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Mutations That Alter a Conserved Element Upstream of the Potato Virus X Triple Block and

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The putative subgenomic RNA (sgRNA) promoter regions upstream of the potato virus X (PVX) triple block and coat protein (CP) genes contain sequences common to other potexviruses. The importance of these sequences to PVX sgRNA accumulation was determined by inoculation of *Nicotiana tabacum* NT1 cell suspension protoplasts with transcripts derived from wild-type and modified PVX cDNA clones. Analyses of RNA accumulation by S1 nuclease digestion and primer extension indicated that a conserved octanucleotide sequence element and the spacing between this element and the start-site for sgRNA synthesis are critical for accumulation of the two major sgRNA species. The impact of mutations on CP sgRNA levels was also reflected in the accumulation of CP. In contrast, genomic minus- and plus-strand RNA accumulation were not significantly affected by mutations in these regions. Studies involving inoculation of tobacco plants with the modified transcripts suggested that the conserved octanucleotide element functions in sgRNA accumulation and some other aspect of the infection process.

INTRODUCTION

Potato virus X (PVX) is the type member of the potexvirus group of plant, plus-strand RNA viruses (Bercks, 1970; Milne, 1988). The flexuous rod-shaped particles contain a 6.4-kb genomic RNA that is capped and polyadenylated. Of the five open reading frames (ORFs) encoded by the PVX genome (Huisman *et al.,* 1988; Skryabin *et al.,* 1988), only the product of ORF1, the viral replicase, is absolutely required for RNA synthesis. Products of the three internal, overlapping reading frames (ORF 2–ORF 4), which are referred to as the triple block (TB) genes, are necessary for virus movement (Beck *et al.,* 1991; Angell *et al.,* 1996). ORF5 encodes the coat protein (CP), which is necessary for encapsidation and for virus movement (Chapman *et al.,* 1992a; Baulcombe *et al.,* 1995; Oparka *et al.,* 1996). PVX infection results in production of various species of PVX RNAs (Dolja *et al.,* 1987) and proteins corresponding to each of the ORFs (Price, 1992). Genomic minus-strand RNAs, genomic and subgenomic plus-strand RNAs, and double-stranded versions of these RNAs have been detected (Dolja *et al.,* 1987). Similar RNA species have been synthesized *in vitro* using membrane-containing extracts derived from infected plants (Doronin and Hemenway, 1996). The PVX replicase is likely to be the only protein translated directly from the genomic RNA, whereas 3′ coterminal subgenomic RNAs (sgRNAs) serve as the templates for translation of other viral proteins (Grama and Maior, 1990; Morozov *et al.,* 1990). The two major sgRNA species of approximately 2.1 and 0.9 kb in length have their 5′ termini upstream of the TB and CP genes, respectively (Dolja *et al.,* 1987; Kim and Hemenway, 1996).

The *cis*- and *trans*-acting regulatory functions and the mechanisms for PVX RNA synthesis are not well-characterized. White *et al.* (1992) determined that a hexanucleotide sequence (5′-ACUUAA-3′) located in the 3′ nontranslated region (NTR) of various potexvirus genomic RNAs is important for the replication of clover yellow mosaic virus. Recently, Sriskanda *et al.* (1996) reported that an 8-nucleotide, U-rich motif located downstream of the conserved hexanucleotide in the 3′ NTR of PVX RNA serves as a target for binding of host proteins and is required for minus-strand RNA synthesis. Deletion of elements in the PVX 5′ NTR inhibits both genomic and sgRNA accumulation, but minus-strand RNA levels are relatively unchanged (Kim and Hemenway, 1996). Additionally, both sequence and structural elements in the 5′ NTR affect plus-strand RNA levels (E. D. Miller and C. Hemenway, unpublished data). Although a study by Chapman *et al.* (1992b) demonstrated that a region around the 5′ end of the CP gene (nt 5586–5666) can direct synthesis of a reporter gene in a PVX vector, detailed analyses of the *cis*-acting elements required for sgRNA synthesis are lacking.

Alignment of several potexvirus RNA sequences has revealed nucleotide conservation upstream of the putative sgRNA initiation codons (Skryabin *et al.,* 1988; Sit *et al.,* 1989; Zuidema *et al.,* 1989; White and Mackie, 1990; Bancroft *et al.,* 1991; Solovyev *et al.,* 1994). Additionally, White *et al.* (1990) observed that the conserved se-

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quences are similar to the hexanucleotide element located near the 3′ end of potexviral genomic RNA and proposed that these elements may represent recognition sites for the replicase. Sequence elements important for sgRNA synthesis of several plant tricornaviruses, some of which are similar to the internal coding regions of tRNA genes, are not represented in potexviral genomes (for review, see Duggal *et al.,* 1994).

In this study, we analyzed the effect of mutations in regions upstream of the two major PVX sgRNAs on the accumulation of plus- and minus-strand PVX RNAs in tobacco plants and protoplasts. Our data indicate that a conserved octanucleotide element, which includes the hexanucleotide element reported by White *et al.* (1992), is important for sgRNA accumulation in protoplasts and may also function in other aspects of PVX infection in plants. Spacing between these conserved elements and the 5′ ends of the sgRNAs, as well as the identity of the start-site nucleotides, may also be important for PVX sgRNA accumulation.

MATERIALS AND METHODS

Materials

All restriction enzymes, S1 nuclease, T7 RNA polymerase, and cap analogue were obtained from New England Biolabs. RQ1 RNase-free DNase I and Sequenase were purchased from Promega and U.S. Biochemicals/Amersham, respectively. Avian myeloblastosis virus reverse transcriptase was purchased from Life Sciences. Reagents for protoplast digestion were purchased from Yakult Honsha Co., Ltd. and Karlan Research Products.

Construction of site-directed mutants

Construction of the PVX wild-type (wt) plasmid, pMON8453, and a replication-defective control plasmid, p32, was described previously (Hemenway *et al.,* 1990; Kim and Hemenway, 1996). Mutations located upstream of the TB and CP sgRNAs were constructed by site-directed mutagenesis of pMON8453 ssDNA templates using synthetic oligonucleotides as described by Kunkel *et al.* (1985). All site-directed mutations were verified by sequencing, and the mutated regions were resected back into the wt background. *Bst*EII and *Afl*II sites were used for resection of TB mutants, and CP mutants were resected using *Bsu*36I and *Nhe*I. The final mutant constructs were confirmed by sequencing through the entire resected regions.

RNA transcription

RNA transcripts were generated from *Spe*I-linearized cDNA templates using T7 RNA polymerase as described by Kim and Hemenway (1996). Briefly, 5 μg of DNA templates were incubated at 37° in a 100-μl reaction containing 40 m*M* Tris–HCl, pH 7.9, 6 m*M* $MgCl_2$, 2 m*M* spermidine, 10 m*M* DTT, 100 μg/ml BSA, 40 units RNasin, 100 μM ATP, CTP, and UTP, 12.5 μM GTP, 100 μM m^7GpppG, and 20 units of T7 RNA polymerase. After 10 min, GTP was added to 50 μM final concentration, and the reaction was incubated for an additional 50 min. Integrity and relative concentration of purified transcripts were determined by agarose gel electrophoresis at 5°.

