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Cis-acting Elements in the 3' Region of *Potato virus X* are Required for Host Protein Binding

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The 3' region of *Potato virus X* (PVX) has the 74 nt 3'-nontranslated region (NTR) that is conserved among all potexviruses and contains several *cis*-acting elements for minus-strand and plus-strand RNA accumulation. Three stem-loop structures (SL1-SL3), especially formation of SL3 and U-rich sequence of SL2, and near upstream elements in the 3' NTR were previously demonstrated as important *cis*-acting elements. To investigate the binding of these *cis*-acting elements within 3' end with host protein, we used the electrophoretic mobility shift assays (EMSA) and UV-cross linking analysis. The EMSA with cellular extracts from tobacco and RNA transcripts corresponding to the 150 nt of the 3' end of PVX RNA showed that the 3' end of PVX formed complexes with cellular proteins. The specificity of protein binding was confirmed through competition assay by using with 50-fold excess of specific and non-specific probes. We also conducted EMSA with RNAs containing various mutants on those *cis*-acting elements (Δ10, SL3B, SL2A and Δ21; J Mol Biol 326, 701-720) required for efficient PVX RNA accumulation. These analyses supported that these *cis*-acting elements are required for interaction with host protein(s). UV-cross linking analysis revealed that at least three major host proteins of about 28, 32, and 42 kDa in mass bound to these *cis*-elements. These results indicate that *cis*-acting elements from 3' end which are important for minus and plus-strand RNA accumulation are also required for host protein binding.

Keywords : 3' *cis*-elements, EMSA, host protein binding, PVX

Potato virus X (PVX), the type member of the Genus *Potexvirus*, is a flexuous rod-shaped virus containing a 6.4 kb plus-stranded RNA genome (Bercks, 1970; Milne, 1988). PVX utilizes transcriptional and translational strategies for its gene expression during replication. Two major and a couple of minor virus-specific RNAs of positive-strand RNAs (subgenomic RNA, sgRNA) are synthesized from which the triple gene blocks (TGBs), ORFs 2-4, that are associated with the function of viral cell-to-cell transport (Beck et al., 1991; Angell et al., 1996), and the coat protein (CP), product of ORF5, involved in both virus movement and encapsidation (Chapman et al., 1992; Oparka et al., 1996) are generated (Morozov et al., 1991; Verchot et al., 1998). It has been reported that the product of ORF4 is synthesized during translation of sgRNA by leaky ribosome scanning strategy (Verchot et al., 1998). For these processes, the initial step in PVX RNA replication is the synthesis of negative-strand RNA from a positive-strand genomic RNA template. Thus, transcription of negative strand RNA is a crucial event in PVX RNA replication.

The 3' non-translated region (NTR) of PVX RNA is shorter than the 5' NTR and contains a poly(A) tract at the end (Bercks, 1970; Huisman et al., 1988; Skryabin, 1988). The terminal 5' and 3' NTRs of the PVX genomic RNA are 84 and 72 nucleotides (nt) long, respectively (Fig. 1). Previous studies have indicated that the 5' and 3'-terminal nt of the PVX genome can form conserved stem-loop (SL) structures (Miller et al., 1998; Pillai-Nair et al., 2003). The SL structure formed by the 5'-terminal nt containing the AC-rich single-stranded region and the stem-loop 1 (SL1) secondary structure is more stable than the structure formed by the 3'-terminal nt. The conservation of these structures among divergent potexviruses suggests that they may function as *cis*-acting signals for the initiation of viral RNA replication or translation. Implication of 5' NTR in the regulation of the synthesis of both genomic RNAs and sgRNAs has been reported (Kim and Hemenway, 1996; Miller et al., 1998).

