6. Both PAV6ΔSG2 and PAV6 replicated similarly in the presence of BMV.GFP (Fig. 6B).

The experiments above show that sgRNA2 inhibits expression of RNA lacking the BTE. To examine whether sgRNA2 inhibits translation of BTE-containing RNA in a BYDV infection, we co-inoculated oat protoplasts with BMV.TEGFP RNA and either PAV6 or PAV6ΔSG2 RNA. PAV6 reduced GFP expression from BMV.TEGFP substantially, whereas PAV6ΔSG2 only slightly reduced the expression level of GFP (Fig. 6C). Northern blot hybridization revealed that PAV6 and PAV6ΔSG2 also inhibited accumulation of RNAs 3 and 4 of BMV.GFP and BMV.TEGFP (Fig. 6D), and that PAV6 showed higher inhibitory effects than PAV6ΔSG2 did (Fig. 6D). Thus, accumulated sgRNA2 in BYDV-infected cells trans-inhibits GFP expression from BMV RNAs that contain or lack a BTE. These experiments do not differentiate whether the reduced GFP levels are due to inhibition of translation, transcription, or replication of the GFP mRNA, or a combination of these events.

Subgenomic RNA2 in BYDV-infected cells inhibits translation of reporter mRNA

To determine whether the inhibition of gene expression by BYDV sgRNA2 is at the level of translation, we tested the effect of BYDV infection on translation of a non-replicating reporter mRNA construct in oat protoplasts. A two-step electroporation method was developed. First oat protoplasts were inoculated with infectious BYDV PAV6 or PAV6ΔSG2 RNA by electroporation. After 24-h incubation to allow genomic RNA replication and accumulation of sgRNAs, protoplasts were electroporated again with reporter cap-fluc-A(60). This is a capped and polyadenylated firefly luciferase gene lacking any viral sequence. The firefly luciferase activity was analyzed after another 4-h incubation. Inoculation of 1 pmol of PAV6 RNA in the first step caused a 60% drop in translation of cap-fluc-A(60), whereas inoculation of PAV6ΔSG2 RNA in first step had no effect on translation (Fig. 7A). Again, PAV6 and PAV6ΔSG2 RNAs accumulated to similar levels (Fig. 7B). Thus, the inhibition of gene expression by BYDV sgRNA2 most likely functions at the level of translation.

Discussion

Subgenomic RNA2 trans-inhibits the accumulation of BYDV RNA

Subgenomic RNAs of positive-sense RNA viruses in many plant virus families and the Nidovirales class and Togaviridae and Nodaviridae families of animal viruses all have been considered to be messenger RNAs required for expression of 3'-proximal viral genes. In this report, we show that sgRNA2 of BYDV has a different function, acting as a trans-inhibitor of RNA replication via the BTE in its 5' end. In three different contexts, nonreplicating 105-nt BTE RNA, replicating BMV RNA, and expressed as sgRNA2 in natural BYDV infection, the BTE inhibited viral RNA accumulation in trans. RNAs containing the nonfunctional TEBF sequence that differs from BTE by only a four base
duplication did not inhibit in trans. In a natural infection, the trans-function of BTE is fulfilled in the context of sgRNA2, which is not detectable until about 10 hpi (data not shown), after translation of RdRp from gRNA. Thus, we propose that BYDV sgRNA2 trans-inhibits translation of BYDV RNAs via its BTE to act as a switch that turns off translation of gRNA. When introduced artificially at the very beginning of infection (co-inoculated with gRNA), sgRNA2 prevents the initial translation of gRNA, blocking infection all together. These results reveal that viral subgenomic RNAs do not always serve as mRNAs, and instead can perform important regulatory functions.