Protoplast isolation and inoculation

Protoplasts from *Nicotiana tabacum* cv. Samsun NN suspension culture (NT1) were prepared according to An (1985) and Fontes *et al.* (1994). Protoplasts were digested with 0.75% cellulase, 0.1% pectolyase Y3, in 20 m*M* 2-(*N*-morpholino)ethanesulfonic acid, 0.4 *M* mannitol, pH 5.5. After digestion, protoplasts were washed twice with 0.4 *M* mannitol, pH 5.5, and resuspended in electroporation buffer containing 0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.11% Na_2HPO_4, 0.4 *M* mannitol, pH 6.5. Approximately, 2×10^6 protoplasts were inoculated with 5 μg of RNA transcripts by electroporation (250 V, 500 μF). After inoculation, each protoplast sample was treated with RNase A (1 μg/ml) to remove uninoculated input RNA and incubated at room temperature under constant fluorescent light for up to 48 hr.

RNA analyses

Total RNA was isolated from inoculated protoplasts using Trizol reagent at 48 hr postinoculation (hpi). For detection of plus- and minus-strand PVX RNAs, total RNA from 4×10^5 and 1.6×10^6 protoplasts was hybridized to ^{32}P-labeled strand-specific probes (1.5×10^4 cpm), P1 and P3 (Fig. 1), respectively, and subsequently digested with S1 nuclease (Kim and Hemenway, 1996). Accumulation of TB and CP sgRNAs was measured by primer extension according to Kim and Hemenway (1996), except that total RNA from 8×10^5 protoplasts was used. Specifically, total RNA was annealed to 0.5 pmol (5×10^5 cpm) of 5′ end-labeled oligonucleotide primers complementary to the TB region (nt 4584–4600; ePtb) or to the CP gene (nt 5710–5725; ePcp). Products from S1 nuclease digestion and from extension reactions were separated on 6%-polyacrylamide sequencing gels and visualized by autoradiography. Probes and primers were determined to be in excess by hybridization of equal amounts of probe/primer to RNA isolated from increasing amounts of infected protoplasts. Signals increased linearly when hybridizations included RNA isolated from 10 to 100% of protoplast samples (data not shown). Relative molar amounts of PVX RNAs in each sample were determined by using Image Quant software on a Molecular Dynamics PhosphorImager. Microsoft Excel was used for statistical calculations. Because of variability inherent in the protoplast experiments, Student's two-tail *t* tests were used to calculate confidence levels for values that

differed from wt. Differences were considered significant if the *P* value was ≤ 0.05.

For Northern blot analysis, RNA from 8×10^5 protoplasts were electrophoresed on a 1% agarose/6% formaldehyde gel and transferred to GeneScreen (Dupont) membrane by electroblotting at 10V for 2 hr in 25 m*M* sodium phosphate buffer, pH 5.5. The membrane was hybridized with ^{32}P-labeled (6×10^7 cpm) full-length, minus-strand RNA transcript for 16 hr at 55° (Sambrook *et al.,* 1989).

Plant inoculations and phenotype analysis

Capped transcripts (5 μg) derived from pMON8453 or from mutant clones were resuspended in 0.1 *M* phosphate buffer (pH 8.0) and inoculated onto two leaves of individual *N. benthamiana* plants (Hemenway *et al.,* 1990). Inoculated plants were maintained at 25° in 14 hr light and at 22° in 10 hr dark, and were monitored for symptom development for up to 28 days postinoculation (dpi). At 7 dpi, 0.15 g tissue from two inoculated or two upper leaves was homogenized in 0.5 ml of buffer containing 10 m*M* HEPES, pH 7.9, 10 m*M* KCl, 0.1 m*M* EDTA, 1 m*M* DTT, and 0.5 *M* PMSF. Samples were centrifuged for 15 min at 10,000 *g* and the supernatants were utilized for protein analysis.

Protein analysis

For analysis of CP accumulation in protoplasts at 48 hpi, 2×10^5 protoplasts (20 μg total protein) were resuspended in 30 μl of Laemmli loading buffer (Laemmli, 1970), electrophoresed on 12%-polyacrylamide/SDS gels, and blotted onto nitrocellulose membrane (Towbin *et al.,* 1979). For analysis of CP accumulation in infected plants, 20 μl of plant extracts (27 μg total protein) were similarly analyzed. The blots were probed with antiserum prepared against purified PVX, and products were visualized using a Biotin/StreptAvidin alkaline phosphatase kit (Amersham).

Computer-generated figures

Images depicted in figures were derived from PhosphorImager files or by scanning autoradiograms with a UMAX Powerlook II scanner and were directly imported into Adobe Photoshop on a Power Macintosh.

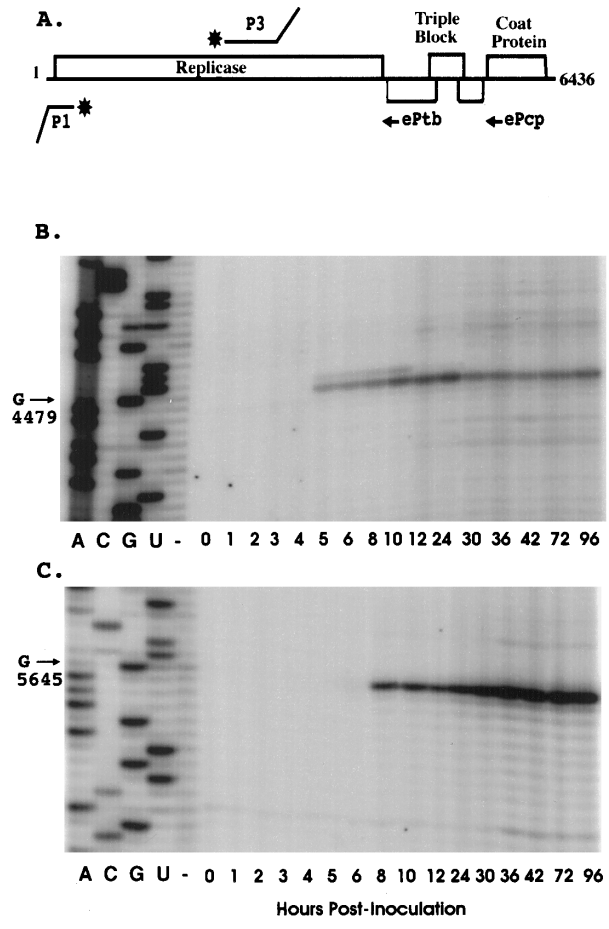

FIG. 1. Detection of PVX sgRNA accumulation. (A) A schematic representation of the PVX genome depicts the five ORFs, which are denoted by open boxes. Relative positions of the probes, P1 and P3, used in the S1 nuclease protection assay for detection of plus-strand and minus-strand RNAs, respectively, 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 ePtb and ePcp, respectively, and represent the locations of primers used for primer extension detection of sgRNA accumulation. (B and C) *N. tabacum* cv. Samsun suspension culture protoplasts (NT1) were inoculated with wt PVX RNA transcripts derived from pMON8453 and were harvested at various hpi. Total RNAs extracted from 8×10^5 protoplasts or 0.5 μg of purified PVX viral RNA (−) were extended with ePtb (B) or ePcp (C) primers, and extension products were separated on 6% sequencing gels. Sequences of PVX RNA (A, C, G, and U) generated with the same primers are indicated. The positions of primary extension products are numbered and marked by arrows.

