Long-distance RNA–RNA interactions and conserved sequence elements affect potato virus X plus-strand RNA accumulation

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ABSTRACT

Conserved octanucleotide sequences located upstream of two major potato virus X (PVX) subgenomic RNAs (sgRNAs), as well as elements in the 5' end of the genome, affect accumulation of sgRNA. To determine if complementarity between these sequences is important for PVX RNA accumulation, we analyzed the effects of mutations within these elements and compensatory mutations in a tobacco protoplast system and in plants. Mutations in the 5' nontranslated region (NTR mutants) that reduced complementarity resulted in lower genomic RNA (gRNA) and sgRNA levels, whereas mutations to the octanucleotide elements affected only the corresponding sgRNA levels. However, for both the NTR and octanucleotide mutants, the extent of reductions in RNA levels did not directly correlate with the degree of complementarity, suggesting that the sequences of these elements are also important. Mutants containing changes in the NTR and compensatory changes in one of the octanucleotide elements restored levels of gRNA and the other sgRNA species with an unaltered octanucleotide element to those of wild-type. Although compensatory changes significantly increased levels of the sgRNA species with the modified octanucleotide element, levels were not restored to those of wild-type. Our data indicate that long distance RNA–RNA interactions and the sequences of the interacting elements are required for PVX plus-strand RNA accumulation.

Keywords: *cis*-acting regulatory elements; plant RNA virus; RNA replication; subgenomic RNA synthesis

INTRODUCTION

Positive-strand RNA viruses replicate by utilizing input genomic RNA (gRNA) as a template for synthesis of a complementary minus-strand RNA, which subsequently serves as a template for synthesis of genomic plus-strand RNA. For some plus-strand RNA virus groups, minus-strand RNA also serves as a template for synthesis of subgenomic RNAs (sgRNAs) that contain genes frequently encoding structural proteins. Several mechanisms have been proposed for sgRNA synthesis in different RNA virus systems. Miller et al. (1985) proposed that brome mosaic virus (BMV) sgRNA synthesis occurred by internal initiation on minus-strand RNA, based on studies utilizing an *in vitro* BMV replicase extract. Using a similar BMV replicase preparation, Adkins et al. (1997) determined that recognition of the subgenomic promoter by RNA-dependent RNA polymerase (RdRp) was sequence specific. A discontinu-

ous RNA transcription process involving long-distance interactions between the terminal leader and the intergenic sequences has been proposed for coronaviruses (Lai et al., 1983; for review see Lai & Cavanagh, 1997). Alternatively, sgRNA synthesis in this system may involve generation of truncated minus-strand RNAs that serve as templates for sgRNA synthesis (Sawicki & Sawicki, 1990; Sethna et al., 1991; Jeong & Makino, 1992). A mechanism involving disruption of minus-strand RNA synthesis may also apply to the plant RNA virus, red clover necrotic mosaic virus (RCNMV). Sit et al. (1998) proposed that a *trans*-acting sequence element (34-nt) present in RNA-2 of RCNMV directly interacts with a complementary region in the RNA-1 sgRNA promoter region to terminate minus-strand synthesis and generate templates for sgRNA synthesis. In general, these examples indicate that *cis*- and *trans*-acting regulatory elements can act at a distance to modulate sgRNA synthesis.

Potato virus X (PVX), the type member of the potexvirus group, is a flexuous rod-shaped virus containing a 6.4-kb plus-stranded RNA genome (Bercks, 1970; Hiebert & Dougherty, 1988). The PVX genome, which is capped and polyadenylated, encodes five open read-

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ing frames (ORFs), as indicated in Figure 1 (Bercks, 1970; Huisman et al., 1988; Milne, 1988). ORF1 encodes the viral replicase protein (165 kDa), which is the only viral protein absolutely required for PVX RNA synthesis. This replicase exhibits the methyltransferase/helicase/polymerase arrangement found in all non-segmented viruses of the Sindbis-like supergroup (Rozanov et al., 1992). The triple gene block (TB), ORFs 2–4, have been shown to be necessary for viral cell-to-cell transport (Beck et al., 1991; Angell et al., 1996), and the product of ORF5, coat protein (CP), is involved in both virus movement and encapsidation (Chapman et al., 1992; Oparka et al., 1996). During PVX infection, genomic-length plus- and minus-strand RNAs, several sgRNAs, and corresponding double-stranded RNAs are produced (Dolja et al., 1987; Price, 1992). The two major sgRNAs are utilized for expression of the first TB gene (ORF2) and CP, respectively, whereas the other two TB genes (ORFs 3 and 4) are expressed from a less-abundant sgRNA (Morozov et al., 1991; Verchot et al., 1998).

