JOURNAL OF VIROLOGY, Aug. 1996, p. 5533–5540 Vol. 70, No. 8
0022-538X/96/$04.00+0

The 5′ Nontranslated Region of Potato Virus X RNA Affects both Genomic and Subgenomic RNA Synthesis

KOOK-HYUNG KIM AND CYNTHIA HEMENWAY*

Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27695-7622

Received 6 February 1996/Accepted 12 May 1996

A tobacco protoplast system was developed to analyze *cis*-acting sequences required for potato virus X (PVX) replication. Protoplasts inoculated with transcripts derived from a PVX cDNA clone or from clones containing mutations in their 5′ nontranslated regions (NTRs) were assayed for RNA production by S1 nuclease protection assays. A time course of plus- and minus-strand-RNA accumulation indicated that both minus- and plus-strand PVX RNAs were detectable at 0.5 h postinoculation. Although minus-strand RNAs accumulated more rapidly than plus-strand RNAs, maximum levels of plus-strand RNAs were 40- to 80-fold higher. On the basis of these data, time points were chosen for determination of RNA levels in protoplasts inoculated with PVX clones containing deletions or an insertion in their 5′ NTRs. Deletions of more than 12 nucleotides from the 5′ end, internal deletions, and one insertion in the 5′ NTR resulted in substantially decreased levels of plus-strand-RNA production. In contrast, all modified transcripts were functional for minus-strand-RNA synthesis, suggesting that elements in the 5′ NTR were not essential for minus-strand-RNA synthesis. Further analysis of the 5′ NTR deletion mutants indicated that all mutations that decreased genomic plus-strand-RNA synthesis also decreased synthesis of the two major subgenomic RNAs. These data indicate that *cis*-acting elements from different regions of the 5′ NTR are required for plus-strand-RNA synthesis and that this process may be linked to synthesis of subgenomic RNAs.

Potato virus X (PVX), the type member of the potexvirus group, is a flexuous rod-shaped virus containing a single, plus-strand RNA of approximately 6.4 kb that is capped and polyadenylated (4, 40). The viral RNA includes 5′ and 3′ nontranslated regions (NTRs) of 84 and 72 nucleotides (nt), respectively, and encodes five open reading frames (ORFs) (25). ORF1 (165 kDa) has homology to replicase genes of other viruses and is the only viral protein absolutely required for PVX RNA synthesis. ORFs 2 to 4, referred to as the triple block (TB) genes, are required for viral cell-to-cell movement (3). The product of ORF5, coat protein (CP), encapsidates viral RNA and is involved in the spread of PVX throughout a plant (2, 9). Each of these gene products and RNAs corresponding to minus-strand genomic RNAs, plus-strand genomic and subgenomic RNAs, and double-stranded versions of these RNAs are detected in infected plants (11, 47). The two major subgenomic RNA species of approximately 2.1 and 0.9 kb in length are likely to initiate upstream of the TB genes and CP, respectively. Membrane-containing extracts derived from PVX-infected tissues also support synthesis of similar RNA products on endogenous templates (12). Aside from these basic features, very little is known about the *cis*-acting elements or mechanisms involved in PVX replication.

Studies of several plant plus-strand RNA viruses have provided evidence for *cis*-acting sequences and/or structures that are necessary for different aspects of RNA synthesis (for a review, see reference 16). Such *cis*-acting elements have been analyzed for several multipartite viruses (6, 8, 13, 14, 17, 19, 22, 23, 30, 42–45, 48, 59, 62–64, 66) and for a few monopartite plant viruses (20, 55, 56, 60, 61, 67). However, few studies have focused on regulatory elements at the 5′ ends of viral RNAs that are specifically required for synthesis of genomic plus-strand RNA (6, 41, 45). RNA sequence elements similar to internal control regions of tRNA gene promoters have been found at the 5′ ends of brome mosaic virus (BMV) RNAs (34) and of other plant viral RNAs (1, 36, 66) and were shown to be important for RNA synthesis (45). Mutations that altered a predicted stem-loop structure in the 5′ NTR of BMV RNA 2 also reduced replication (43). Although PVX RNA does not contain internal control region-like sequences, it is likely that specific sequence and/or structural elements in the 5′ NTR and potentially elsewhere in the PVX genome are required for genomic RNA synthesis.

The PVX 5′ NTR is likely to contain multiple *cis*-acting regulatory signals. In translational studies, Smirnyagina et al. (53) defined the 5′ NTR as two regions, the α sequence containing nt 1 to 41 (excluding the cap structure), which is AC rich, and the β sequence containing nt 42 to 83. Elements within these regions modulate efficient translation and enhance translation of reporter genes in vitro (53, 57, 69) and in vivo (46). *cis*-acting elements in the PVX 5′ NTR are believed to function in assembly, on the basis of data indicating that approximately the first 47 nt in the 5′ NTR of another potexvirus, papaya mosaic virus, are required for this process (51). Although the 5′ NTR of PVX is also likely to contain elements necessary for RNA replication, such elements have not been studied. Consequently, we developed a tobacco protoplast PVX replication system to study *cis*-acting elements in this region that are involved in PVX RNA synthesis. An S1 nuclease protection assay was optimized for detection of plus- and minus-strand RNAs synthesized in protoplasts inoculated with transcripts derived from wild-type (wt) and mutated PVX cDNA clones. Using this system, we determined that multiple elements in the PVX 5′ NTR are required for synthesis of both genomic and subgenomic RNAs.

* Corresponding author. Mailing address: Department of Biochemistry, Box 7622, North Carolina State University, Raleigh, NC 27695-7622. Phone: (919) 515-5719. Fax: (919) 515-2047.

MATERIALS AND METHODS

Materials. All restriction enzymes, exonuclease III, nuclease S1, and cap analog were purchased from New England Biolabs. Enzymes utilized in tran-

scription and sequencing reactions were obtained from Promega and U.S. Biochemicals, respectively. Reagents for protoplast growth and digestion were purchased from Sigma and Yakult Honsha Co., Ltd., respectively. The source of α-thio-dXTPs was Pharmacia.