It has also been reported that the sequence at the 5' end of the genome interacts with the conserved element upstream of sgRNA between the nucleotide at the 5' end and that

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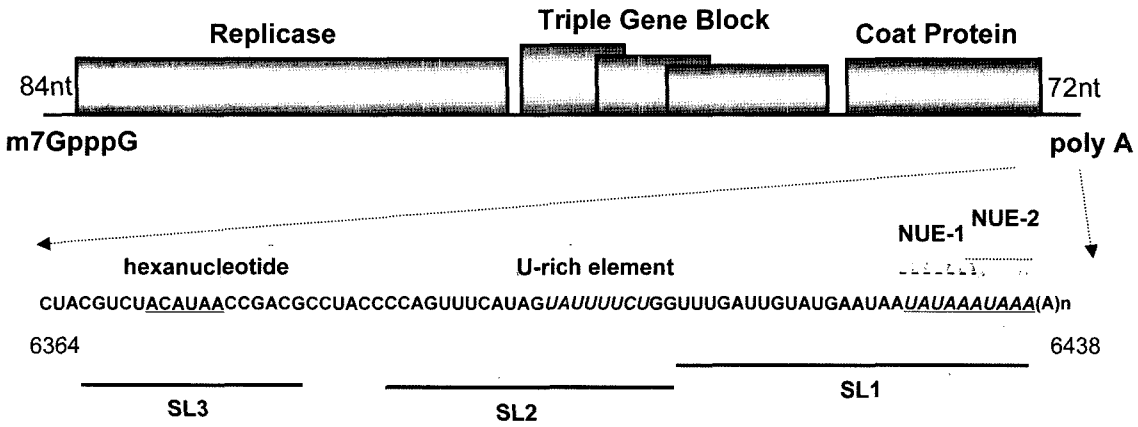

Fig. 1. Schematic diagram of the PVX genome organization and 3' RNA sequences showing hexanucleotide, U-rich sequence, and the two near upstream elements (NUEs). RNA sequences involved in forming stem-loop structures (SL1-3) present in the 3' end of the genome (Pillai-Nair, 2003) are depicted.

upstream of sgRNA (Kim & Hemenway, 1999). The functional significance of the SL1, including tetra loop (TL) and SC motifs and the C-C mismatch has been investigated by generating several modifications within SL1 and inoculating transcripts that contained each of these mutations into *Nicotiana benthamiana* plants (Miller et al., 1999). The top region (SC, LC, SD, and TL) of SL1 also plays a key role in binding to PVX CP subunits and host proteins, consequently, is important in initiating virus-like particle formation and many aspects of virus replication (Kwon et al., 2005; Kwon and Kim, 2006). It has previously been reported that the single-stranded region at the 5' end and the SL1 has binding activity to the host extract (Kim et al., 2002). Thus, the 5' region of PVX may interact with other viral *cis*-acting elements, viral gene products, and several host proteins that control RNA synthesis and viral replication.

Specific binding of host cell proteins (28 and 32 kDa) to regions within the 3' NTR of PVX RNA genome and their possible role in RNA replication have been reported (Sriskanda et al., 1996). Furthermore, they found that an 8-nt U-rich motif important for (−)-strand RNA accumulation within the PVX 3' NTR was required for binding and PVX replication by deletion mapping and site-directed mutations. The regulation of viral RNA replication or transcription may involve several cellular proteins that specifically recognize either terminal elements and/or sequences elsewhere in the genome. Recently, the importance of sequences and structures in the PVX 3' NTR for accumulation of viral RNAs has also been reported (Pillai-Nair et al., 2003). It has been identified that the multiple sequence elements throughout the 3' NTR that were required for minus-strand RNA accumulation, as well as a short region near the 3' terminus that was dispensable and localized elements important for optimal plus-strand RNA accumulation. Altogether, these results indicate that the 3' NTR is required for multiple aspects of virus replication.

It is also likely that the binding of cellular proteins with the PVX regulatory sequences and/or RdRp may also be involved in regulating PVX replication. RNA-protein interactions play key role in an enormous variety of important biological processes. Recent studies with other plus-strand RNA viruses have characterized several cellular proteins that bind specifically to viral RNA and play a key role in viral RNA replication (for a review see, Lai, 1998). Cellular proteins that specifically bind to other regulatory sequence elements were observed for other viral systems (Blackwell and Brinton, 1995; Diez et al., 2000; Furuya and Lai, 1993; Ito and Lai, 1997; Li et al., 1997; Shi, et al., 1996; Wang and Zhang, 1999; Zhang and Lai, 1995).