We have previously shown that conserved octanucleotide sequence elements located upstream of the two major PVX sgRNAs are important for sgRNA accumulation in protoplasts and potentially some other aspect of the infection process in plants (Kim & Hemenway, 1997). We also found that multiple sequence and structural elements in the 5' nontranslated region (NTR) of the PVX RNA affect both gRNA and sgRNA accumulation (Kim & Hemenway, 1996; Miller et al., 1998). Levels of sgRNAs were reduced in protoplasts inoculated with mutants containing modifications in the 5' NTR elements, despite no reduction in minus-strand RNA accumulation. These results suggested that initiation of PVX sgRNA synthesis is not solely dependent upon the presence of minus-strand RNA and the appropriate local signals. Additionally, we observed that there is complementarity between the octanucleotide elements and the 5' terminal sequences and suggested that complementarity may contribute to regulation of sgRNA synthesis (Kim & Hemenway, 1997). In this study, we have analyzed the functional significance of this complementarity by inoculation of transcripts con-

taining mutations at the 5' end of the genome, within the conserved octanucleotide sequences, or compensatory changes in both regions onto *Nicotiana tabacum* protoplasts and plants. Our data indicate that complementarity between the 5' terminus (or 3' terminus of minus-strand RNA) and the conserved octanucleotide elements is important for both genomic and subgenomic plus-strand RNA accumulation. In addition, it appears that a long-distance interaction is a prerequisite for all plus-strand RNA accumulation and that subsequent sgRNA accumulation further depends on the sequences of the octanucleotide elements.

RESULTS

Alteration of conserved octanucleotide elements and reduction in complementarity affect sgRNA accumulation

To determine the role(s) of complementarity and conserved sequences in PVX RNA accumulation, we analyzed protoplasts and plants inoculated with transcripts containing mutations in the octanucleotide elements upstream of the TB and CP genes. The extent of complementarity was determined by direct alignment of the defined regions, without allowing for gapping. As shown in Figure 2A,B, the complementarity was reduced by 3 bp in mutants TB8 and CP8. For mutants TB9, TB10, and CP9, potential pairing was increased by one G:U pair, along with conversion of two existing A:U pairs to G:U pairs, resulting in a small increase in predicted stability (data not shown) compared to wild-type pairing. Modifications in mutant CP10 converted three A:U pairs to G:U pairs, but did not alter the number of potential pairs. All three mutations introduced in the element upstream of the CP gene (CP8–10) changed amino acid residues near the C terminus of the third TB gene as follows: Leu67 to Lys in CP8, Leu67 to Asn in CP9, and Ser68Phe69 to ArgAsn in CP10. However, it is unlikely that these codon changes will affect PVX replication because deletion of TB genes did not affect PVX replication in infected cells (Kim & Hemenway, unpubl. data). Two additional sets of mutants, TB4 and CP4 and TB6 and CP6, were used as control transcripts for detection of RNA accumulation. Mutants TB4 and CP4, which reduced complementarity by three potential pairs, were previously shown to greatly decrease sgRNA accumulation, while mutants TB6 and CP6, which changed sequences immediately upstream of the sgRNA start sites, had no effect on sgRNA levels (Kim & Hemenway, 1997). RNA accumulation in protoplasts inoculated with the mutant transcripts were always compared to levels in cells infected with wild-type transcripts or the replication defective negative control (p32).

Regardless of the mutations introduced, no significant changes in genomic minus- and plus-strand

FIGURE 1. Genome organization of PVX. The five ORFs encoded by the PVX genome are denoted by open boxes. Relative positions of the probes, P1 and P3, used in the S1 nuclease protection assay for detection of plus-strand and minus-strand RNAs, respectively, are indicated; asterisks denote the ^{32}P -labeled positions of these probes. Two arrows drawn below the first of the TB ORFs and the CP ORF are labeled pr1 and pr2, respectively, and represent the locations of primers used for primer extension detection of sgRNA species.

FIGURE 2. Effects of mutations at the terminus or within conserved elements on complementarity and sgRNA accumulation. The potential base pairing between the conserved octanucleotide elements (in bold text) and the 3' end of minus-strand RNA are shown for wild-type and mutant RNAs. Positions of the 5' ends of two major sgRNAs are denoted by V and potential base pairs are denoted by bold dots. Changes in complementarity resulting from modifications to the 5' NTR (mutants NTR1–5) are shown above the wild-type pairing for TB (A) and CP (B) sgRNAs. Modifications to the conserved elements upstream of the TB (A; mutants TB4 and TB8–10) and CP genes (B; mutants CP4 and CP8–10) are shown below the wild-type pairing. Protoplasts inoculated with replication-defective (p32), wild-type (wt), or mutant transcripts were harvested at 48 h postinoculation and were analyzed for TB (C,E) and CP (D,F) sgRNA levels by primer extension. Extension products isolated from 8×10^5 protoplasts were separated on 6% sequencing gels. Only primary extension products are shown.