RESULTS

Timecourse of TB and CP sgRNA accumulation in protoplasts

The timecourse for accumulation of the TB and CP sgRNAs in protoplasts was determined to evaluate the relative accumulation of these RNAs and to ascertain an appropriate timepoint for subsequent mutational studies. *N. tabacum* NT1 cell suspension protoplasts were inoculated with capped transcripts derived from the wt PVX cDNA clone, pMON8453. Total RNA was extracted at various times after inoculation and the accumulation of TB and CP sgRNAs was determined by primer extension analysis using primers, ePtb and ePcp, which are complementary to nt 4584 to 4600 and 5710 to 5725, respectively (Fig. 1A). Purified genomic RNA was also extended using the same primers to determine the positions of background stops generated by genomic plus-strand RNA in inoculated protoplasts.

As previously demonstrated (Kim and Hemenway,

1996), the major extension products for ePtb (Fig. 1B) and ePcp (Fig. 1C) were 7 and 5 nt upstream of the initiation codons for the first of the TB proteins and CP, respectively. These positions are likely to correspond to the start-sites for transcription, assuming that these products did not arise from cleavage of transcripts initiated upstream. Subgenomic RNA for TB was detectable as early as 5 hpi, and reached maximum levels at 12–24 hpi. Then the levels were reduced by approximately 50% and maintained for up to 96 hpi. Onset of accumulation of CP sgRNA was detectable slightly later, at 8 hpi, and peaked at 36–42 hpi. These levels were maintained for up to 96 hpi, which was the last timepoint analyzed. This pattern of accumulation was also observed when CP levels were monitored over time (data not shown). These data indicate that accumulation of the TB sgRNA occurred prior to that of CP sgRNA, but the maximum levels of the TB sgRNA were lower than those observed for CP sgRNA. Based on the data obtained from several timecourse studies, we chose to isolate RNA from inoculated protoplasts at 48 hpi for subsequent analyses of *cis*-acting elements that affect sgRNA accumulation.

Accumulation of genomic plus- and minus-strand RNA is not substantially affected by mutation of sequences upstream of the TB and CP genes

To evaluate the effects of mutations in regions upstream of the TB and CP genes on PVX RNA accumulation, levels of genomic plus- and minus-strand RNAs, as well as sgRNAs were analyzed. As shown in Fig. 2, alignments of these regions indicated an identical octanucleotide element upstream of both PVX TB and CP genes, which is also present in many of the other potexviruses. The conserved hexanucleotide element discussed (White *et al.,* 1992) is within this octanucleotide sequence. A more detailed comparison of these regions for our PVX sequence is illustrated in Fig. 3. The sequence upstream of the AUG for the first of the TB genes (A) contains an octanucleotide element immediately downstream of the UAA termination codon for the PVX replicase gene. In contrast, a similar element upstream of the CP gene (B) lies within the C-terminal coding region of the third TB gene. The distance between the conserved octanucleotide element and the transcription start-site is 14 nt for both TB and CP genes.

Four basic types of site-directed mutations were introduced into these regions (Fig. 3). Four mutations (TB1–TB4 and CP1–CP4) altered one or more nucleotides within the octanucleotide element. Because this element lies within the coding sequence of the third TB gene, the CP1, CP3, and CP4 mutations were designed to conserve codons. Mutant CP2 produces a codon change from serine to threonine. Although both of these codons have polar, uncharged sidechains, they may differentially affect protein structure and/or function. Although coding

Upstream of Triple Gene Block

PVX	CUUUAACC **GUUAAGUU** ACCUUAUA -	12 nt - AUG
BAMV	AUUCUCUA **GUUAAGU**A ACCUAUUA -	15 nt - AUG
CCMV	GUGACAUG A**UUAAGUU** GGGUGCCU -	22 nt - AUG
CYMV	GAACACUU **GUUAAGUU** UGGUGGUA -	24 nt - AUG
FXMV	GGUUUUGA **GUUA**G**G**G**U** AACUCCAC -	17 nt - AUG
LVX	AAGAAGGG **GUUAAGUU** ACCGCUGA -	6 nt - AUG
NMV	CGCUACCG **GUUAAGUU** CAUUGCCU -	16 nt - AUG
PMV	GUUCCAUA **GUUAAGU**C AGGAGGAG -	30 nt - AUG
PlAMV	GUGACUUA **GUUAAGUU** UGGAGGAU -	24 nt - AUG
PAMV	UCUAGGAG **GUUAAGUU** ACCUUUAA -	7 nt - AUG
SMYEAV	GAUUAGGG **GUUAAGUU** ACCAUUCU -	31 nt - AUG
WCIMV	GACUACGG **GUUAAG**AG ACCUUAAG -	5 nt - AUG

Upstream of Coat Protein Gene

PVX	GUUGAAAC **GUUAAGUU** UCCAUUGA -	10 nt - AUG
BAMV	UAGGGUUU **GUUAAGUU** UCCCUUUU -	19 nt - AUG
CCMV	CCAUACGG **GUUAAGUU** UGCCUAAG -	4 nt - AUG
CYMV	CAACCACG **GUUAAGUU** ACCCAAAA -	14 nt - AUG
CyMV	UGGCGAGG **GUUAAGUU** ACCACAAU -	14 nt - AUG
FXMV	UUGAGGGU **GUUA**G**G**G**U** AACCAACC -	53nt - AUG
LVX	GCAUCGGG **GUUAAGUU** GCCAGAAA -	16 nt - AUG
NMV	GGAACGGG **GUUAAGUU** UCCUUUAU -	54 nt - AUG
PMV	UACACGGG C**UUA**G**G** AA CUAGCUUC -	25 nt - AUG
PlAMV	CCCUAGAG **GUU**U**AAG**U UGGAACAG -	28 nt - AUG
PAMV	UGGAACGG **GUUAAGUU** UCCAUUUA -	46 nt - AUG
SMYEAV	AUUUUAGC **GUUAA**U**U**A CCGCUGCA -	56 nt - AUG
WCIMV	AACCACGG **GUUAAGUU** UACCAUCU -	12 nt - AUG