Our approach to studying PVX RNA replication is to identify the *cis*-acting signal for RNA replication and the proteins which recognize these signals. In this study, we used electrophoretic mobility shift assay (EMSA) and UV cross-linking studies to investigate binding of cellular proteins to the 3' end of PVX RNA. We found that three major host proteins of about 28, 32, and 42 kDa in mass bind to sequences in the 3' region of the PVX genome and that the minimal binding sequences is within the 3' proximal 150 nucleotides of the PVX RNA genome comprising a conserved hexanucleotide sequence element in the loop of SL3, and the CU nucleotide in a U-rich sequence within SL2.

Materials and Methods

Materials. All restriction enzymes, modifying enzymes, polymerases, and m7GpppG cap analogue were purchased from New England BioLabs. RNasin and RQ1 RNase-free DNase and Sequenase were obtained from Promega and U.S. Biochemicals, respectively. Deoxy-, dideoxy- and

ribonucleotides were purchased from Boehringer Mannheim and oligonucleotides were synthesized by Sigma-Genosys. Avian myeloblastosis virus reverse transcriptase and Trizol reagents were from Life Sciences. Reagents for protoplast preparation were obtained from Yakult Honsha Co., Ltd and Karlan Research Products.

Plasmid construction and RNA transcription. The construct, p10, was generated by digestion of the parent plasmid, pMON 8453 (Hemenway et al., 1990) with *Xho*I and *Sph*I and ligated into pGEM3Zf(−) (Promega, USA) digested with *Sal*I and *Sph*I. The ligated plasmid (p10) contained only the 3' 150 nt of PVX genome downstream of the bacteriophage T7 promoter. This plasmid was then linearized with *Spe*I before being used as a template for *in vitro* synthesis of positive-strand RNAs containing 150 nt of PVX 3' end. 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. For synthesis of a nonspecific competitor, plasmid pGEM3Zf(−) linearized with *Pvu*II was used to generate 268-nt RNA containing cloning sites and internal vector sequences as described previously (Kim et al., 2002). The previously generated 3' mutants that contain deletions or site-directed mutations were used for *in vitro* binding (Pillai-Nair et al., 2003). Radio-labeled (^{32}P) and unlabeled RNAs were prepared and gel purified as described previously (Kim and Hemenway, 1996; Kim et al., 2002; Kwon et al., 2005).

Preparation of plant cell extracts and EMSA. *N. tabacum* cv. Samsun suspension cell (NT-1) protoplasts were prepared as described previously (Kim and Hemenway, 1997), and subsequently used for inoculation experiments or as sources of S100 protein extracts (Kim and Hemenway, 1997; Kim et al., 2002; Sriskanda et al., 1996). The total protein concentration of each extract was determined by Bradford analysis (Bio-Rad). The EMSA experiments were carried out as described by Kim et al. (2002). For competition experiments, various amounts of unlabeled RNA and DNA were added to the reaction mixture 10 min prior to or simultaneously with the addition of labeled probe and incubated on ice for 10 min and analyzed on a 4% nondenaturing polyacrylamide gels. The relative densities of bands corresponding to RNA-protein complexes were determined by using ImageQuant software on a PhosphorImager (Molecular Dynamics). *In vitro* binding experiments with wild-type and mutant transcripts were performed separately at least three times with different transcript preparations.

UV cross-linking of RNA-protein complex. For cross-linking studies, the RNA-protein binding reactions were incubated in microtitre plates and subsequently irradiated with a hand-held UV lamp (Fisher Scientific) on ice (254 nm, 1 cm distance) for 15 min. Non-crosslinked RNAs were removed from samples by incubation with RNase A (1 mg/ml) at 37°C for 30 min. Proteins cross-linked to radioactive RNA fragments were analyzed as previously described (Kim et al., 2002).