Maintenance of plants. *Nicotiana benthamiana* plants for protoplast studies were grown in magenta boxes containing Murashige and Skoog basal medium supplemented with 3% sucrose and 0.8% agar. Plants were maintained at 25°C for 12 h in light and at 22°C for 12 h in darkness.

Construction of deletion mutants. The PVX cDNA clone pMON8453 was the starting material for the construction of mutants. This clone was derived by insertion of an *Spe*I restriction site immediately downstream of the poly(A) tail in the clone pMON8660 (24). Internal deletion mutants were created by digestion of pMON8453 with *Mun*I (nt 46 in the PVX genome) and exonuclease *Bal* 31 (49). The products were treated with mung bean nuclease to create blunt ends and self ligated. The extents of deletions in recombinants were determined by sequencing.

The starting plasmid for the 5′ deletion mutants was p31. This plasmid was created by insertion of a *Sac*I restriction site upstream of the PVX 5′ end in pMON8453. Deletions were introduced by exonuclease III digestion of α-thio-dXTP-containing templates (28). Single-stranded p31 DNA was annealed with primer complementary to nt 196 to 215 of the PVX genome and extended with Sequenase in the presence of dXTPs and one α-thio-dXTP. Extended DNA samples were digested with *Sac*I, treated with mung bean nuclease to remove the 3′ overhangs, and digested with 20 U of exonuclease III. After treatment with T4 DNA polymerase to create blunt ends, the resulting population of deleted DNAs was digested with *Bsi*WI (203 nt from the 5′ end of the PVX genome) and electrophoresed on 5% polyacrylamide gels. Corresponding deleted fragments were excised from gels, purified, and resected into wt p31 DNA that was treated with *Sac*I, T4 DNA polymerase, *Bsi*WI, and bacterial alkaline phosphatase. Deletion endpoints in resected plasmids were verified by sequencing.

A control plasmid, p32, which was used to generate transcripts defective for RNA synthesis, was obtained by deleting the *Bsi*WI-*Afl*II fragment from pMON8453. This plasmid contains a deletion of nt 203 to 4157 in the replicase gene.

Synthesis of RNA transcripts in vitro. DNA templates (5 μg) were linearized by digestion with *Spe*I and were incubated at 37°C in a 100-μl reaction mixture containing 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 100 μg of bovine serum albumin (BSA) per ml, 40 U of RNasin, 100 μM ATP, CTP, and UTP, 12.5 μM GTP, 100 μM m^7GpppG, and 20 U of T7 RNA polymerase. After 10 min, GTP was added to make a 50 μM final concentration, and the reaction mixture was incubated for an additional 50 min. After removal of the template by digestion with 2.5 U of RQ1 DNase, RNA transcripts were purified by phenol-chloroform extraction and ethanol precipitation. Transcribed full-length negative RNAs used as control RNAs for S1 nuclease protection assays were prepared similarly, but without the cap addition. Integrities and relative concentrations of purified transcripts were analyzed by agarose gel electrophoresis at 5°C.

Protoplast isolation and inoculation. Protoplasts were obtained from leaves of 2-month-old *N. benthamiana* plants. Aliquots of 5×10^5 protoplasts were inoculated with 5 μg of capped transcripts or 1 μg of purified PVX RNA in 40% polyethylene glycol (molecular weight, 1,540) and 3 mM $CaCl_2$ (37). After inoculation, protoplasts were washed two times with 1 ml of 10% mannitol and resuspended in 1 ml of incubation solution (0.2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4$, 10 mM $CaCl_2$, 0.16 mg of $CuSO_45H_2O$ per liter, 10% [wt/vol] mannitol, and 1 μg of gentamicin sulfate per ml). 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 h.

Construction of DNA probes for RNA analyses. Strand-specific probes for quantitation of plus- and minus-strand RNAs (Fig. 1) were prepared by labeling the 5′ ends of double-stranded DNA fragments with [γ-^{32}P]ATP and by subsequent purification through strand-separation gels (15). Fragments used for plus-strand-RNA-specific probe P1 were obtained by digesting p13 [PVX nt 1 to 203 in pGEM3zf(−) (Promega)] with *Bsi*WI and *Bsa*AI. The resulting 608-bp fragment contains 203 nt complementary to the 5′ end of PVX plus-strand RNA and 405 nt of noncomplementary sequence derived from the vector. Minus-strand probe P2 was obtained by digesting p36 with *Mun*I and *Ase*I. p36 was first constructed by inserting a *Sac*I-*Bam*HI fragment containing the PVX 5′ 493 nt into pMON921 (7). The *Mun*I-*Ase*I fragment from p36 contains 493 nt complementary to the 3′ end of minus-strand PVX RNA and 253 nt of noncomplementary sequence derived from the vector. A second minus-strand-specific probe, P3, was obtained by digestion of p40 (PVX nt 1 to 3349 in pMON921) with *Bst*EII and *Bsa*BI, resulting in a fragment containing 614 nt complementary to part of PVX ORF1 (nt 2736 to 3349) and 548 bases of noncomplementary sequences.

S1 nuclease analyses. Total RNA was extracted from inoculated protoplasts at various hours postinoculation (h.p.i.) with Trizol reagent according to the manufacturer's instructions (Gibco BRL). RNA samples were analyzed for the accumulation of viral genomic plus- and minus-strand RNAs by S1 nuclease analysis (5). For quantitation of plus-strand RNAs, approximately 10^4 cpm of probe P1 was mixed with total RNA from 2.5×10^5 protoplast cells or 10 ng of in vitro-generated RNA transcripts in hybridization buffer [400 mM NaCl, 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 1 mM EDTA, 10 μg of *Escherichia coli* tRNAs, 80% formamide]. Samples were incubated at 100°C for 5 min and subsequently at 37°C for 16 h. Digestion with nuclease S1 was initiated by the addition of buffer containing 25 mM NaCl, 3 mM sodium acetate (pH 4.6), 0.1 mM $ZnSO_4$, 2 μg of salmon sperm DNA per ml, and 25 U of S1 nuclease (Boehringer Mannheim), and the reaction mixture was incubated at 25°C for 30 min. Digestion products were precipitated, electrophoresed on 6% polyacrylamide sequencing gels, and visualized by autoradiography.