FIG. 2. Alignment of the putative promoter sequences for several potexvirus sgRNAs. Shown are sequences from the genomic RNAs of our PVX strain (unpublished data), bamboo mosaic virus (BaMV) (Lin *et al.,* 1994a), cassava common mosaic virus (CCMV) (Calvert *et al.,* 1995), clover yellow mosaic virus (CYMV) (Sit *et al.,* 1990), cymbidium mosaic virus (CyMV) (Chia *et al.,* 1992), foxtail mosaic virus (FMV) (Bancroft *et al.,* 1991), lily virus X (LVX) (Memelink *et al.,* 1990), narcissus mosaic virus (NMV) (Zuidema *et al.,* 1989), papaya mosaic virus (PMV) (Sit *et al.,* 1989), plantago asiatica mosaic virus (PlAMV) (Solovyev *et al.,* 1994), potato aucuba mosaic virus (PAMV) (Xu *et al.,* 1994), strawberry mild yellow edge-associated virus (SMYEAV) (Jelkmann *et al.,* 1992), and white clover mosaic virus (WCIMV) (Forster *et al.,* 1988). Proposed consensus sequences are shown in bold. AUG denotes initiation codons for the first TB protein (top panel) and CP (bottom panel).

constraints were not an issue for the octanucleotide element upstream of the TB genes, mutations introduced were identical to those placed in this element for the CP gene. Mutations TB5 and CP5 changed the G residues at the putative transcription start-sites for TB and CP sgRNAs to A and U, respectively. The result of mutations TB6 and CP6 was the creation of a *Bgl*II site, which resulted in changing 5/6 and 4/6 nt upstream of the putative 5′ ends of TB and CP sgRNAs, respectively. In the CP6 mutation, the UGA termination codon for the third TB gene was converted to a UAG termination codon. Three nt were deleted in mutants TB7 and CP7 to reduce spacing between the octanucleotide element and the 5′ ends of the sgRNAs.

Transcripts derived from wt and mutated cDNA clones were inoculated into *N. tabacum* NT1 cell suspension protoplasts and analyzed for PVX RNA accumulation at

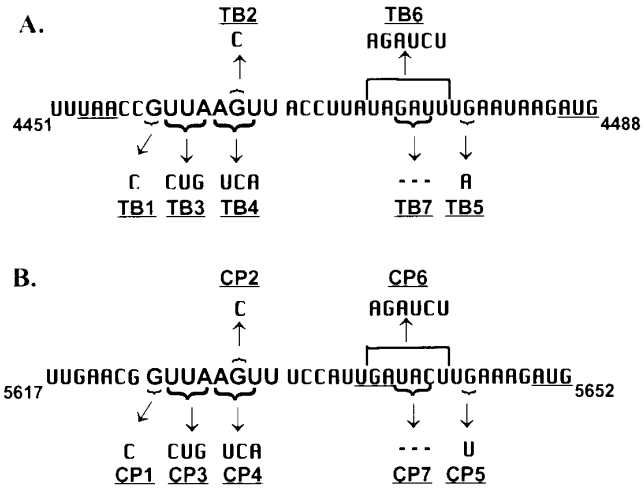

FIG. 3. Site-directed mutations in regions upstream of PVX sgRNAs. Sequences in PVX plus-strand RNA located upstream of the first of the TB genes (A) and the CP gene (B) are indicated, with the conserved 8-nt elements shown in large type face. Arrows indicate site-directed mutations or deletions (--) in the TB (TB1–TB7) and CP (CP1–CP7) upstream regions of wt sequences.

48 hpi. Total RNAs were extracted from protoplasts, hybridized to probes P1 and P3 (Fig. 1), and digested with S1 nuclease for detection of plus- and minus-strand RNA, respectively. Data shown in Fig. 4 represent typical autoradiograms of the S1 analyses (A and B) and graphic displays of relative average levels for three experiments (C and D). Minus-strand RNA accumulation (Figs. 4A and 4C) in mutant-inoculated protoplasts reached levels from 85–159% of levels detected in wt-inoculated protoplasts. Based on statistical analyses, only the reduction to 88% in protoplasts inoculated with TB3 was significant at the 95% confidence level. The increase in average minus-strand levels above wt levels observed for several of the mutants were not significant. Additional experiments will be required to determine if such increases would be significant with a larger sample size. Plus-strand RNA levels (Figs. 4B and 4D) were approximately 69–123% compared to wt levels. Although several of the mutants exhibited approximately a 30% reduction in plus-strand RNA relative to wt levels, only the decrease observed for mutant TB5 was significant. No protected fragments corresponding to minus- or plus-strand PVX RNAs were detectable in protoplasts inoculated with p32 transcripts, which serve as a nonreplicating, input RNA control. These data indicate that modifications of the octanucleotide element and sequences between this element and the 5′ end of the sgRNAs did not substantially alter accumulation of PVX genomic plus- and minus-strand RNAs.

Modification of octanucleotide elements affects sgRNA accumulation

The effects of the TB 1–4 and CP 1–4 mutations on sgRNA accumulation were analyzed by primer extension. Total RNAs extracted from protoplasts at 48 hpi were hybridized to end-labeled primers, ePtb (Fig. 5A) and ePcp (Fig. 5B), for extension of TB and CP sgRNAs, respectively. Quantification of relative average values from