Results and Discussion

To identify cellular proteins that interact with the 3' end of PVX genomic RNA, an *in vitro* EMSA and UV cross-linking analyses were used to study RNA-protein interactions. The 3' end of the PVX genome is identical in all sequenced PVX strains and were previously determined to contain at least three well-defined stem-loop structures (SL1, SL2, and SL3 in the 3' to 5' direction), based on computer predicted folding and solution structure probing (Fig. 4B; Pillai-Nair et al., 2003).

The 3' region of PVX RNA interacts with cellular proteins in the S100 extracts. EMSA of RNA probes derived from the 3' end (150 nt) of PVX RNA in the presence of protoplast S100 extracts was used to determine whether proteins bind to regulatory elements in the 3' region of the PVX genome. This probe was synthesized *in vitro* by using T7 RNA polymerase as described in Materials and Methods. Cytoplasmic S100 extracts prepared from *N. tabacum* protoplasts were incubated with RNA probe, and then the RNA-protein complexes were electrophoresed on 5% native polyacrylamide gel as described previously (Kim et al., 2002). One major RNA-protein complex was detected (Fig. 2). No obvious differences were observed in the number of or the electrophoretic mobilities of the RNA-protein complexes that formed with extracts from uninfected or PVX RNA-infected NT-1 cells at 24 hours post-inoculation (hpi, Fig. 3A). The major RNA-binding proteins are cellular in origin and their binding to PVX RNA does not seem to require viral factors. When increasing amounts of the S100 extracts or labeled PVX 3' 150 nt RNA were incubated with the labeled PVX 3' 150 nt RNA or with the NT-1 cell extracts, formation of RNA-protein complexes was proportionally increased (Fig. 2, panels A and B, respectively). Approximately 2.5 μg of total cell protein in S100 extracts was required to detect RNA-protein complex (Fig. 2A).

Nucleotides located near the 3' end of plus-strand RNA are necessary for synthesis of genomic minus- and plus-strand RNAs (Pillai-Nair et al., 2003; Sriskanda et al., 1996). Formation of SL3 was required for accumulation of minus-strand RNA, whereas SL1 and SL2 formation were

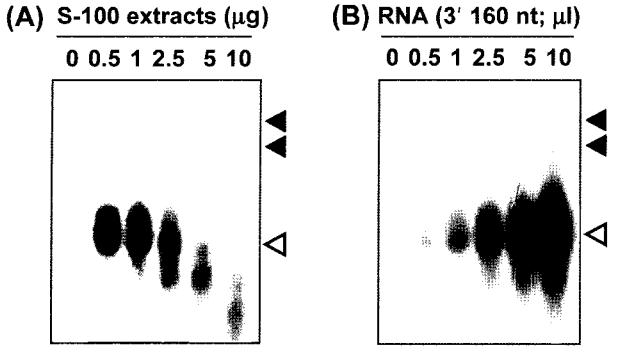

Fig. 2. Electrophoretic mobility shift assay (EMSA) of RNA-cellular protein complexes with increasing amount of either S100 extracts (A) or RNA probes (B). RNA-cellular protein complexes were separated on 4% non-denaturing polyacrylamide gel. Arrowheads indicate position of RNA probe (open arrowhead) RNA-cellular protein complexes (closed arrowheads).