Initial stages of the protocol for minus-strand-RNA analysis were performed according to the methodology of Ishikawa et al. (27). Briefly, total RNAs from 1.5×10^6 protoplasts cells were hybridized with 1 μg of purified PVX RNA in 30 μl of annealing buffer (300 mM KCl, 50 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and treated with 1 μg of RNase A per ml at 30°C for 60 min to remove excess plus-strand RNAs. Annealed RNAs were recovered by the addition of 14 μl of 1% sodium dodecyl sulfate (SDS) and 4.2 μl of proteinase K (20 mg/ml) for 30 min at 30°C, extraction with phenol-chloroform, and precipitation. These products were then mixed with 10^4 cpm of minus-strand RNA probe P2 (for time course studies) or P3 (for mutational analyses) in hybridization buffer and analyzed by S1 nuclease quantitation as described above. Relative molar amounts of PVX RNAs in each sample were obtained by using an Image Quant PhosphorImager (Molecular Dynamics).

Detection of subgenomic RNAs by primer extension. Total RNA from 5×10^5 protoplasts inoculated with water or transcripts were annealed to 0.5 pmol of 5′ end-labeled oligonucleotide primers complementary to the TB region (nt 4584 to 4600) or to the CP gene (nt 5710 to 5725). Extensions were performed at 42°C for 15 min in buffer containing 50 mM Tris-HCl (pH 7.5), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates, 100 μg of BSA per ml, and 10 U of avian myeloblastosis virus reverse transcriptase (Gibco BRL) (18, 21). Products were separated on 6% sequencing gels and visualized by autoradiography.

Coat protein detection. At 36 h.p.i., 10^5 protoplasts were resuspended in 30 μl of Laemmli loading buffer (29), electrophoresed on SDS–12% polyacrylamide gels, and blotted onto a nitrocellulose membrane (58). The blots were probed with antiserum prepared against purified PVX, and products were visualized with a Biotin-StreptAvidin kit (Amersham).

Computer-generated figures. Autoradiograms depicted in the figures were photographed with a Kodak DCS200 digital camera and directly imported into Adobe Photoshop on a Power Macintosh.

RESULTS

Development of S1 nuclease protection assay for PVX replication. Analysis of *cis*-acting sequences in the 5′ NTR that have an impact on RNA synthesis first required development of a sensitive strand-specific detection system. An S1 nuclease protection assay was utilized to detect the accumulation of PVX RNAs in protoplasts inoculated with PVX RNA or PVX transcripts. Three strand-specific probes were constructed: P1 for plus-strand RNAs and P2 and P3 for minus-strand RNAs (Fig. 1A). The specificity of each probe in the S1 nuclease protection assay was confirmed by hybridization to purified PVX RNA or transcribed minus-strand PVX RNA. As shown in Fig. 1B, probe P1 specifically hybridized to plus-strand PVX RNA and protected the predicted 203-nt RNA fragment from S1 nuclease digestion (lane 3). When this probe was hybridized to RNA isolated from PVX RNA-inoculated protoplasts harvested at 18 h.p.i., it also protected plus-strand PVX RNA (lane 5). In contrast, this probe did not hybridize to minus-strand RNAs (lane 4) or to cellular RNAs isolated from water-inoculated protoplasts (lane 6).

Detection of minus-strand PVX RNA was more difficult, since the presence of excess molar amounts of plus-strand RNAs decreased levels of hybridization of the P2 or P3 probes to their complementary, minus-strand RNA sequences (data not shown). To circumvent this problem, we employed a method described by Ishikawa et al. (27) which involves prehybridization of minus- and plus-strand RNAs and subsequent removal of excess plus-strand RNA by RNase A digestion. Undigested hybrid RNAs were denatured, and minus-strand RNAs were detected by S1 nuclease analysis. Analysis of probe P2 specificity (Fig. 1B) indicated that it hybridized to minus-strand RNA transcripts (lane 8) but not to PVX RNA (lane 9). This probe also protected the expected 493-nt fragment of minus-strand RNA isolated from PVX RNA-inoculated protoplasts (lane 10). Similarly, probe P3 was specific for minus-