RNA levels were observed by S1 nuclease protection (Table 1). In contrast, substantial reductions in TB sgRNA accumulation were observed for mutants TB4 (2%), TB8 (13%), TB9 (30%), and TB10 (33%), as shown in Figure 2C and Table 1. Similar reductions in CP sgRNA were observed with mutants CP4 (1%), CP8 (17%), CP9 (26%), and CP10 (27%) (Fig. 2D), which were mirrored by corresponding reductions in CP accumulation (data not shown). The greatest reductions in RNA levels were observed in protoplasts inoculated with TB8 and CP8 mutants that decrease complementarity by 3 bp. Sequence modifications in the conserved octanucleotide elements (TB9, TB10, CP9, and CP10) that maintained or increased complementarity and/or stability significantly reduced sgRNA accumulation, but larger decreases in sgRNA levels were observed when the modifications (TB4, TB8, CP4, and CP8) reduced potential pairing and stability. For all of

these mutants, modifications to sequences upstream of one gene did not significantly alter accumulation of the other major sgRNA species.

As we previously observed for mutants CP4 and TB4 (Kim & Hemenway, 1997), TB8–10 and CP8–10 transcripts did not result in infection after inoculation onto *Nicotiana benthamiana* plants (Table 2). Regardless of the effect of the mutation on sgRNA accumulation in protoplasts, none of the mutants containing modifications to the conserved octanucleotide elements were able to infect plants.

Mutations in the 5' NTR that reduce complementarity affect both gRNA and sgRNA accumulation

To further analyze the role of complementarity in sgRNA accumulation, sequences at the 5' end of the genome

TABLE 1. Effects of mutations in the 5' end of the genome and upstream of the sgRNA initiation site on PVX RNA accumulation

Mutants	Genomic ^{a,b}		Subgenomic ^{a,b}	
	(-)RNA	(+)RNA	TB	CP
p32	0	0	0	0
wild-type	100 ± 0	100 ± 0	100 ± 0	100 ± 0
NTR1	88 ± 7	74 ± 13 ^c	73 ± 12 ^c	98 ± 8
NTR2	96 ± 8	58 ± 16 ^c	62 ± 8 ^c	65 ± 4 ^c
NTR3	96 ± 7	38 ± 11 ^c	42 ± 14 ^c	42 ± 10 ^c
NTR4	81 ± 1	33 ± 9 ^c	30 ± 16 ^c	30 ± 14 ^c
NTR5	93 ± 13	45 ± 15 ^c	28 ± 11 ^c	23 ± 5 ^c
TB4	129 ± 14	78 ± 23	2 ± 2 ^c	114 ± 14
TB6	118 ± 7	116 ± 12	90 ± 7	115 ± 18
TB8	117 ± 8	92 ± 24	13 ± 7 ^c	109 ± 22
TB9	114 ± 7	99 ± 23	30 ± 4 ^c	108 ± 11
TB10	115 ± 6	118 ± 23	33 ± 3 ^c	107 ± 9
cTB4	121 ± 7	107 ± 8	60 ± 4 ^c	93 ± 7
cTB10	97 ± 11	105 ± 19	59 ± 1 ^c	122 ± 17
CP4	111 ± 17	84 ± 13	78 ± 1 ^c	1 ± 1 ^c
CP6	115 ± 18	110 ± 4	97 ± 10	74 ± 14
CP8	93 ± 10	126 ± 19	95 ± 12	17 ± 8 ^c
CP9	91 ± 12	84 ± 10	100 ± 12	26 ± 4 ^c
CP10	89 ± 8	85 ± 6	101 ± 1	27 ± 4 ^c
cCP4	97 ± 10	87 ± 8	92 ± 8	51 ± 7 ^c
cCP10	95 ± 12	90 ± 21	87 ± 11	50 ± 7 ^c

^aAccumulation of genomic (-)/(+)strand and sgRNAs were obtained by S1 nuclease protection and primer extension analyses, respectively.

^bEach value is the mean compared to that of wild-type and standard error compiled from at least three independent experiments.