The inhibitory effects of the BTE and sgRNA2 on BYDV replication and transcription were dose-dependent (Figs. 2B, 4B). The dose dependency up to 10-fold excess supports our hypothesis that BTE and sgRNA2 trans-inhibited translation of capped and uncapped mRNAs by competing for translation initiation factor(s) (Wang et al., 1997). The BTE inhibits translation of capped, polyadenylated non-viral RNA in vitro (Wang et al., 1997) and in vivo (Fig. 7). Added eIF4F reversed the trans-inhibition effect caused by BTE in vitro (Wang et al., 1997), and the BTE specifically binds eIF4F and eIFiso4F (E. Allen, E. Pettit, and W.A. Miller, unpublished data). Moreover, our preliminary data suggest that sgRNA2 (via its BTE) inhibits translation of cellular mRNAs (R. Shen, W. Staplin, unpublished data).

Surprisingly, the replication of BYDV was not inhibited as much when the molar ratio of BTE/PAV6 was increased to 20:1 and 40:1. One possible explanation for this result is that, at these higher concentrations, the BTE may base pair to the viral 5' UTR by the kissing stem-loop interaction in trans instead of in cis. Thus, high concentrations of added BTE may stimulate translation by delivering translation factors to the 5' UTR in trans. This process would be possible only if the BTE in the 105 nt or sgRNA2 5' UTR context has a higher affinity for factors than the BTE in the 3' UTR context, so that at the highest concentrations of added BTE, there are still free factors available to bind BTE105 but which are too low in concentration to bind the BTE within BYDV genomic RNA to stimulate translation in cis.

A second possibility for the decreased inhibition at high BTE/sgRNA2 is that, at the highest concentrations, the BTE may not fold into a functional secondary structure, preventing trans-inhibition that occurs at lower concentrations.

It is noteworthy that the dose–response curve resembles the “bell-shaped” double-stranded RNA activated PKR antiviral response in mammals. dsRNA induces the response, but excessively high concentrations of dsRNA prevent dimerization of PKR necessary for autophosphorylation to initiate shut-off of translation (Davis and Watson, 1996; Hunter et al., 1975). A PKR system may also exist in plants (Bilgin et al., 2003) so we cannot rule out the possibility that the BTE may induce a PKR-like translational shutdown, as is the case for the highly structured 3’ UTRs of some tumor-suppressing genes (Nussbaum et al., 2002).

**Subgenomic RNA 2 trans-inhibits gene expression from RNAs containing or lacking the BTE**

Wild-type PAV6 trans-inhibited GFP expression from an unrelated virus, BMV with or without the BTE, whereas PAV6SG2 did not (Figs. 6A and C). Thus, the decreased expression levels of GFP were caused by BYDV sgRNA2. There are at least two explanations for the differential effect of PAV6 and PAV6SG2 on the translation of GFP from BMV. The first is that specific BTE secondary structure present only in the sgRNA2 context, but not gRNA and sgRNA1 due to position effect, and is required for the trans-inhibition function. A more likely reason for the lack of trans-inhibition by the BTE in the gRNA and sgRNA1 contexts (PAV6SG2) is that gRNA and sgRNA1 are normally present at >10-fold lower concentrations than sgRNA2. Furthermore, most gRNA may be sequestered in virions.

**Feedback regulation of BYDV gene expression by its sgRNA2**

The correlation between the stimulatory function of the BTE in cis and ability to inhibit virus replication in trans provides strong evidence that the same factors are used in trans-inhibition and cis-stimulation of translation. Competition studies showed that both sgRNA2 and the BTE trans-inhibited translation of gRNA in vitro (Wang et al., 1999). Here, we showed that sgRNA2 trans-inhibited translation of reporter mRNA (Fig. 7) and GFP expression from BMV in vivo (Fig. 6). These data suggest that the BTE and sgRNA2 trans-inhibit BYDV RNA replication and transcription by inhibiting translation of genomic RNA which prevents the production of the RdRp. However, we cannot rule out the possibility that sgRNA2 may also trans-inhibit RNA replication or transcription directly.