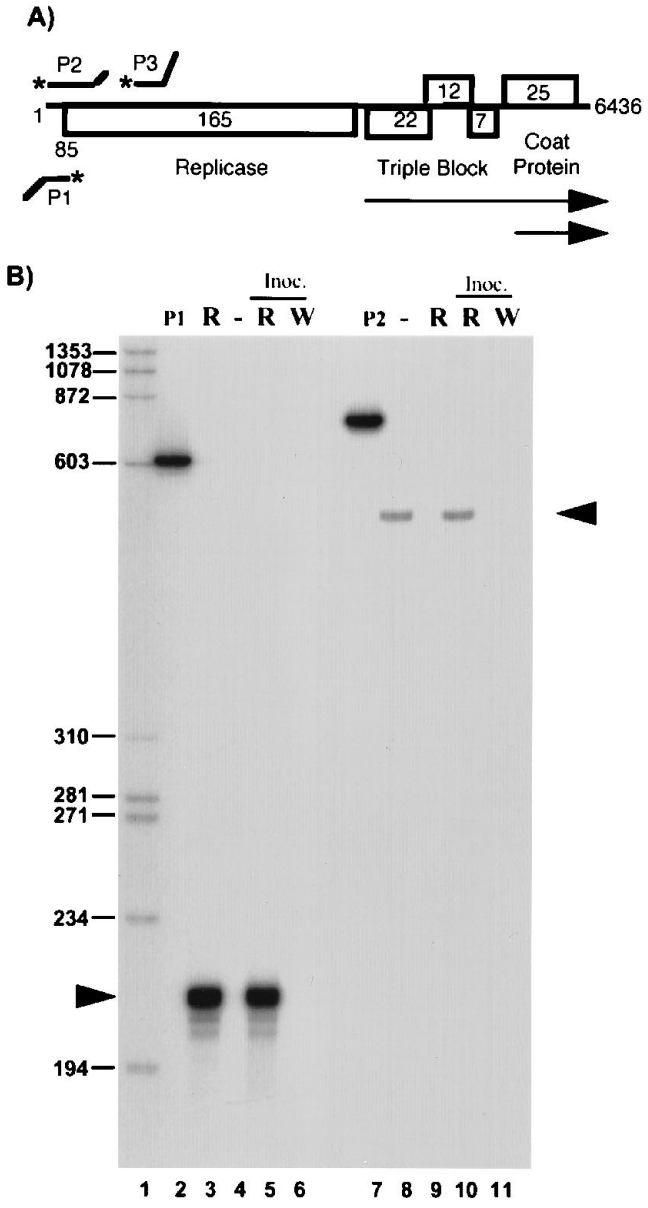

FIG. 1. Analysis of PVX RNA synthesis by S1 nuclease protection assay. (A) Schematic representation of the PVX genome. The five ORFs are denoted by open boxes and are labeled according to the sizes of the predicted polypeptides. Relative positions for the plus-strand RNA probe (P1) and minus-strand RNA probes (P2 and P3) are indicated, with asterisks denoting ^{32}P-labeled positions. The two arrows drawn below approximate regions of the genome corresponding to the two largest subgenomic RNAs. (B) Probe P1 was used to detect plus-strand RNA (lanes 2 to 6), and probe P2 was used for minus-strand RNA detection (lanes 7 to 11). Lane 1 contains ϕX174 DNA digested with *Hae*III; lanes 2 and 7 contain unhybridized probes P1 and P2, respectively; lanes 3 and 9 contain products protected by hybridization to PVX RNA; lanes 4 and 8 contain products protected by hybridization to a minus-strand RNA transcript; lanes 5 and 6 contain products protected by hybridization to total RNA isolated from 2.5×10^5 *N. benthamiana* protoplasts inoculated (Inoc.) with PVX RNA (R) and water (W), respectively; lanes 10 and 11 contain products protected by hybridization to total RNA isolated from 1.5×10^6 protoplasts inoculated with PVX RNA and water, respectively. The protected fragments after S1 nuclease digestion are indicated by arrowheads. Molecular weight markers are noted at the left.

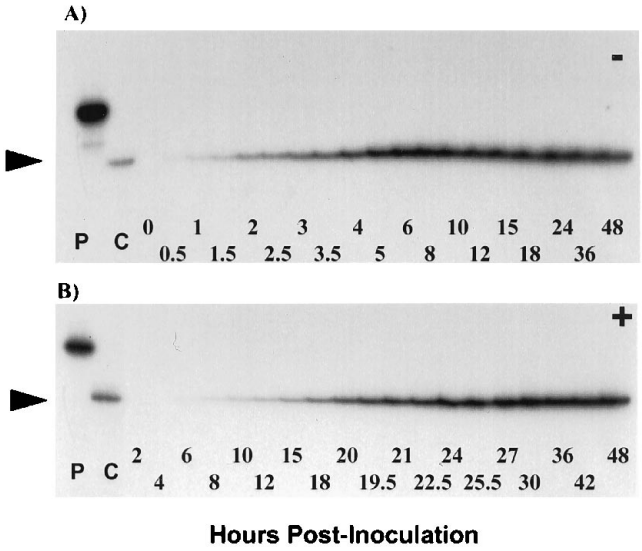

FIG. 2. Accumulation of PVX minus- and plus-strand RNAs in *N. benthamiana* protoplasts inoculated with PVX RNA. Protoplasts were inoculated with purified PVX RNA, and total RNAs were extracted at each time point indicated at the bottom of each panel. For the detection of minus-strand RNA accumulation (−), probe P2 and total RNAs from 1.5×10^6 protoplasts were hybridized and digested with S1 nuclease. Accumulation of plus-strand RNAs (+) was determined similarly with probe P1 and total RNAs from 2.5×10^5 protoplasts. Protected fragments were separated on a 6% sequencing gel and exposed to X-ray film for 48 h for minus-strand RNA detection and for 10 h for plus-strand RNA detection. Lane P contains probe alone without any treatment. Lane C contains protected fragments obtained by hybridization to minus-strand transcript RNA (A) or to PVX RNA (B).

strand PVX RNA (data not shown). Although six times more protoplasts were used for minus-strand than for plus-strand RNA detection in this analysis, the intensities of protected plus-strand RNA fragments (lane 5) were significantly greater than those of minus-strand RNA fragments (lane 10). Thus, the accumulation levels of genomic plus- and minus-strand RNAs were substantially different at 18 h.p.i., on the basis of the intensities of the protected fragments.

These data indicate that the single-stranded DNA probes used in the S1 nuclease protection assay clearly distinguished genomic plus- and minus-strand RNAs synthesized in infected protoplasts.