^cThe value is significantly different ($T = 0.05$) from the wild-type.

were changed. All of these mutations (NTR1–5) were designed to destabilize the proposed complementarity by 2 to 3 bp (Fig. 2A,B). Genomic minus-strand RNA accumulation was not significantly affected by these mutations (Table 1). In contrast, genomic plus-strand RNA levels were reduced in protoplasts inoculated with each of these mutants, and values obtained from NTR3 (38%), NTR4 (33%), and NTR5 (45%) were significantly different from that of wild-type. Levels of TB and CP sgRNAs in these protoplasts were also reduced, and these reductions mirrored the reduction in genomic plus-strand RNA for all the NTR mutants, with the exception of NTR1 (Fig. 2E,F; Table 1). For protoplasts inoculated with this mutant, gRNA and TB sgRNA levels were reduced to approximately 70%, whereas levels of CP sgRNA were 98% of those in protoplasts inoculated with wild-type transcripts. In protoplasts inoculated with mutant NTR5, accumulation of gRNA and CP sgRNA was reduced to 45% and 23%, respectively.

All of the NTR mutants were able to infect *N. benthamiana* plants (Table 2). However, they exhibited a delay of symptom development, reduced symptom severity and lower levels of PVX CP compared to plants inoculated with wild-type transcripts. These results suggest that sequences at the 5' end of the genome that are complementary to the conserved elements are important for optimum plus-strand RNA accumulation.

Modifications to these elements reduced the rate of infection in plants, which likely reflects reduced plus-strand RNA levels and/or rate of accumulation.

Modifications in the 5' NTR that compensate mutant octanucleotide sequences restore gRNA and sgRNA accumulation in protoplasts, but not infectivity in plants

Because protoplasts inoculated with NTR mutants (NTR1–5) exhibited reduced level of genomic and subgenomic plus-strand RNA, it remained to be determined if this reduction was due to a change in the primary sequence and/or altered the complementarity to the conserved elements. Two of the NTR mutants, NTR4 and NTR5, contain changes in the 5' sequence that were complementary to the alterations in the conserved elements of TB4 or CP4 and TB10 or CP10, respectively. Four additional clones were constructed by adding mutations found in NTR4 to those in TB4 or CP4 (cTB4, cCP4) and those found in NTR5 to those in TB10 or CP10 (cTB10, cCP10) to determine if modifications in the two regions would be compensatory (Fig. 3A,B).

Minus-strand RNA accumulation in protoplasts inoculated with cTB4, cTB10, cCP4, or cCP10 transcripts was similar to wild-type levels and those found in protoplasts inoculated with the single mutants from which they were derived (NTR4, TB4, CP4, NTR5, TB10, and CP10; Table 1). Although both NTR4 and NTR5 significantly reduced plus-strand gRNA levels (33% and 45%,

TABLE 2. Infectivity on *N. benthamiana* plants

Mutants	Symptoms ^a	CP accumulation ^b
p32	0/9	–
wild-type	9/9	+
NTR1	9/9	+
NTR2	9/9	+
NTR3	7/9	+
NTR4	6/9	+
NTR5	3/9	+
TB4	0/9	–
TB8	0/9	–
TB9	0/9	–
TB10	0/9	–
CP4	0/9	–
CP8	0/9	–
CP9	0/9	–
CP10	0/9	–
cTB4	0/9	–
cTB10	0/9	–
cCP4	0/9	–
cCP10	0/9	–

^aThe number of plants showing symptoms at 28 days postinoculation out of nine plants inoculated.

^bCP accumulation was determined by Western analysis at 7 days postinoculation.

FIGURE 3. Effects of compensatory mutations on accumulation of PVX sgRNAs. Potential pairing for transcripts derived from cTB4 and cTB10 are compared to wild-type pairing in **A**. The cTB4 transcript contains the NTR4 mutations at the terminus and TB4 changes in the octanucleotide element; cTB10 transcripts include the NTR5 terminus and TB10 octanucleotide element. Shown in **B** are potential pairings for cCP4 and cCP10 transcripts, which contain NTR4/CP4 and NTR5/CP10 mutations, respectively. For all of the compensatory mutants, changed sequences are underlined. Primer extension analysis for detection of TB (**C**) and CP (**D**) sgRNAs was as described in the legend to Figure 2.

respectively), these levels were restored to wild-type levels in protoplasts inoculated with cTB4, cCP4, cTB10, and cCP10 (Table 1). These data suggest that complementarity between the 5' region and the conserved elements is important for optimum gRNA accumulation.