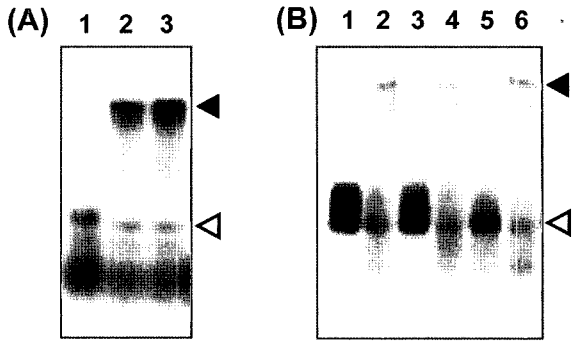

Fig. 3. Binding and competition assay of the 3' 150 nt RNA probe and cellular protein complex. (A) Five μg of NT-1 S100 extracts from healthy and PVX-infected NT1 protoplasts (lanes 2 and 3, respectively) were mixed with the 3' 150 nt RNA probe. Panels (B) represents EMSA of [^{32}P]-labeled the 3' 150 nt RNA and S100 NT-1 extracts that were pre-incubated with unlabeled 3' 150 nt RNA in 50 molar excess, relative to labeled RNA probe (lane 3), 10 mg of *E. coli* tRNA, salmon sperm DNA, and BSA (lane 4-6, respectively). Unlabeled plasmid *E. coli* tRNA, salmon sperm DNA, or BSA were used as non-specific competitor. Lanes 1 and 2 represent RNA probe and RNA probe incubated with 5 mg of S100 extracts, respectively. Arrowheads indicate position of RNA probe (open arrowhead) RNA-cellular protein complexes (closed arrowheads).

less important. However, sequences within all of these predicted structures were required for minus-strand RNA accumulation, including a conserved hexanucleotide sequence element in the loop of SL3, and the CU nucleotide in a U-rich sequence within SL2 (Pillai-Nair et al., 2003). It is possible that these regions, i.e. SL1-3 and conserved hexanucleotide sequence element at the 3' end, may be involved in and/or necessary for protein binding during replication.

To access the specificity of the interactions between the cellular proteins and the PVX 3' 150 nt RNA, competition analyses were conducted with unlabeled PVX 3' 150 nt RNA as the specific competitor and *E. coli* tRNA, plasmid pBS(+) DNA, or BSA were used as non-specific competitors. The addition of 50-fold molar excess of the unlabeled PVX 3' 150 nt RNA caused almost complete inhibition of the formation of RNA-protein complexes when incubated with the NT-1 cell extracts prior to the addition of the [^{32}P]-labeled PVX 3' 150 nt RNA (Fig. 3B, lane 3). However, no or little inhibition of the complex formations were detected when *E. coli* tRNA, plasmid pBS(+) DNA or BSA were used as a competitor (Fig. 3B, lanes 4-6). These results showed that non-homologous competitor were unable to compete for binding to cellular proteins with the PVX 3' 150 nt RNA probe even at 50-fold molar excess, whereas the homologous competitor significantly reduced the formation of RNA-protein complexes at 50-fold molar excess. These results indicate that the interactions between cellular proteins and PVX 3' end of PVX RNA are specific.

Localization of host protein binding motif at the 3' end of PVX genomic RNA. The 3' NTR of PVX RNA was predicted to contain three SL structures, denoted SL1, SL2 and SL3 in the 3' to 5' direction (Pillai-Nair et al., 2003). To more precisely localize the required sequence or element within the 3' 150 nt of the PVX genomic RNA, five sets of mutant transcripts including two previously constructed mutants, Δint and Δ10 (Pillai-Nair et al., 2003), were generated for binding reactions (Fig. 4A). Deletion of the sequence between the hexanucleotide and eight nucleotide U-rich regions in the Δint mutant, was created by site-directed mutagenesis. The smaller deletion, Δ10, extends through the hexanucleotide sequence. The larger deletion, Δ21, lacks most of the U-rich element and the sequence between the U-rich element and putative near upstream elements (NUEs), except for five nucleotides at the 3' region. Mutants SL3B and SL2A contains site-directed nt changes in SL3 and SL2 regions, respectively (Fig. 4A, shown in bold). The SL3B (GACG to CUGC in SL3 stem region) mutation was predicted to change the optimal, wild-type secondary structure of SL3 and alter the free energy of the SL structure. As shown in Fig. 4B (Pillai-Nair et al., 2003), change of the 5' side of the stem (CC to GG) and 3' side of the stem (GG to CC) of SL2 in mutant SL2A was predicted to maintain the optimal, wild-type secondary structure of SL2 and does not alter the free energy of the SL2 structure.