Accumulation of plus- and minus-strand PVX RNAs in protoplasts. A time course of plus- and minus-strand PVX RNA accumulation in protoplasts inoculated with PVX RNA was determined to select optimum times for detection of these species in subsequent mutational studies. As shown in Fig. 2A, accumulation of genomic minus-strand RNA was detectable as early as 0.5 h.p.i., peaked at 6 to 8 h.p.i., and decreased somewhat by 48 h.p.i. Genomic plus-strand RNA was detectable at 6 h.p.i. (Fig. 2B) and reached maximal levels at 24 to 27 h.p.i. Plus-strand RNA levels were maintained up to 48 h.p.i., which was the last time point checked. Although not illustrated in Fig. 2B, detectable levels of plus-strand RNA appeared as soon as 0.5 h.p.i., when higher amounts of protoplast products were analyzed by S1 nuclease digestion (data not shown). However, accurate quantitation of plus-strand RNAs at time points before 2 to 6 h.p.i. was complicated by residual input RNA.

These data illustrate that the accumulation of minus-strand RNA occurred earlier than that of plus-strand RNA. This relative pattern of accumulation was consistent in three different experiments using different preparations of protoplasts. Since the predigestion of excess plus-strand RNA with RNase A may also remove some portion of minus-strand RNA, the levels of minus-strand RNA accumulated may not represent absolute levels. Although probes P2 and P1 could be end labeled to the same specific activity and more protoplasts were

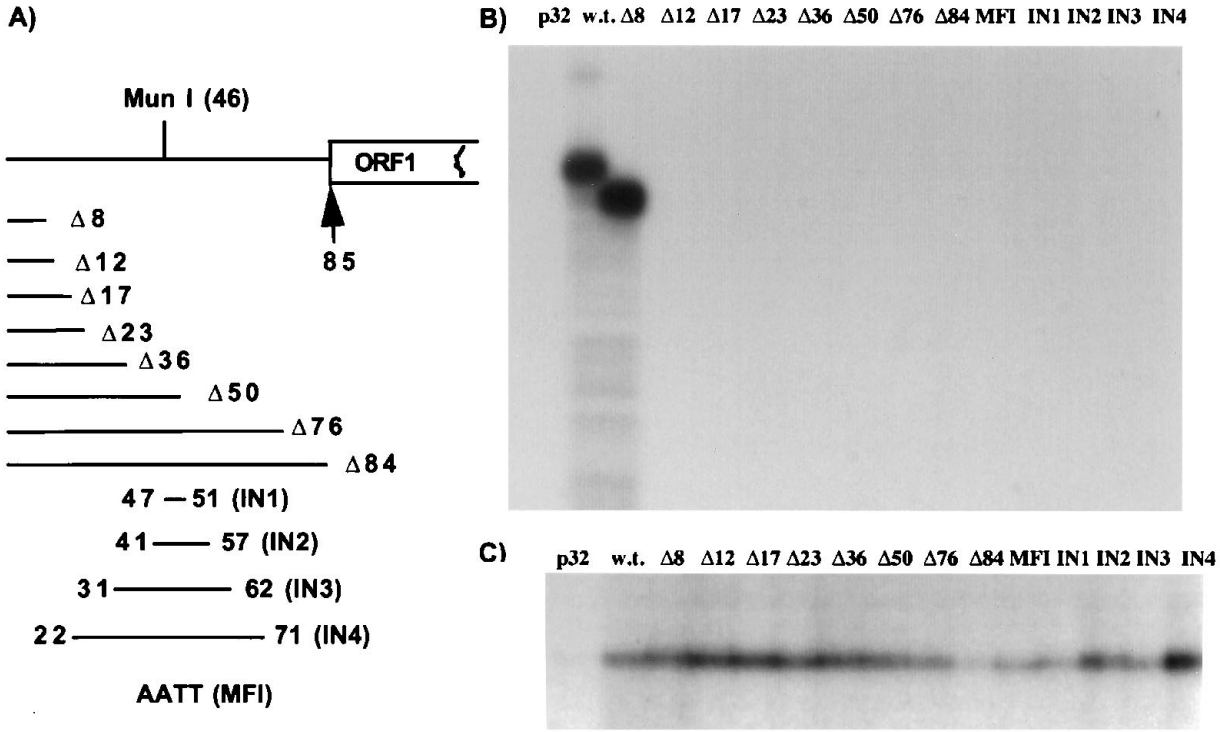

FIG. 3. Effects of 5′ NTR mutations on PVX RNA synthesis. (A) Schematic representation of mutations made in the 5′ NTR of the PVX genome. The initiation codon of ORF1 (replicase) at nt 85 is indicated by an arrow, and a *Mun*I restriction site (at nt 46) in the 5′ NTR is marked. Deletions in the 5′ NTR are indicated with lines, and the extents of deletion (Δ) are represented at the right. AATT (MFI) indicates an insertion of four extra nucleotides at the *Mun*I site. (B and C) Accumulation of plus- and minus-strand RNA, respectively, in protoplasts inoculated with control transcripts p32 and pMON8453 (w.t.) or transcripts containing mutations in the 5′ NTR (Δ8, Δ12, Δ17, Δ23, Δ36, Δ50, Δ76, Δ84, MFI, IN1, IN2, IN3, and IN4). Extracted total RNAs from 2.5×10^5 (for plus-strand RNA) or 1.5×10^6 (for minus-strand RNA) protoplasts were hybridized to probes P1 and P3, respectively, and analyzed by S1 nuclease protection assay. Protected fragments were separated on 6% sequencing gels and exposed to film for 10 h (plus-strand RNA) or 48 h (minus-strand RNA).

used for minus-strand-RNA detection, longer exposures were required for minus-strand-RNA gels to obtain autoradiograms with intensities similar to those obtained for plus-strand-RNA detection. Thus, the relative levels of plus-strand-RNA accumulation are greater than for minus-strand RNA. Given these results, time points of 8 and 24 h.p.i. were chosen for detection of minus- and plus-strand-RNA synthesis, respectively.