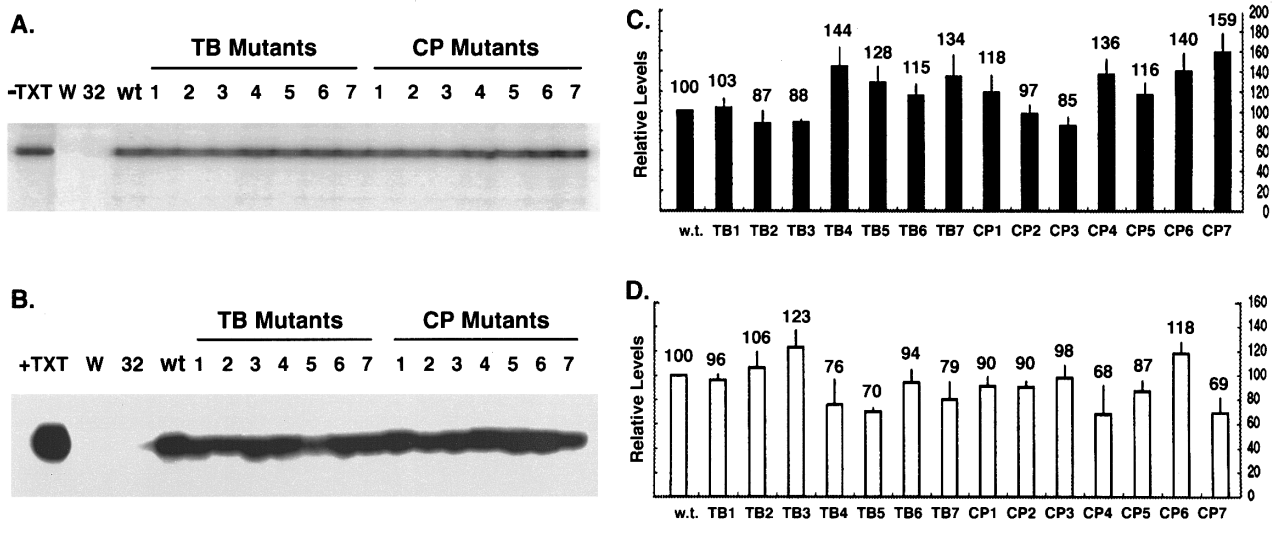

FIG. 4. Effects of mutations on genomic plus- and minus-strand RNA accumulation in protoplasts. *N. tabacum* NT1 cell suspension protoplasts were inoculated with water (W), control transcripts derived from p32 (replication defective control) and pMON8453 (wt), and with mutant transcripts (TB1-7 and CP1-7). Total RNAs were isolated from 1.6×10^6 protoplasts for minus-strand RNA analysis (A) or 4×10^5 protoplasts for plus-strand RNA detection (B) and were hybridized to strand-specific probes P3 and P1, respectively. Hybridized RNAs were analyzed by S1 nuclease protection assay and separated on 6% sequencing gels. The first lanes in A and B contain protected fragments obtained by hybridization to minus- (−TXT) and plus-strand (+TXT) RNA transcripts, respectively. C and D illustrate relative average values for genomic minus- and plus-strand RNA accumulation that were obtained by quantitating the signal in each lane using a Molecular Dynamics, Inc. PhosphorImager. The mean values from three independent experiments (different protoplasts and different transcript preparations) are shown, along with standard error bars.

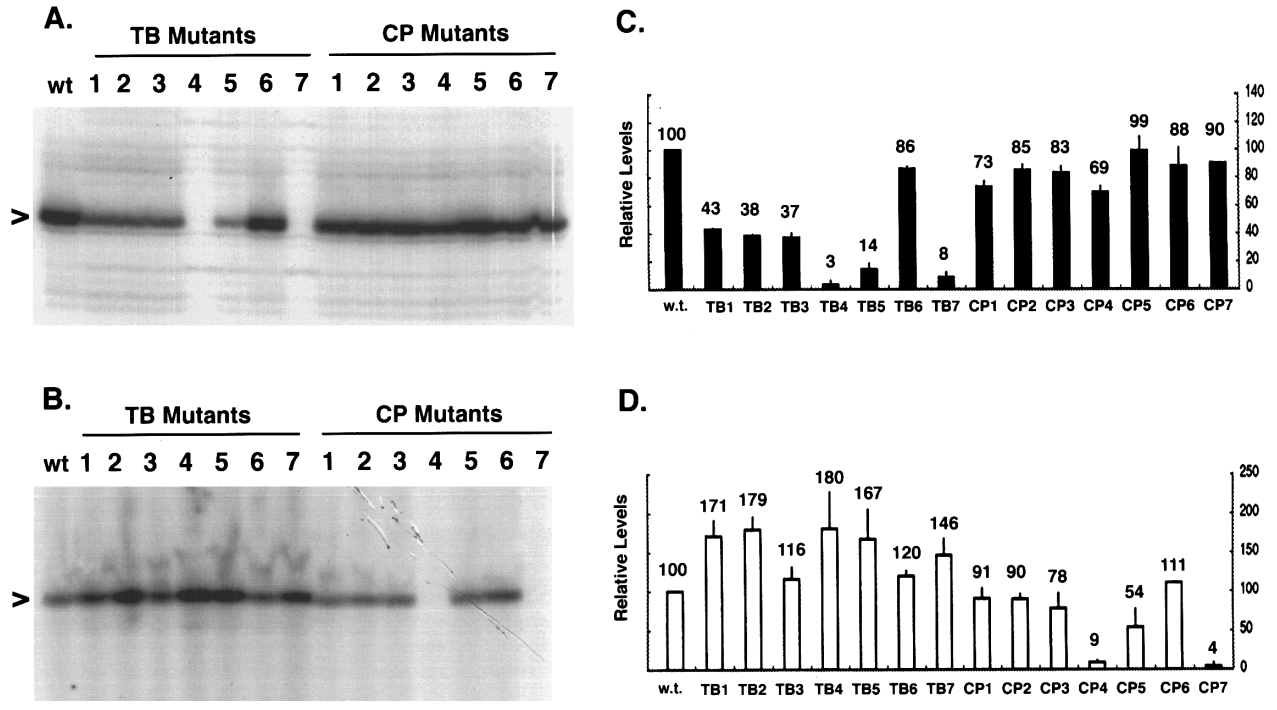

FIG. 5. Effects of mutations on sgRNA accumulation. Total RNAs extracted from 8×10^5 protoplasts inoculated with wt or mutant transcripts (TB1-7, CP1-7) were annealed to ePtb (A) or ePcp (B) for primer extension. Products were separated on 6% sequencing gels, and positions of primary extension products are indicated by arrows. A quantitative comparison of the TB and CP extension products is depicted in C and D, respectively; values were calculated as described in the legend for Fig. 4.

three primer extension experiments are shown in Figs. 5C and 5D.

All modifications to the octanucleotide element upstream of the TB genes significantly decreased accumulation of TB sgRNA (Figs. 5A and 5C). In protoplasts inoculated with mutants TB1, TB2, and TB3, corresponding sgRNA levels were 43, 38, and 37%, respectively, compared to that of wt. The greatest reduction in TB sgRNA accumulation to 3% of wt levels was observed in protoplasts inoculated with octanucleotide mutant TB4. In contrast, the TB1–4 mutations resulted in levels of CP sgRNA that were generally greater than those in protoplasts inoculated with wt transcripts (Figs. 5B and 5D). However, only the 79% increase in CP sgRNA observed with mutant TB2 was significant. To address the correlation between CP sgRNA levels and production of CP, protoplasts inoculated with TB mutants were also assayed for CP production by Western blotting. As shown in Fig. 7A, approximate wt levels of CP were produced in protoplasts inoculated with TB1–TB3 transcripts, whereas CP levels were somewhat reduced in TB4-inoculated protoplasts. Because of the variability inherent in these experiments, larger sample sizes will be needed to determine if small differences in RNA and CP levels are consistent and significant. It can be concluded from these that the octanucleotide element upstream of the TB genes is important for accumulation of TB sgRNA.