Protoplasts inoculated with cTB4 exhibited TB sgRNA levels that were 60% of wild-type, which was significantly increased above the 2% levels detected in protoplasts inoculated with TB4 (Fig. 3C, Table 1). Similarly, TB sgRNA levels in protoplasts inoculated with mutant cTB10 were 59% compared to wild-type and represented a significant increase over 33% observed for TB10. Conversion of the sequence at the 5' end of the genome in cCP4 and cCP10 restored CP sgRNA accumulations to 51% and 50% compared to that of wild-type, and these levels were increased over the 1% and 27% observed for CP4 and CP10, respectively (Fig. 3D, Table 1). In addition, changes in the levels of CP sgRNA accumulation mirrored the corresponding changes in CP accumulation (data not shown). Although these compensatory changes at the 5' end significantly increased accumulation of the sgRNA residing downstream of the modified octanucleotide element and indicate the importance of complementarity in RNA accumulation, these levels were still lower than that of wild-type. In contrast, the levels of the other sgRNA residing downstream of an unmodified octanucleotide element were restored by these compensatory changes and these levels did not differ significantly from wild-type. These data indicate that complementarity be-

tween the terminus and one of the octanucleotide elements was important for genomic and sgRNA accumulation, but that the sequences of the conserved octanucleotide elements were critical for optimal sgRNA accumulation.

The importance of the conserved element sequence was further supported by plant infection data (Table 2). Although the NTR4 and NTR5 mutants decreased complementarity to the conserved element and exhibited reduced gRNA and sgRNA levels in protoplasts, they were infectious on plants. In contrast, the corresponding cTB4, cTB10, cCP4, and cCP10 mutants were unable to infect plants despite their increased RNA levels in protoplasts. These data indicate that although complementarity affects RNA accumulation, the conserved element is also required for some aspect of the infection process in plants.

DISCUSSION

Previous deletion studies indicated that multiple sequence elements within the PVX 5' NTR are required for both genomic and sgRNA accumulation (Kim & Hemenway, 1996). Additionally, sgRNA levels are regulated by conserved octanucleotide elements upstream of the two major sgRNA species and these elements exhibit complementarity to the 5' terminus of PVX RNA (Kim & Hemenway, 1997). In this report, mutational analyses of the conserved octanucleotide elements and terminal sequences were utilized to determine if inter-

actions between distant regions are necessary for PVX plus-strand RNA accumulation.

Alterations to the conserved element upstream of one sgRNA species that reduced the extent of complementarity (mutants CP4, CP8, TB4, and TB8) substantially lowered the corresponding sgRNA levels, and did not significantly affect accumulation of the other sgRNA or genomic-length minus- and plus-strand RNAs. When modifications were introduced into the octanucleotide elements that maintained or increased the predicted complementarity, with replacement of some A:U pairs with G:U pairs (mutants CP9, CP10, TB9, and TB10), we also observed reductions in the corresponding sgRNA levels, but to a lesser extent. Although compensatory changes at the terminus did increase sgRNA levels for octanucleotide mutants with either decreased or increased complementarity, these levels were restored to no more than 50–60% of wild-type. These data indicate that complementarity contributes to sgRNA accumulation, but that the sequences of the octanucleotide elements are critical for optimal accumulation of the corresponding sgRNAs. This octanucleotide sequence requirement was also indicated in the plant infection data, where none of the octanucleotide or compensatory mutants was able to elicit infection in *N. benthamiana* plants. Additional experiments will be required to determine the relative importance of each residue within these octanucleotide elements under similar conditions of predicted pairing and/or stability.

All of the 5' NTR mutations were predicted to have reduced pairing with the octanucleotide elements (6/11 or 7/11 pairs) and all exhibited some reduction in plus-strand RNA levels. RNA levels in the protoplasts inoculated with the NTR1 mutant that has only 2 nt changes from the wild-type sequence (7/11 predicted pairs) were minimally affected. For the other NTR mutants (NTR2–5) containing 3 or 4 nt changes, the reductions in plus-strand RNA levels were greater. In addition, the extent of reduction was similar for all the plus-strand RNA species measured, indicating that the terminal sequences and/or potential interactions with these sequences are a prerequisite for all plus-strand RNA accumulation. The conservation of GU-rich terminal nucleotides at the 3' termini of different potexvirus minus-strand RNAs (Fig. 4) may indicate that sequence in this region is important for RNA accumulation. The first 3 nt are identical for several potexvirus sequences and the subsequent 8-nt elements that are complementary to the conserved octanucleotide elements upstream of the sgRNA species are also similar among these viruses. Alternatively, the abundance of GU-rich residues, rather than the precise sequence, at the termini may provide alternative pairing possibilities with the conserved downstream elements. Previously, we found that deletion of the first 8 nt from the 5' end (mutant $\Delta 8$) did not affect PVX genomic or sgRNA accumulation (Kim & Hemenway, 1996). Although the predicted pair-