Sequences in the 5′ NTR are critical for genomic plus-strand RNA synthesis but not for minus-strand RNA synthesis. The importance of *cis*-acting elements in the PVX 5′ NTR for viral replication was determined by inoculating protoplasts with transcripts containing modifications in this region. As shown in Fig. 3A, several transcripts containing deletions and one containing an insertion were used in these studies. Exonuclease III was used to generate deletions from the 5′ end of the genome (Δ8, Δ12, Δ17, Δ23, Δ36, Δ50, Δ76, and Δ84), and internal deletions (IN1, IN2, IN3, and IN4) were obtained by digestion with nuclease *Bal* 31 at the *Mun*I site (nt 46). A 4-nt insertion was generated by fill-in of the *Mun*I site (MFI).

The impact of these mutations on plus-strand-RNA synthesis was measured by hybridization of inoculated-protoplast RNA with probe P1 and subsequent S1 nuclease digestion (Fig. 3B). No protected fragment was observed when protoplasts were inoculated with transcripts derived from a negative control, p32. This clone contains a deletion of nt 203 to 4138 in the PVX replicase gene and is defective in RNA synthesis. Transcripts from p32 also served as an input RNA control to ensure that the products of S1 nuclease digestion were not protected by input inoculum RNAs. Only protoplasts inoculated with transcripts derived from the wt clone, pMON8453 (referred to as w.t. in the figures), and mutant Δ8 accumulated detectable levels of plus-strand RNAs. The difference in the mobilities of the protected fragments in these two lanes corresponds to the fact that Δ8 transcripts are 4 nt smaller than the wt transcripts. In contrast, none of the other mutated transcripts supported synthesis of genomic plus-strand RNAs in protoplasts. In addition, similar results were obtained when these mutant transcripts were tested for infectivity in *N. benthamiana* plants. Only plants inoculated with Δ8 transcripts showed a symptom development similar to that of wt-inoculated plants (data not shown).

Unlike the dramatic effects of 5′ NTR mutations on genomic plus-strand-RNA synthesis, all mutants synthesized detectable levels of genomic-length minus-strand RNA (Fig. 3C). Mutants Δ8, Δ12, Δ17, Δ23, and IN4 synthesized levels of genomic minus-strand RNA similar to or greater than wt levels. Mutants Δ36 and IN2 synthesized approximately 60 to 70% of wt levels, whereas Δ50, Δ76, IN1, IN3, and MFI synthesized 20 to 40% of wt levels. It is interesting that even mutant Δ84, containing a deletion of the entire 5′ NTR, synthesized detectable levels of genomic-length minus-strand RNAs. The protected products for this minus-strand-RNA analysis do not differ in size, because a probe internal to ORF1, P3, was used for hybridization. Similar relative levels of minus-strand RNA were detected in four different experiments. Also, relative levels of ORF1 (replicase) translated in vitro from mutant transcripts were reflective of differences in minus-strand-RNA levels observed in vivo (data not shown), suggesting that minus-strand-RNA levels may vary because of differences in translation of replicase.

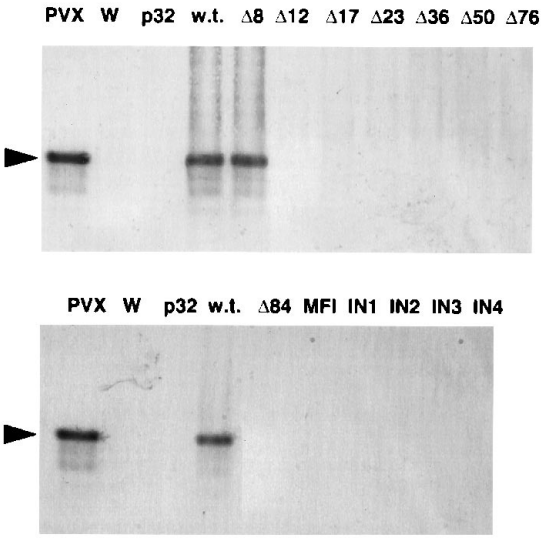

FIG. 4. Accumulation of CP in protoplasts inoculated with transcripts containing mutations in the 5′ NTR. Total protein was isolated from *N. benthamiana* protoplasts 2 days after inoculation with control transcripts (water [W], p32, and wt) or modified transcripts (Δ8, Δ12, Δ17, Δ23, Δ36, Δ50, Δ76, Δ84, MFI, IN1, IN2, IN3, and IN4). Samples were electrophoresed on SDS–12% polyacrylamide gels, blotted to nitrocellulose, and probed with antisera to PVX. The first lane in both gels contains 50 ng of PVX; the arrowhead notes the position of authentic PVX CP.