In contrast to effects of mutations in the octanucleotide element upstream of TB on TB sgRNA accumulation, only the CP4 mutation resulted in a significant reduction in CP sgRNA accumulation to 9% of wt levels. CP sgRNA levels in protoplasts inoculated with mutants CP1–CP3 were not significantly different than wt levels. A correlation between CP sgRNA and CP accumulation was observed (Fig. 7B). CP levels in protoplasts inoculated with mutants CP1–CP3 were similar to those in wt-inoculated protoplasts, whereas CP was not detectable in protoplasts inoculated with mutant CP4. In general, levels of TB sgRNAs were significantly decreased by 15–30% in protoplasts inoculated with the CP1–CP4 mutants (Figs. 5B and 5D). These data indicate that modifications to the octanucleotide element differentially affect TB and CP sgRNA accumulation.

Because protoplasts inoculated with CP4 exhibited reduced CP sgRNA and CP levels, we additionally analyzed the CP mutants by Northern blotting to ensure that the near wt levels of plus-strand RNA detected by S1 analyses reflected intact plus-strand RNA. As indicated in Fig. 6, CP sgRNA levels were substantially reduced in protoplasts inoculated with mutant CP4, which is consistent with the primer extension data. In addition, all CP mutants exhibited similar levels of intact genomic-length, plus-strand RNA, regardless of the CP sgRNA levels. Thus, the signals detected for plus-strand RNA by S1 analysis of the CP mutants were representative of full-length products. Northerns were not further utilized for

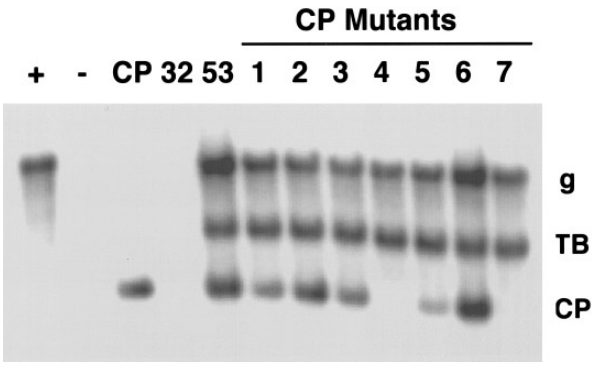

FIG. 6. Northern blot analysis of CP mutants. Total RNAs isolated from 8×10^5 protoplasts inoculated with transcripts from a replication defective clone (32), wt clone (53), and mutants CP1–CP7 were electrophoresed on a 1% agarose/6% formaldahyde gel. Transcript controls (20 ng) corresponding to CP, full-length minus-strand (−), and full-length plus-strand (+) PVX RNA were also loaded. The gel was blotted to GeneScreen membrane and probed with ^{32}P-labeled, full-length minus-strand transcript (5×10^6 cpm/lane). Positions corresponding to genomic-length PVX RNA (g), TB sgRNA (TB), and CP sgRNA (CP) are noted at the right side of the autoradiogram.

our studies because the S1 and primer extension methods were more reproducible and quantitative.

Alterations in the regions between the octanucleotide elements and the translation initiation codons have differing effects on sgRNA accumulation

To begin to analyze the importance of sequences located between the octanucleotide conserved sequence and the translation initiation codons, three additional mutant sets were inoculated into protoplasts. Of the mutations in this region, TB6 and CP6 had no substantial effect on sgRNA accumulation. The TB6 mutation containing 5-nt changes produced approximately 86% of wt TB sgRNA (Figs. 5A and 5C). Slightly higher than wt CP sgRNA (Figs. 5B and 5D) and somewhat reduced CP levels (Fig. 7A) were observed. Again, a larger sample size will be necessary to assess the significance of these smaller differences in RNA and CP levels. In protoplasts inoculated with the CP6 mutant, levels of CP sgRNA, CP, and TB sgRNA did not differ significantly from corresponding wt levels (Figs. 5C, 5D, and 7B). These data suggest that the altered residues were not critical for either TB or CP sgRNA accumulation or were changed to acceptable replacement nucleotides.

The TB5 and CP5 mutations were designed to change the putative 5′ G of the corresponding sgRNAs to an A and U, respectively. As indicated in Fig. 5, TB sgRNA levels were significantly reduced to 14% of wt in protoplasts inoculated with mutant TB5 (Figs. 5A and 5C), but levels of CP sgRNA (Figs. 5B and 5D) and CP (Fig. 7A) were not significantly affected. Modification to the 5′ nt of the CP sgRNA in mutant CP5 resulted in approximately a 50% reduction of the average CP sgRNA levels (Figs. 5B and 5D) and a decrease in CP accumulation (Fig. 7B), but the sgRNA levels did not differ significantly from wt. TB sgRNA accumulation (Figs. 5A and 5C) was not affected by the CP5 mutation. Thus, modification of the putative 5′ G of the TB sg RNA to an A resulted in approximately a sevenfold reduction of TB sgRNA accumulation, whereas conversion of the 5′ G of CP sgRNA to a U did not significantly alter CP sgRNA levels.

Greater than 10-fold reductions in sgRNA accumulation were observed in protoplasts inoculated with the TB7 and CP7 mutants, which contain deletions of 3 nt between the octanucleotide element and the putative 5′ end of the sgRNAs. In protoplasts inoculated with mutant TB7, accumulation of TB sg RNA (Figs. 5A and 5C) was reduced to 8% of wt levels. Although average CP sgRNA levels (Figs. 5B and 5D) were slightly higher than wt levels in these samples, these differences were not significant. Similarly, CP sgRNA levels (Figs. 5B and 5D) were reduced to 4% of wt accumulation in protoplasts inoculated with mutant CP7. This significant reduction in CP sgRNA was also accompanied by a consistent, corresponding reduction in CP accumulation (Fig. 7B), but did not affect TB sgRNA accumulation (Figs. 5A and 5C). Thus, nt 4474–4476 and 5640–5642 are critical for accumulation of TB and CP sgRNA, respectively. Given that mutants TB6 and CP6, which included nucleotide changes in the same positions, had little effect on sgRNA accumulation, our data suggest that spacing is critical or that there is flexibility in the sequence tolerated in this region.

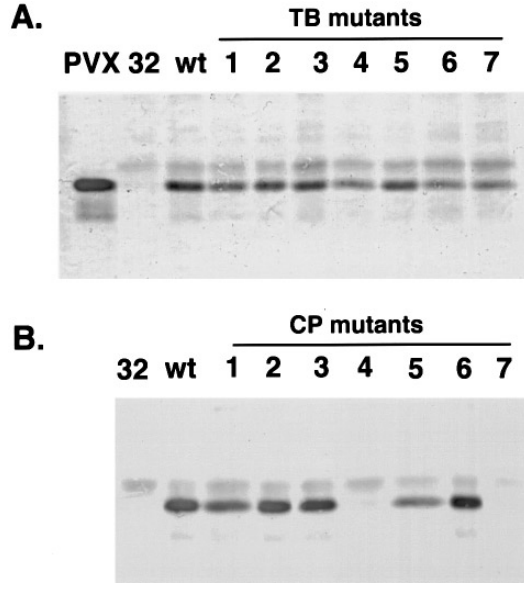

FIG. 7. Accumulation of CP in protoplasts inoculated with modified transcripts. Total protein was isolated at 48 hpi from protoplasts inoculated with control transcripts derived from p32 (32) and pMON8453 (wt), or modified transcripts (TB1-7, A; CP1-7, B). Samples (20 μg protein) were electrophoresed on 12% SDS–polyacrylamide gels, blotted to nitrocellulose, and probed with antisera to PVX. CP was detected using an alkaline phosphatase reaction. PVX (50 ng) was loaded as a marker for position of CP migration in A.