All RNA transcripts generated shifted complexes when incubated with the NT-1 extract in EMSA, with the abundance of the shifted complexes decreasing with more extensive 3' deletions (Fig. 5). Though the level of binding activity was reduced using Δ21 RNA probe compared to

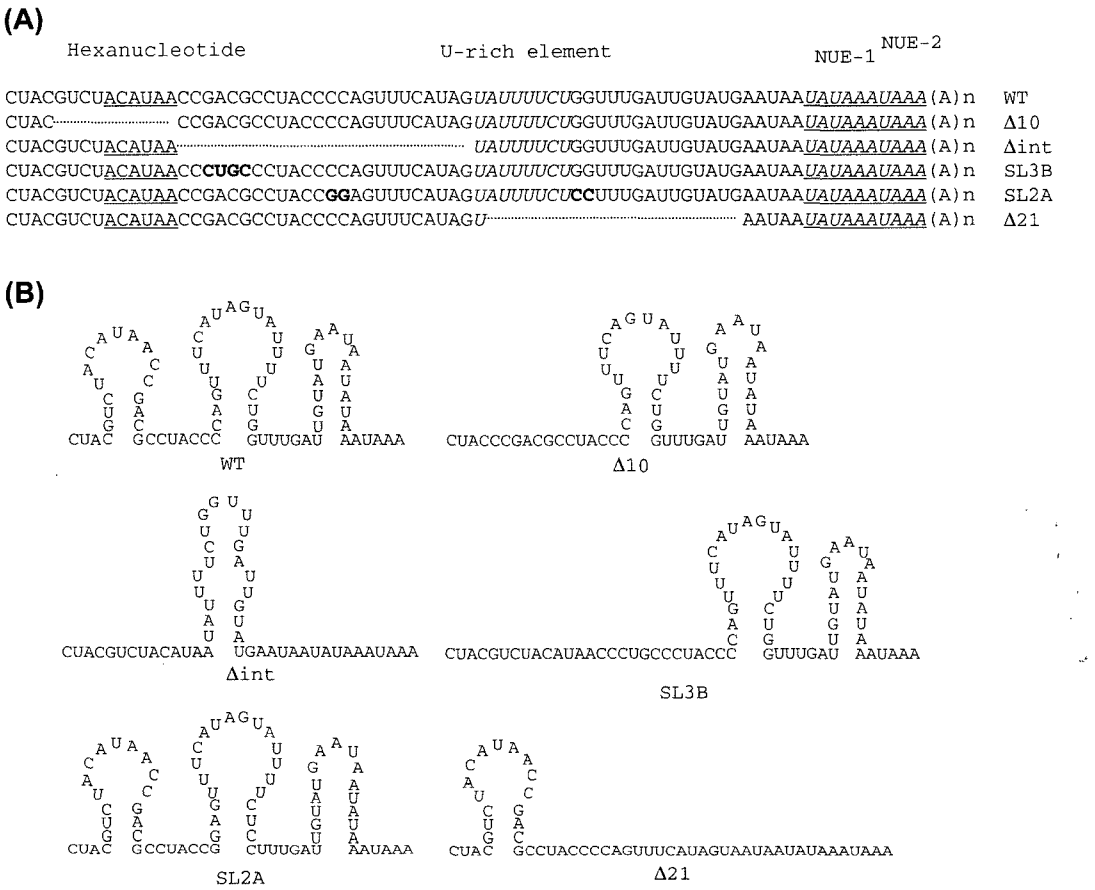

Fig. 4. Construction of deletion and site-directed mutation (A) and the predicted optimal secondary structures (B). Deleted sequences are noted by dotted lines. Site-directed nucleotide substitutions were indicated as bold characters. The conserved hexanucleotide (underlined), U-rich element (italicized), and NUEs (italicized and underlined) are also depicted.