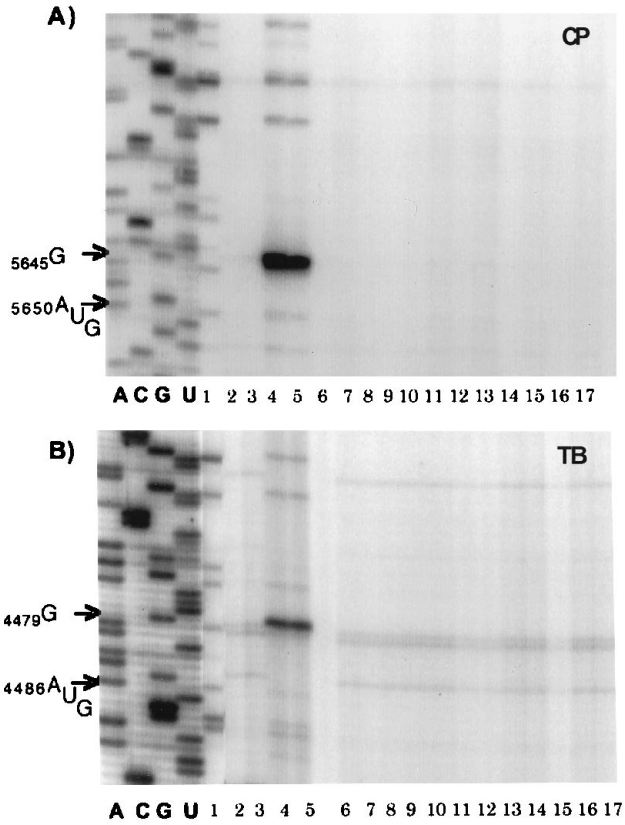

FIG. 5. Analysis of subgenomic RNA accumulation in inoculated protoplasts by primer extension. Total RNAs extracted from 5×10^5 protoplasts (lanes 2 to 17) or 0.5 μg of purified viral RNA (lane 1) was annealed to 5′ end-labeled CP (A) or TB (B) primers complementary to nt 5710 to 5725 and 4584 to 4600, respectively. Extension products were separated on 6% sequencing gels and compared with a sequence of PVX RNA (A, C, G, and U) generated with the same primers. Lanes 2 to 17 contain extension products detected in protoplasts inoculated with water, p32, wt, Δ8, Δ12, Δ17, Δ23, Δ36, Δ50, Δ76, Δ84, MFI, IN1, IN2, IN3, and IN4, respectively. The positions of primary extension products and initiation codons for the gene encoding CP and the first of the TB genes are noted and numbered at left.

These data indicate that the first eight nucleotides of the PVX 5′ NTR were not essential for genomic plus-strand-RNA synthesis but that sequences between nt 8 and 12 were necessary for this process. Sequences downstream of this point were also critical for generation of plus-strand RNA. In contrast, these 5′ NTR sequences were not absolutely required for minus-strand-RNA synthesis, indicating that requirements for genomic plus- and minus-strand-RNA synthesis were different. It can also be concluded that minus-strand-RNA accumulation in this system does not require amplification of the input plus-strand template.

***cis*-acting elements in the 5′ NTR also have an impact on subgenomic RNA synthesis.** Protoplasts inoculated as described above were also analyzed for CP production to indirectly determine the possible roles of the 5′ NTR on PVX subgenomic-RNA synthesis. The Western blot (immunoblot) in Fig. 4 shows that CP was detected only in protoplasts inoculated with wt or Δ8 transcripts. The absence of CP in protoplasts inoculated with all other mutant transcripts indicated that elements critical for genomic plus-strand-RNA synthesis potentially had an impact on the production of the most abundant subgenomic RNA species.

To further substantiate this observation, levels of accumulation of PVX CP subgenomic RNA and the other major subgenomic RNAs containing TB and CP genes were determined by primer extension analyses (Fig. 5). Total RNAs extracted from protoplasts at 24 h.p.i. were hybridized with primers complementary to the CP gene and to the TB region (containing sequences complementary to nt 5710 to 5725 and 4584 to 4600, respectively). Since genomic plus-strand RNA will also hybridize to these primers, purified PVX RNA was extended to determine positions of background stops generated by this RNA species. As shown in Fig. 5A, protoplasts inoculated with wt transcripts (lane 4) produced a predominant extension product at nt 5645 (5 nt upstream from the CP initiation codon), which was not present in PVX RNA extension products (lane 1). Since other minor extension products are similar in position and intensity in both purified PVX RNA and RNA isolated from wt-inoculated protoplasts, the major product observed at nt 5645 represents the extension product of CP subgenomic RNA. This CP subgenomic RNA extension product was also detected in protoplasts inoculated with Δ8 transcripts (Fig. 5A, lane 5) but not with any other of the mutant transcripts (lanes 6 to 17) or in samples from water-inoculated (lane 2) or p32-inoculated (lane 3) protoplasts.

Analysis of the second-most predominant subgenomic RNA species that contains both TB and CP genes (Fig. 5B) indicated that the major extension product in the wt-inoculated sample (lane 4) was at nt 4479 (7 nt upstream of the initiation codon for the first of the triple-block proteins). This extension product was specific for infected protoplasts and was not observed in the purified PVX RNA extension reaction (lane 1). Again, only Δ8 (Fig. 5B, lane 5) supported accumulation of this subgenomic RNA. These results clearly demonstrate that similar 5′ NTR elements are essential for both genomic and subgenomic plus-strand-RNA synthesis and indicate that these two processes may be linked. These data also define the 5′ ends of the two major PVX subgenomic RNA species.

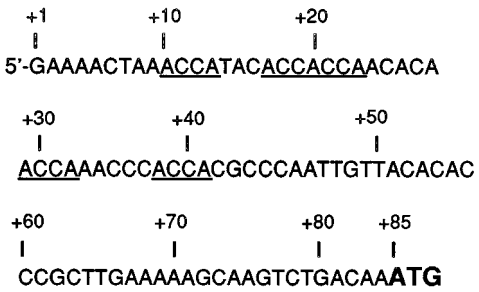

FIG. 6. Nucleotide sequence of the 5′ NTR of PVX RNA. The repeating ACCA motifs are underlined, and the initiation codon for ORF1 at +85 is in boldface type.

DISCUSSION

To study the *cis*-acting elements required for synthesis of PVX RNA in vivo, we developed a sensitive S1 nuclease protection assay for detection of both plus- and minus-strand RNAs. This method was highly specific for PVX genomic plus-strand and minus-strand RNAs produced in *N. benthamiana* protoplasts inoculated with infectious PVX transcripts. The time course studies indicated that both RNA species were detectable by 0.5 h.p.i., but minus-strand RNA accumulated to maximum levels earlier than did plus-strand RNA. Despite this more rapid accumulation of minus-strand PVX RNA, the relative levels of minus-strand RNA were 20- to 40-fold lower than plus-strand RNA. Similar studies with tobacco mosaic virus indicated that maximum accumulation of tobacco mosaic virus minus-strand RNAs in protoplasts preceded that of plus-strand RNAs, but plus-strand RNAs accumulated to 100-fold or greater levels (27). This asymmetry in minus- and plus-strand-RNA accumulation has been studied in detail for several plant plus-strand-RNA viruses (6, 10, 35, 65).