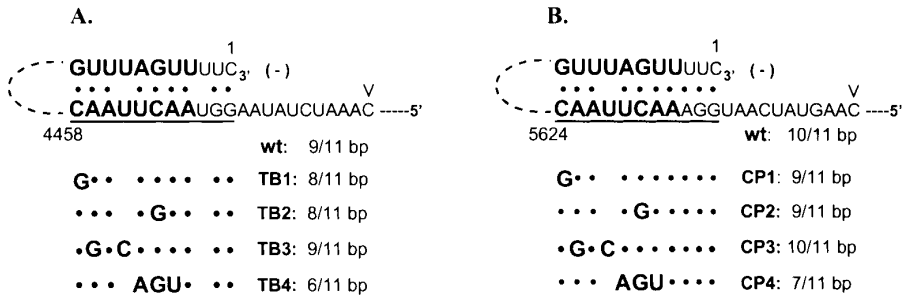

FIG. 8. Complementarity between conserved octanucleotide elements and the 3′ end of PVX minus-strand RNA. The regions around the conserved octanucleotide elements upstream of the TB (A) and CP (B) genes are shown paired to the complementary sequence at the 3′ end of minus-strand RNA. Complementarity for the wt minus-strand RNA is indicated at the top of each panel, and the 11-nt underlined region contains the conserved octanucleotide element. Positions of the 5′ ends of the sgRNAs are denoted by "V" and potential base-pairs are denoted by bold dots. The predicted complementarity for each of the octanucleotide mutants is indicated in each panel. The numbering refers to nt positions on the corresponding genomic RNA.

From these data, we conclude that modification to the octanucleotide elements and deletion of three nucleotides between these elements and the putative transcription initiation sites had the greatest effect on sgRNA accumulation. Modification of the putative 5′ start-sites differentially affected RNA accumulation, with the greatest reduction observed in TB sgRNA levels.

Only TB6 and CP6 mutant transcripts are able to infect plants

To analyze the effects of mutations on PVX infection *in vivo,* where both TB and CP products would be critical for cell-to-cell movement, transcripts containing modifications were inoculated onto *N. benthamiana* plants. Infection was monitored by evaluation of symptom development and detection of CP in inoculated and upper leaves (Table 1). Plants inoculated with wt transcripts or with mutant transcripts, TB6 or CP6, showed systemic symptoms by 5 to 7 dpi, and accumulated PVX CP in inoculated and upper leaves by 7 dpi. In contrast, plants inoculated with all other mutant transcripts and the replication-defective control transcripts (p32) did not develop symptoms for up to 28 dpi and did not contain detectable levels of CP at 7dpi.

Thus, despite the fact that mutants CP1, CP2, CP3, and CP6 produced near wt levels of both sgRNA species and CP in NT1 protoplasts, only the CP6 transcripts were infectious on plants. These data suggested that elements in the regions upstream of the sgRNAs may be important not only for sgRNA accumulation, but may also be involved in other aspects of infection in plants. Alternatively, perhaps subtle differences in sgRNA and corresponding protein levels or kinetics of accumulation are critical for infection *in planta.*

DISCUSSION

To begin to understand the process of PVX sgRNA synthesis, we measured the accumulation of the two predominant sgRNA species in tobacco protoplasts inoculated with infectious transcripts derived from a wt PVX cDNA clone or from cDNAs containing modifications in putative subgenomic promoter regions. Time-course studies indicated that TB sgRNA accumulated a few hours prior to CP sgRNA, but the relative levels of the TB sgRNA were approximately 2-fold lower than those for CP sgRNA at 12 hpi, and 10-fold lower at 42 hpi. Chapman *et al.* (1992a) reported a similar accumulation pattern for these two PVX sgRNAs, and this general pattern of expression has also been reported for several other plant viruses that express multiple sgRNAs (Watanabe *et al.,* 1984; Lehto *et al.,* 1990; Johnston and Rochon, 1995).

TABLE 1

Infectivity on *N. benthamiana* Plants

Mutants	Symptoms[a]	CP accumulation[b]	
		Inoculated	Upper
p32	0/11	−	−
wt	10/11	+	+
TB1	0/11	−	−
TB2	0/11	−	−
TB3	0/11	−	−
TB4	0/11	−	−
TB5	0/11	−	−
TB6	10/11	+	+
TB7	0/11	−	−
CP1	0/11	−	−
CP2	0/11	−	−
CP3	0/11	−	−
CP4	0/11	−	−
CP5	0/11	−	−
CP6	9/11	+	+
CP7	0/11	−	−

[a] Symptom development was observed for 28 days postinoculation (dpi). A total of 11 plants were inoculated.

[b] CP accumulation was determined by Western analysis of total proteins extracted from inoculated and upper leaves at 7 dpi.

Our data showed that a conserved octanucleotide element upstream of the TB and CP genes is important for sgRNA accumulation and that modifications in this region differentially affect levels of the corresponding RNAs. Alteration of positions 5–7 in these elements (mutants TB4 and CP4) drastically decreased the corresponding sgRNA levels, indicating that these positions may function similarly for both genes. Interestingly, positions 5 and 7 in this element are the most variable among the potexvirus elements. In contrast, alterations in positions 1, 6, or both 2 and 4 of this element reduced TB sgRNA accumulation approximately twofold, but did not significantly alter CP sgRNA accumulation. If the reduced TB sgRNA accumulation is a result of reduced synthesis, it is possible that a twofold reduction in CP sgRNA synthesis may not be detected if the stability of this sgRNA species is inherently greater. Alternatively, some positions in the octanucleotide element may function differently upstream of the two genes.

Mutations designed to alter the sequence between the conserved element and the translation initiation codons of the TB and CP genes also exhibited differing effects. Changing the 5′ G of the TB sgRNAs to an A (mutant TB5) reduced the synthesis of TBsg RNA to 14% of wt levels, whereas alteration of the 5′ G of the CP sgRNA (mutant CP5) did not significantly reduce this RNA species. It may be that the optimum nt for the start of sgRNA transcription is dependent on the context of a given template. Deletion of nt 4474–4476 and 5640–5642 upstream of the TB and CP start-sites (mutants TB7 and CP7), respectively, greatly reduced accumulation of the corresponding sgRNAs. Because sequence changes (mutants TB6 and CP6) in these nucleotides did not affect sgRNA accumulation, these data suggested that the correct distance between the octanucleotide sequence and the putative start-site is required. Reductions in sgRNA accumulation in response to alteration of putative sgRNA start-sites and sequences immediately upstream have also been demonstrated for brome mosaic virus (Johnston and Rochon, 1995) and alfalfa mosaic virus (van der Vossen *et al.,* 1995).