PVX	3' - CUU UUGAUUUG GUAUG
BaMV	3' - CUU UUGGUGAG GUUUG
CCMV	3' - GUU UUGUUUUG UCUUG
CYMV	3' - CUU UUGUUUUG CUUUU
FMV	3' - CUU UUGAGAAG GCUUU
NMV	3' - CUU UUGUUUGG UUGUG
PMV	3' - CUU UUCUUUGU GUUUC
PIAMV	3' - CUU UUGUUUGG AUGUG
PAMV	3' - CUU UUGUUUUG UUGUU
SMYEA	3' - CUU AUAGGUUG UGUGA
WCIMV	3' - CUU UUGUUCUG CUCUG

FIGURE 4. Comparison of 3' terminal sequences on potexvirus minus-strand RNAs. Sequences from gRNAs of our PVX strain (Hemenway et al., 1990), bamboo mosaic virus (BaMV; Lin et al., 1994), cassava common mosaic virus (CCMV; Calvert et al., 1995), clover yellow mosaic virus (CYMV; Sit et al., 1990), foxtail mosaic virus (FMV; Bancroft et al., 1991), narcissus mosaic virus (NMV; Zuidema et al., 1989), papaya mosaic virus (PMV; Sit et al., 1989), plantago asiatica mosaic virus (PIAMV; Solovyev et al., 1994), potato acuba mosaic virus (PAMV; Xu et al., 1994), strawberry mild yellow edge-associated virus (SMYEA; Jelkmann et al., 1992), and white clover mosaic virus (WCIMV; Forster et al., 1988) are shown. The sequences that are complementary elements to the octanucleotide are shown in bold.

ing between the terminus and the TB octanucleotide element of this deletion mutant is 7/11 bp compared to 9/11 for the wild-type pairing, this level of complementarity may be sufficient as long as particular nucleotides are paired. In contrast, deletion of 12 nt from the 5' end (mutant $\Delta 12$), predicted to further reduce complementarity to 4/11 bp, resulted in no detectable plus-strand RNA accumulation.

Regardless of the extent to which terminal sequence and/or precise nucleotides involved in pairing affect interactions, the compensatory mutant data strongly indicate that plus-strand RNA accumulation is dependent on an interaction between the terminus and the downstream conserved elements. When mutations in the 3' terminal element are compensated by changes to one of the subgenomic elements (mutants cTB4, cTB10, cCP4, and cCP10), levels of gRNA are restored to wild-type levels. The sgRNA species downstream of the altered octanucleotide element is increased, but is not restored to wild-type because of the importance of the octanucleotide sequence. In contrast, the sgRNA derived from the unaltered element is also restored to wild-type in the compensatory mutants, despite the reduced complementarity to the altered 3' terminal element. Thus, complementarity and interaction between the terminus and at least one downstream element may be required for initiation of plus-strand RNA synthesis. Once this event occurs, sgRNAs will accumulate optimally if the octanucleotide elements are unchanged.

The positions of known and putative *cis*-acting regulatory elements on PVX gRNA and corresponding

minus-strand RNA are shown in Figure 5A. Two elements similar to the conserved octanucleotide elements upstream of the TB and CP genes can be found upstream of the second of the TB genes at position 5011 and within the 3' region of the replicase gene at nt 3379, but similar additional elements are found in only a few other potexviruses. Dolja et al. (1987) observed that PVX infected plants contained several minor RNA species (3.6, 3.0, 1.8, and 1.4 kb) in addition to the more abundant 2.1- and 0.9-kb products associated

with TB and CP sgRNAs, respectively. Whether or not these minor RNA species are derived from transcription at these additional elements remains to be determined. Support for the functional significance of the element at nt position 5011 in PVX RNA comes from data by Verchot et al. (1998), which indicates that the proteins encoded by the second and third of the TB genes are translated from a 1.4-kb sgRNA *in vivo*. Elements complementary to the conserved octanucleotide elements are also found at the termini. The im-