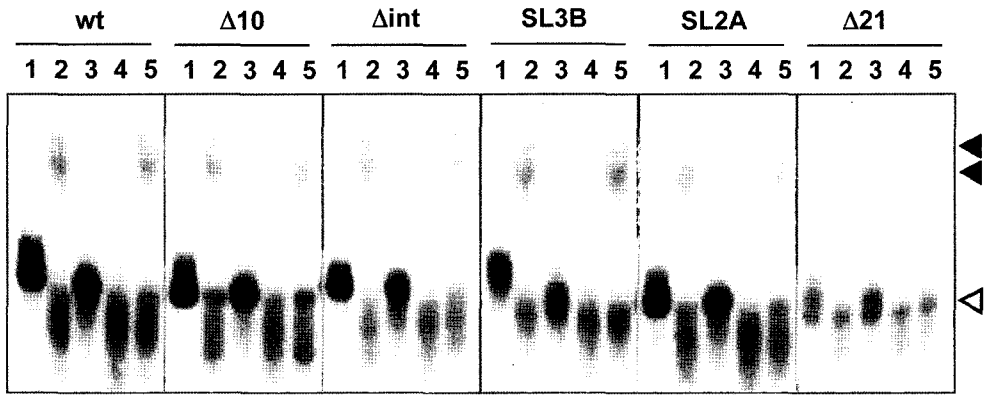

Fig. 5. Effects of mutations introduced into the 3' end of PVX genomic RNA on cellular protein binding and competition. [^{32}P]-labeled transcripts corresponding to the 3' 150 nt, Δ10, Δint, SL3B, SL2A, and Δ21 RNAs were each mixed with water (lane 2), 50 molar excess of unlabeled RNA (lane 3), 10 mg of *E. coli* tRNA, and BSA (lanes 4 and 5, respectively). In lane 1, no S100 extract was added. Closed arrowheads indicate position of RNA-cellular protein complexes.

that of using 3' 150 nt RNA, Δ21 3' PVX plus-strand RNA still showed shifted bands (Fig. 5). This result demonstrates that the 3' end 150 nucleotide of PVX plus-strand RNA contains binding domains for cellular proteins. RNA transcript containing SL3 structure destabilization also revealed binding activity to the host extracts. Similarly site-directed mutations introduced at the SL2 region did not significantly affected cellular protein binding (Fig. 5). Since

the intensity of the shifted bands obtained from using SL3B and SL2A RNA as probes for gel-retardation assay appears to be similar than that obtained from using 3' end 150 nt containing intact SL1 to SL3 (Fig. 5), it may be that RNA structure may not significantly contribute to cellular protein binding although each mutation may contributes binding of different cellular proteins. When we used unlabeled RNA as a competitor for binding, it significantly reduced cellular protein binding both in EMSA and in UV cross-linking assays (Figs. 5 and 6).

Since, our goal is to identify cell proteins that bind specifically to the 3' end of PVX RNA, extracts from uninfected NT-1 cells were used for UV cross-linking studies. [^{32}P]-labeled RNA transcripts were generated, gel purified and incubated with S100 NT-1 cell extracts, and exposed to UV irradiation as described previously (Kim et al., 2002). UV cross-lined RNA-protein complexes were treated with RNase A, and then analyzed by 12% SDS-PAGE. More than three major RNA-protein complexes were detected with apparent molecular mass of approximately 28, 32, and 42 kDa (p28, p32, and p42, respectively) by autoradiography (band marked by arrowheads in Fig. 6). No band was detected when NT-1 S100 extracts were pre-incubated with proteinase K before RNA-protein binding or prior to SDS-PAGE (data not shown). No distinct difference was observed in UV cross-linking pattern between healthy and PVX-infected S100 extracts (Fig. 6, panel A). These results indicate that the RNA binding activity was due to a protein in NT-1 S100 extracts.