Many of the mutants displayed some common properties. Generally, modification to sequences upstream of one gene did not substantially alter accumulation of the other major sgRNA species, indicating that the elements function independently. If sgRNA synthesis is sequential from the 3′ end of the minus-strand, these data suggested that CP sgRNA synthesis is not dependent upon prior synthesis of the TB sgRNA. However, we cannot rule out that alteration of TB sgRNA synthesis may change the timing of CP sgRNA accumulation and vice versa. For most mutants, genomic plus- and minus-strand RNA accumulation were not significantly altered. We were surprised to observe this trend for mutants CP4 and CP7, which also exhibited inhibition in CP production. Chapman *et al.* (1992a) previously demonstrated that deletion of PVX CP sequence and/or inhibition of translation of CP resulted in a large decrease in genomic plus-strand RNA levels and a smaller change in sgRNA levels, as determined by Northern blotting. They proposed that CP was required to stabilize the genomic RNA or affects the asymmetry between plus- and minus-strand RNA synthesis. It is possible that these different observations may be a function of the techniques used for evaluating and quantitating RNA levels. However, it is more likely that the data reflect the different modifications introduced into the PVX genome. Although reduced CP levels may lead to reduced genomic plus-strand accumulation, the overall effect on genomic RNA levels may be determined by the extent to which various aspects of RNA synthesis or the coordination of this process are affected by a given mutation. A correlation between sgRNA synthesis and levels of viral RNA was reported for the coronavirus, mouse hepatitis virus, where inhibition of sgRNA synthesis from a defective-interfering (DI) RNA resulted in increased DI replication in *cis* (Jeong and Makino, 1992), apparently due to increased minus-strand accumulation (Lin *et al.,* 1994b). Thus, the increased levels of PVX minus-strand RNA observed in protoplasts inoculated with CP7 may lead to additional synthesis of genomic plus-strand RNA that counteracts the absence of CP. Alternatively, because TB sgRNA levels were not affected in protoplasts inoculated with the CP4 or CP7 mutants, perhaps very low levels of CP are generated by internal initiation of translation on the TB sgRNA and these levels of CP are sufficient to maintain levels of genomic RNA. Regardless of the mechanism, our data indicate that a large decrease in sgRNA accumulation somehow compensates for the absence of CP, and the end result is no substantial decrease in genomic RNA.

Only the TB6 and CP6 mutants, which exhibited wt levels of all RNA species in protoplasts, were able to infect plants. Given that both CP and TB are required for cell-to-cell movement (Beck *et al.,* 1991; Chapman *et al.,* 1992b; Angell *et al.,* 1996; Oparka *et al.,* 1996), one might expect that TB1–5 and TB7 mutants, which resulted in decreased levels of TB sgRNA, would not infect plants. Similarly, the CP4 and CP7 mutants that did not accumulate CP sgRNA or CP were not infectious on plants. However, it is not clear why mutants CP1–3 and CP5, which expressed near wt levels of TB and CP sgRNAs and CP, were not able to infect plants. The CP2 mutation, which changed a serine codon to threonine near the C-terminus of the third TB protein, may have altered structure and or function of this protein and subsequent movement of the virus. It is possible that modifications in CP1–3 and CP5 disturbed the timing of CP sgRNA and CP accumulation, which may be critical for coordination of replication and cell-to-cell movement. Alternatively these sequence elements and/or affiliated structures may affect interac-

tions with essential viral and/or host proteins that are required for movement or virion assembly. Sit *et al.* (1994) reported that the 5′ 38–47 nt of another potexvirus, papaya mosaic virus, supported assembly of particles, but sequences necessary for the assembly of PVX have not been studied. Encapsidation of sgRNAs has been reported for a few potexviruses, and data presented by Price (1993) suggested that PVX preparations include significant populations of smaller particles that contain RNAs similar in size to sgRNAs. Additional studies will be needed to determine if elements upstream of the sgRNAs and elsewhere in the genome are involved in PVX assembly and/or movement.

Mechanisms proposed for sgRNA synthesis of several plant viruses invoke internal initiation on minus-strand RNA, and this process requires signals upstream of the sgRNAs (Miller *et al.,* 1985; for review, see Duggal *et al.,* 1994). We showed previously that multiple elements in the 5′ region of the genomic RNA, or corresponding 3′ region of minus-strand RNA, are important for both genomic and sgRNA accumulation (Kim and Hemenway, 1996). Data presented here indicated that elements upstream of the TB and CP genes also affect levels of the corresponding sgRNAs. It remains to be determined how these multiple elements in different regions of the genome regulate subgenomic RNA synthesis. For coronaviruses, long-distance interactions between terminal sequences and internal elements have been proposed to function in a discontinuous transcription process for sgRNA synthesis (for review, see Lai, 1990). This process requires several elements that include the 72- to 77-nt leader sequence, intergenic sequences, and 270 nt at the 3′ end of the genome (Makino *et al.,* 1991; Liao and Lai, 1994; Lin *et al.,* 1996; Zhang and Lai, 1996).

For PVX, as well as other potexviruses, there is complementarity between the conserved octanucleotide element upstream of the sgRNAs and the 5′ end of the genome. This complementarity can be modeled on minus-strand to involve an interaction between the octanucleotide elements and sequences at the 3′ end (Fig. 7). Upstream of TB sgRNA, 9 of 11 residues are predicted to base pair, and the interaction upstream of the CP sgRNA would include 10 base pairs. Alignment of these regions for mutants CP1, CP2, TB1, and TB2 indicates one less base pair than for wt, whereas CP3 and TB3 pairing are similar to wt because of a GU pair. Protoplasts inoculated with these mutants did not exhibit substantial reductions in sgRNA accumulation, indicating that changes altering only one base pair or the nt involved in pairing may not be critical for sgRNA synthesis. In contrast, alignment of TB4 and CP4 transcripts indicates 3 fewer base pairs than wt, and protoplasts inoculated with these mutants exhibited 10-fold reduction in sgRNA accumulation. Thus, sufficient base pairing may be needed to facilitate sgRNA synthesis, and this interaction may function by positioning the replication machinery. However, it is likely that such an interaction is not the only factor in regulation of sgRNA synthesis because data presented herein showed that sgRNA accumulation can be inhibited by modification to sequences other than the conserved element. In addition, previous mutational analyses (Kim and Hemenway, 1996) indicated that multiple elements near the 5′ terminus and internal to the 5′ NTR are necessary for plus-strand RNA accumulation. Studies are underway to further define *cis*- and *trans*-acting functions required for PVX sgRNA synthesis.

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