Combined with previously reported results, we propose a feedback regulation mechanism (Fig. 8): in the earliest stage of BYDV infection, viral RdRp is translated via BTE-mediated cap-independent translation of gRNA (Stage 1, Fig. 8). The RdRp then carries out viral RNA replication and sgRNA synthesis (Stage 2, Fig. 8). Viral RNAs accumulate with sgRNA2 becoming particularly abundant. Accumulated sgRNA2, via its 5’ BTE, trans-inhibits translation of BYDV RdRp from gRNA (Stage 3, Fig. 8). This switches gRNA from its mRNA function to its replicase template function and also allows it to be encapsidated, that is, gRNA is available for replication by the existing RdRp and for encapsidation (Stage 4, Fig. 8).

It is noteworthy that BYDV RNA accumulates in the absence of sgRNA2 (Figs. 6B, 7B, PAV6SG2), thus sgRNA2 is not essential for virus replication at least for 72 h in protoplasts. Moreover, the mechanism requires replication before the sgRNA2-mediated switch-off of translation. Thus, sgRNA2 may serve subtle regulatory roles, such as enhancing a different translational switch-off mechanism mediated by the replicase itself, that was
propose this previously (Barry and Miller, 2002). The effect of sgRNA2 on fitness of BYDV in plants and in the field remain to be tested.

A new regulatory role for a viral subgenomic RNA

We show here that RNA harboring the BTE trans-inhibits translation of other BTE-containing RNAs in vivo, as well as translation of RNA lacking the BTE. Thus, the BTE serves as a riboregulator, as proposed from previous in vitro translation experiments (Wang et al., 1999). Substantial evidence indicates that sgRNA2 is not translatable in vivo (A. Rakotondrafara, personal communication). In vitro data also showed that inhibition of translation by sgRNA2 does not require expression of ORF 6 (Wang et al., 1999). Thus, if sgRNA2 is not translatable, it must serve a non-mRNA function, such as a riboregulator.

Other trans-regulatory RNAs of RNA viruses have been reported. A 34-nt sequence in RNA2 of Red clover necrotic mosaic virus trans-activates synthesis of sgRNA from RNA1 by base pairing to RNA1 (Sit et al., 1998). Replication of FHV RNA2 is dependent on the synthesis of subgenomic RNA from RNA1 (Albarino et al., 2003; Eckerle and Ball, 2002). FHV RNA2 then down-regulates translation of subgenomic RNA from RNA1 (Zhong and Rueckert, 1993).

With regard to trans-regulation of translation, Adenovirus virus-associated (VA) RNAs (Mathews and Shenk, 1991) and Epstein–Barr virus EBER RNAs (Bhat and Thimmappaya, 1983; Clarke et al., 1990) protect against dsRNA-activated inhibitor (DAI)-mediated phosphorylation of eIF-2α by binding DAI (Sharp et al., 1993). Like BYDV sgRNA2, VA and EBER RNAs are nontranslated. As a 3′ UTR-derived trans-inhibitor of translation, sgRNA2 sequence resembles the cellular tumor suppressor genes for which the term riboregulator was first coined. The 3′ UTRs of alpha-tropomyosin (Rastinejad et al., 1993) and other (Manjeshwar et al., 2003) mRNAs alone have tumor suppressor activity. The former acts by PKR-mediated inhibition of translation (Davis and Watson, 1996). More recently, large classes of noncoding microRNAs (miRNA) have been found that regulate gene expression either by inducing mRNA degradation (RNAi) or by blocking translation (Bartel, 2004; Carrington and Ambros, 2003). Recently, Epstein–Barr virus has been shown to express miRNAs that target both host and viral mRNAs (Pfeffer et al., 2004). While its mechanism of action remains to be fully elucidated, sgRNA2 of BYDV appears to be a new example of a trans-regulatory RNA.