FIGURE 5. Elements and interactions involved in PVX plus-strand RNA accumulation. A map of known and potential *cis*-acting elements required for PVX plus-strand RNA accumulation is shown in **A**. Positions and sequences of *cis*-acting elements defined here and previously (Kim & Hemenway, 1997; White et al., 1992), as well as potential regulatory elements of similar sequence (denoted by ?), are indicated on both the PVX plus-strand RNA (top sequence) and corresponding minus-strand RNA (bottom sequence). A model for interactions on minus-strand RNA that are required for plus-strand RNA synthesis is shown in **B**. The black hatched box corresponds to the element (5'-GUUUAGUU-3') located 4 nt from the 3' end of minus-strand RNA and the gray hatched boxes depict the TB and CP octanucleotide elements (3'-CAAUUCAA-5'). The putative octanucleotide element near ORF 3 is marked by a gray hatched box with a ? below it. Also shown are the products of plus-strand RNA synthesis, including gRNA, TB sgRNA, CP sgRNA, and the putative sgRNA initiating near ORF 3 (indicated by a ?).

portance of the element 4 nt from the 5' end of plus-strand RNA or corresponding 3' end of minus-strand RNA was demonstrated by the NTR mutant data presented herein. A similar element present at the 3' end of plus-strand RNA or 5' end of minus-strand RNA was shown by White et al. (1992) to be important for RNA accumulation of another potexvirus, clover yellow mosaic virus. Although they reported that mutation or deletion of the six central nucleotides within this element decreased D-RNA accumulation, they did not determine if the alterations differentially affected minus- and/or plus-strand D-RNA levels or if modifications to this element in gRNA would have similar effects on genome replication. They postulated that the location of this element on the 3' end of the gRNA and similar elements upstream of the sgRNAs may function for binding of the replication machinery. Not shown in Figure 5 is a stem-loop structure (SL1) spanning nt 32–105 in the 5' region of the PVX genome (Miller et al., 1998). Modifications to specific sequence and structural features of SL1 result in decreased genomic and sgRNA levels, although the terminal sequences are not changed. Thus, multiple elements on either strand may serve various functions during PVX replication.

Miller et al. (1985) observed that BMV sgRNA synthesis in the BMV template dependent replication system required only local regulatory elements for internal initiation on input minus-strand RNA, but this process may require additional elements *in vivo* (Huntley & Hall, 1993). Although our data do not rule out that initiation of PVX sgRNA synthesis occurs internally on minus-strand RNA, this process cannot proceed unless terminal elements are also present and interact with regions elsewhere. One model for these interactions on minus-strand RNA is illustrated in Figure 5B. In this model, initiation of plus-strand RNA synthesis requires an interaction between the 3' terminal element and a complementary element elsewhere on the minus-strand RNA. Although our data suggest that the two sites upstream of the TB and CP genes act as partners in this process, we cannot rule out use of other potential sites of similar sequence (noted by question marks in Fig. 5B).

Once this process is initiated, sgRNA synthesis can also proceed and may be enhanced by further interactions between the terminal element and octanucleotide elements upstream of the sgRNAs. Our data indicate that if one of the internal octanucleotide elements is modified, only the corresponding sgRNA will be reduced and gRNA is unaffected because other potential pairing partners are present elsewhere. In contrast, if the 3' terminal element is modified, all plus-strand RNA accumulation is similarly affected, potentially because of a reduced rate of initiation. Thus, the initiation events for synthesis of gRNA would be a prerequisite for synthesis of all plus-strand RNA species, and the extent of subsequent sgRNA accumulation would

additionally depend on the sequence of the corresponding octanucleotide elements. As an alternative to modeling the interactions on minus-strand RNA, similar interactions could occur within the gRNA to generate truncated species during minus-strand RNA synthesis. These truncated minus-strand RNAs (minus sgRNAs) would then serve as templates for plus-strand sgRNA synthesis, as suggested for coronaviruses (Sawicki & Sawicki, 1990; Sethna et al., 1991) and for the plant virus, RCNMV (Sit et al., 1998). The presence of double-stranded versions of the two major sgRNAs in PVX-infected cells (Dolja et al., 1987; Price, 1992) supports this type of mechanism.

Another potential role for the conserved elements is that they serve as signals for pausing and/or termination of the replication machinery and that interactions between elements modulate this process. Using the adenovirus major late gene arrest site containing sequences that block pol II elongation, Reeder & Hawley (1996) demonstrated that sequences near the 5' terminus of the transcript pair with the sequences upstream of the arrest site to relieve arrest. They proposed that this interaction somehow modifies the polymerase to move from a paused to elongation state. In some procaryotic and eucaryotic DNA-dependent RNA polymerase transcription systems, transcripts contain GU-rich sequences in the paused regions (for reviews, see Reeder & Lang, 1997; von Hippel, 1998). For PVX, the 3' terminus of the newly synthesized minus-strand RNA contains the GU-rich element, GUUUAGUU (Fig. 4). This element and surrounding GU-rich sequences may promote pausing/termination and allow for the machinery to prepare for initiation of plus-strand RNA synthesis. Once plus-strand RNA synthesis is initiated, elongation continues until the GU-rich conserved octanucleotide elements upstream of the TB and CP genes are synthesized and signal pausing/termination, allowing for reinitiation of the replication machinery or loading of additional replication complexes to initiate sgRNA synthesis. In addition, there are GU-rich sequences near the 3' terminus of plus-strand RNA (not shown in Fig. 5) that might regulate termination and subsequent polyadenylation of the gRNA.