To define the sequences within the 3' terminal 150 nt required for binding with these cellular proteins, mutant transcripts containing deletion and site-directed mutations (Δ10, Δint, SL3B, SL2A, and Δ21) were used as probes in UV cross-linking studies. Four mutant transcripts including Δ10, Δint, SL3B, and SL2A abolished p42 binding. p32 binding ability was lost only Δint mutant. Interestingly SL3B and Δ21 transcripts showed strong RNA-binding activity with p28 and p32 (Fig. 6B). Surprisingly, deletions of 21 nt (Δ21) which lacks most of the U-rich element and the sequence between the U-rich element and putative near upstream elements (NUEs), did not abolish all protein binding, although it showed less binding ability in EMSA (Figs. 5 and 6). These results indicated that the nucleotides located at the 3' end of PVX plus-strand RNA contained several crucial RNA elements for cellular protein bindings. Altogether, these results indicate that different RNA sequence elements as well as specific SLs and/or sequences therein possibly serve as binding sites for host proteins.

Our previous data suggested that the sequences located at the 3' end of the positive-strand PVX RNA differentially regulate PVX genomic (+)- and (−)-strand RNA replications (Pillai-Nair et al., 2003). Nucleotide sequences in these

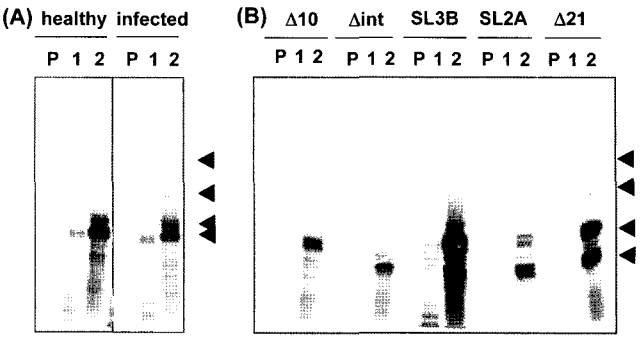

Fig. 6. UV cross-linking assay with the PVX deletion and site-directed mutants. The experiments were carried out with [^{32}P]-labeled 3' 150 nt of PVX genomic RNA probes and S100 cellular extracts. Each RNA was mixed with 50 molar excess of unlabeled RNA (lane 1) and water (lane 2). In lane P, no S100 extract was added. Closed arrowheads indicate major cross-linked cellular proteins with the molecular mass of ca. 28, 32, 42, and 54-kDa bands.

regions might be involved in RNA-RNA and RNA-protein interactions that are required for PVX replication. Sriskanda et al. (1996) showed that two different cellular proteins, p32 and p28, bind to the 3' end of the PVX genomic RNA and that an 8 nt U-rich sequence (UAUUUUCU) within the 3' NTR is required for binding. They also showed that mutations introduced in nt 2-6 of this sequence affected both the binding of cellular proteins and virus replication. In our experiments, however, deletion of 21 nt including most of U-rich sequence (7 out of 8 nt) maintained strong binding ability to p28 and p32 (Fig. 6). It is possible that the deletion of additional nt beyond the U-rich sequence might affected binding affinity of cellular proteins. Though observations reported in this study suggested the involvement of several cellular proteins in the specific recognition of the 3' end of the PVX genome, the identity and function of this cellular protein is still not clear. Such viral RNA-cellular protein interactions have been reported for several plus-strand RNA viruses (Huang and Lai, 2001; Kusov et al., 1996; Liu et al., 1997; Nakhasi et al., 1990; Nuesch et al., 1993; Pardigon and Strauss, 1992). Previously, binding of cellular proteins to the 5' end of PVX RNA was reported using EMSA and UV cross-linking analyses (Kim et al., 2002). It was shown that a 54 kDa cellular protein binds to sequences in the 5' region of the PVX genome and that the minimal binding sequences is within the 5' proximal 46 nucleotides of the PVX RNA genome. Since the binding of the cellular protein to the 5' end of the plus-strand RNA and virus replication on inoculated protoplasts were closely co-related, it is tempting to speculate that essentiality of sequences on 5' end of plus-strand RNA is in part due to the binding of the cellular protein. It is possible that different cellular proteins recognize and bind PVX regulatory

elements located at the PVX 5' and 3' ends, and elsewhere on PVX RNAs for virus replication. Future experiments will involve characterization of the each cellular protein interaction with PVX RNA and/or with viral and other cellular proteins.

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