Materials and methods

Plasmids

Infectious BYDV-PAV genomic RNA was transcribed from the full-length clone, pPAV6 (Di et al., 1993). The sgRNA2 knockout mutant clone of BYDV-PAV, pPAV6ΔSG2, referred to previously as SG2G/C (Koev and Miller, 2000), differs from pPAV6 by a G to C mutation at position 4810, which prevents sgRNA2 synthesis. pTE and pTEBF are clones for T7 transcription of the 105-nt BTE RNA (TE105) and its nonfunctional mutant TEBF (Wang et al., 1997). pSG2 and pSG2BF allow T7 transcription of the 869-nt sgRNA2 and its nonfunctional mutant sgRNA2BF, respectively (Wang et al., 1999). Both pTEBF and pSG2BF contain a GATC duplication in the BanHI site in the BTE. This duplication destroys the cap-independent translation function of the BTE (Wang et al., 1997, 1999).

BMV RNA clones were kindly provided by A.L.N. Rao (University of California, Riverside). pT7B1, pT7B2, and pT7B3 are clones for T7 transcription of BMV RNA1, RNA2, and RNA3, respectively (Dreher et al., 1989). pT7B3EGFP is a clone of BMV RNA3 with the coat protein gene replaced by an enhanced green fluorescent protein (GFP) gene (Rao, 1997). pT7B3EGFP is a clone of BMV RNA3 with the coat protein gene replaced by an enhanced green fluorescent protein (GFP) gene (Rao, 1997). To construct pT7B3TEGF for T7 transcription of BMV.TEGFP RNA3, the 109-nt fragment corresponding to the BTE (nt 4809–4918) was amplified from pPAV6 by PCR using the upstream primer, 5′-GGAGATCTATGTTCTTTATCGATATTTATGTTAGAACAAACACAA-3′, and the downstream primer, 5′-CCCCTAGGTCGACATTGGGCCCATAACGAAATACA-3′. The PCR products were cut with BglII and SalI (restriction sites are in italics), then ligated with pT7B3EGFP that had also been digested with BglII and SalI. The same
strategy was used to clone pT7B3TEBFGFP, except the template for PCR was pSG2BF. The pT7B3TEGFP and pT7B3TEBFGFP constructs were verified by sequencing at the DNA Sequencing and Synthesis Nucleic Acid Facility of Iowa State University on an ABI377 sequencer (Applied Biosystems, Foster City, CA).

RNA preparation and infection of protoplasts

The capped and uncapped RNAs were synthesized by in vitro transcription by using the T7 mMESSAGE mMACHINE or MegaScript kits (Ambion, Austin, TX) as per manufacturer’s instructions. For transcription of infectious RNAs, BYDV constructs were linearized with BamHI and infectious clones of BMV, and Andy Ball for helpful discussion. This research was funded by grants from USDA/ NRI (2001-35319-10011) and NIGMS (GM067104).

Two-step electroporation

In the first step, oat protoplasts were inoculated with infectious BYDV PAV6 or PAV6 = C6SG2 RNA by electroporation as described by Dinesh-Kumar and Miller (1993), except that voltage was 280V. After incubation for 24 h at room temperature, cells were pelleted at 100 x g for 4 min, washed in electroporation buffer, centrifuged again at 100 x g for 4 min, then electroporated in the presence of the appropriate RNA at 280V.

Analysis of GFP expression

Oat protoplasts were analyzed for GFP expression 24, 48, 72, and 96 h after inoculation by flow cytometry by using an ELITE ESP fluorescence-activated cell sorter (Beckman-Coulter, Anaheim, CA) at the Cell and Hybridoma Facility of Iowa State University. All data presented in this report were obtained from at least three independent experiments.

Northern blot hybridization

Total RNA was extracted from protoplasts by using the RNeasy plant RNA isolation kit (QIAGEN, Los Angeles, CA) as per manufacturer’s instructions. Protoplasts were collected at indicated times post-inoculation, RNA extracted, and analyzed by Northern blot as described previously (Koev et al., 1999). A 32P-labeled probe complementary to the full-length GFP gene sequence RNA to detect recombinant BMV RNAs 3 and 4.

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References