Our data indicate that both the sequences of regulatory elements and the complementarity between terminal sequences and internal conserved elements are necessary for plus-strand RNA accumulation, suggesting that long-distance RNA–RNA interactions and sequence recognition by components of the replication complex are required. Viral and/or host proteins may modulate this process by stabilizing or destabilizing potential RNA–RNA pairing through RNA–protein and protein–protein interactions. Experiments are underway to further define the *cis*- and *trans*-acting elements and interactions that regulate PVX RNA synthesis, and to distinguish among the mechanisms proposed in this study.

MATERIALS AND METHODS

Materials

All restriction enzymes, modifying enzymes, polymerases, and m⁷GpppG cap analog were purchased from New England BioLabs. RNasin and RQ1 RNase-free DNase were obtained from Promega. Deoxy-, dideoxy- and ribonucleotides were purchased from Boehringer Mannheim and oligonucleotides were synthesized by Genosys. Reagents for protoplast preparation were obtained from Yakult Honsha Co., Ltd. and Karlan Research Products.

Site-directed mutagenesis and in vitro transcription

Mutations were introduced into the PVX cDNA clone, pMON8453 (Hemenway et al., 1990) using the muta-Gene in vitro mutagenesis kit (Bio-Rad; Kunkel, 1985). The incorporation of the desired mutant was confirmed by sequencing, and a region containing the mutation was resected back into a wild-type pMON8453 clone. The resulting clones were sequenced through the entire resected fragment and flanking regions to ensure that only the mutations introduced were present. In vitro transcription reactions were performed as described previously (Kim & Hemenway, 1996). The quality and relative concentrations of transcripts were checked by electrophoresis on a 1% agarose gel at 4 °C, and visualized by ethidium bromide staining.

Preparation and inoculation of protoplasts

Protoplasts from a rapidly dividing *N. tabacum* suspension cell line, NT-1, were prepared as described previously (Kim & Hemenway, 1997). Five micrograms of transcripts in 0.4 mL of electroporation buffer (0.8% NaCl, 0.02% KCl, 0.02% KH₂PO₄, 0.11% Na₂HPO₄, 0.4 M mannitol, pH 6.5) were added to 0.4 mL aliquots of 2×10^6 protoplasts, and subjected to 250 V at a capacitance of 500 μ F with a BioRad Gene Pulser. After incubation on ice for 15 min, protoplast samples were diluted with 8 mL of culture medium containing 0.4 mg/mL 2,4-D, 0.4 M mannitol, and 10 μ g/mL RNase A. Inoculated protoplasts were then allowed to incubate for 48 h at 25 °C on a 14-h light and 10-h dark cycle. After incubation, cells were washed and incubated with an additional 10 μ g/mL of RNase A for 60 min to digest the remaining input RNA. Protoplast experiments with wild-type and mutant transcripts were performed separately at least three times with different transcript preparations.

RNA and protein analyses

Total RNA was isolated from inoculated protoplasts using Trizol reagent (Life Technologies) at 48 h postinoculation. S1 nuclease protection assays were used for detection of minus- and plus-strand PVX RNAs, with single-stranded DNA probes, P3 and P1, respectively (Fig. 1). Accumulation of TB and CP sgRNAs was measured by primer extension with pr1 and pr2, respectively, according to methods described previously (Kim & Hemenway, 1996, 1997). Products from S1 nuclease digestion and from extension reactions were separated on 6%

sequencing gels and visualized by autoradiography. Relative molar amounts of PVX RNAs in each sample were determined by using Image Quant software on a Molecular Dynamics Phosphorimager. Differences in RNA accumulation were subjected to Student *t*-tests and were considered significant if within a confidence interval of 95% and showing 1.5-fold or more change compared to wild-type.

For protein analysis, 10⁵ protoplasts were resuspended in 30 μ L of Laemmli loading buffer (Laemmli, 1970), electrophoresed on 12% SDS-polyacrylamide gels, and blotted onto nitrocellulose membrane (Towbin et al., 1979). The blots were probed with antiserum prepared against purified PVX, and products were visualized using a Biotin/StreptAvidin kit (Amersham).

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