Analysis of transcripts containing deletions in the PVX 5′ NTR indicated that *cis*-acting elements in this region were required for the synthesis of genomic, plus-strand PVX RNA. Although deletion of 8 nt from the 5′ end did not affect plus-strand-RNA accumulation, 5′ coterminal deletions of 12 nt or more inhibited this process. In addition, transcripts containing an insertion or internal deletions in their 5′ NTRs did not support genomic RNA synthesis. Since the internal 5′ NTR deletions do contain nt 8 to 12, these data indicate that PVX plus-strand-RNA synthesis requires elements from different regions of the 5′ NTR. As indicated in Fig. 6, the PVX 5′ NTR is AC rich and contains several ACCA repeats. Although different potexviruses do not have extensive sequence homology in their 5′ NTRs, all contain AC-rich leader sequences. It remains to be determined if an ACCA motif found between nt 10 to 13, which is repeated five times throughout the PVX 5′ NTR, is a critical sequence in this region. Similar to the PVX system, tobacco mosaic virus RNA contains an approximately 70-nt, AC-rich 5′ NTR with several repeated AAC elements (38). Deletions throughout this region indicated that only nt 2 to 8 were indispensable for replication (55). However, deletions extending from nt 10 to 31 or nt 13 to 31 showed a decrease in RNA accumulation. Since the latter deletions and other large deletions removed several of the AAC elements, Takamatsu et al. (55) postulated that several such elements may contribute to replication.

In contrast to effects of 5′ NTR deletions on PVX plus-strand-RNA synthesis, minus-strand RNA was produced by all mutant transcripts, with differences in levels of minus-strand RNAs among the different mutants. Transcripts with deletions of up to 76 nt from the 5′ end and two internal mutations (IN2 and IN4) produced minus-strand-RNA levels similar to or greater than those produced by wt transcripts, whereas deletion of the entire 5′ NTR (Δ84), internal deletions (IN1 and IN3), and the 4-nt insertion at the *Mun*I site (MFI) resulted in lower levels of minus-strand RNA. These differences may be attributed to deletion of elements in the 5′ NTR that have an impact on minus-strand-RNA synthesis but are more likely due to altered translation of PVX replicase. The latter possibility is supported by data indicating that lower levels of in vitro-translated replicase from these mutant transcripts correlated with the relative levels of corresponding minus-strand RNA produced for each mutant. Other studies on effects of changes in the 5′ NTR of PVX RNA on translation in vitro are consistent with these data (57). For example, a modified PVX leader with an insertion at the *Mun*I site exhibited a decreased level of translation relative to that of the authentic PVX leader. Similarly, our MFI transcripts produced very low levels of replicase in vitro and of minus-strand RNA in vivo. Thus, although all of our mutant transcripts can support translation of replicase in vitro, differences in translation may account for differences in minus-strand-RNA accumulation in vivo.

The most striking result of these PVX replication studies was that inhibition of genomic plus-strand-RNA accumulation was accompanied by inhibition of subgenomic plus-strand-RNA production. Given that minus-strand RNA did accumulate in protoplasts inoculated with mutant transcripts, these data suggest that PVX subgenomic RNA cannot be generated by internal initiation on minus-strand RNA when genomic plus-strand-RNA synthesis does not proceed. These data are in contrast to those reported by Miller et al. (39) for BMV subgenomic RNA synthesis in a template-dependent BMV replicase extract. They demonstrated that synthesis of BMV subgenomic RNA 3 occurred by internal initiation on minus-strand RNA 3, even when such templates contained deletions in sequences corresponding to the 5′ sequences of RNA 3. However, a study by Huntley and Hall (26) indicated that minus-strand RNA corresponding to an internal region of BMV RNA 3 was not sufficient for synthesis of subgenomic RNA in protoplasts that were coinoculated with BMV RNAs 1 and 2. Thus, the mechanism for subgenomic RNA synthesis in an in vitro system, in which syntheses of the various RNA species are not coupled, appears to differ from the coupled process occurring in vivo. Although *cis*-acting elements will clearly vary among different viruses, mechanisms for subgenomic RNA synthesis may also differ. Like potexviruses, coronaviruses contain a single genomic RNA that is capped and polyadenylated, and replication involves synthesis of genomic and subgenomic plus-strand RNAs (for a review, see reference 31). Both *cis*- and *trans*-acting elements in the 5′ NTR and elements upstream of the subgenomic RNAs of mouse hepatitis virus are necessary for subgenomic RNA synthesis (33). This process involves fusion of noncontiguous sequences from the 5′ leader sequence to the subgenomic mRNA by a discontinuous transcription process and results in subgenomic RNA products with leaders that are similar to sequences at the 5′ end of the viral RNA (32, 50, 54). It remains to be determined if synthesis of PVX subgenomic RNAs involves a discontinuous transcription process that requires multiple *cis*- and/or *trans*-acting elements.

As part of the PVX 5′ NTR analysis, the 5′ ends of the two predominate subgenomic RNAs were mapped. The 5′ end of the subgenomic RNA containing TB and CP genes was 7 nt upstream of ORF2. The primer extension product for the smallest subgenomic RNA species, ending 5 nt upstream of the CP initiation codon, confirms data reported by Skryabin et al.

(52). Although the leader sequences have not been determined for potexviral subgenomic RNAs, there are upstream sequence elements that are conserved among different viruses in this group (67, 68). Such regions have been proposed to serve as subgenomic promoter elements but have not been functionally defined. Future experiments will determine if sequences and/or structures in the 5′ NTR, upstream of the TB and CP genes, or elsewhere in PVX RNA are critical for coregulation of genomic and subgenomic RNA synthesis and if they can be altered to uncouple these processes.

ACKNOWLEDGMENTS

We thank L. Hanley-Bowdoin, S. A. Lommel, and P. Wollenzien for critical readings of the manuscript.

This research was supported by Public Health Service grant GM 49841 to C.H.

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