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Role of Intergenic and 3'-Proximal Noncoding Regions in Coat Protein Expression and Replication of *Barley yellow dwarf virus* PAV

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***Barley yellow dwarf virus* PAV (BYDV-PAV) has a 5.7-kb positive-sense single-stranded RNA genome that contains six open reading frames (ORFs). BYDV-PAV produces three subgenomic RNAs (sgRNAs). The largest of which encodes the coat, 17-kDa, and readthrough proteins from two initiation codons. To investigate the role of intergenic and 3'-proximal noncoding regions (NCRs) in coat protein (CP) expression and BYDV-PAV replication, a full-length infectious cDNA of the RNA genome of an Illinois isolate of BYDV-PAV was constructed downstream of the *Cauliflower mosaic virus*-35S promoter. Linear DNA molecules of these cDNAs were infectious, expressed the 22-kDa CP, and produced both genomic RNA and sgRNAs in ratios similar to those observed in protoplasts inoculated with viral RNA. The portion of 5' NCR of sgRNA1 between ORFs 2 and 3 was not required for, but enhanced translation of CP from ORF3. Mutants containing deletions in the NCR downstream of ORF5 failed to replicate in oat protoplasts. These results indicate that an intact 3' NCR is required for BYDV-PAV replication.**

Keywords : BYDV-PAV, *Cauliflower mosaic virus* 35S promoter, deletion analysis, infectious clones.

Barley yellow dwarf virus (BYDV)-PAV is a member of the *Luteoviridae* family of plant viruses. Other monocot-infecting members of the family include BYDVs MAV, RMV, and SGV and *Cereal yellow dwarf virus*-RPV (D'Arcy and Mayo, 1997). BYDV-PAV has a 5.7-kb positive-sense, single-stranded (ss) RNA genome that contains six open reading frames (ORFs) (Miller and Rasochova, 1997). Based on point mutation and deletion analyses, only ORFs 1 and 2, which are thought to encode an RNA-dependent-RNA polymerase, have been shown to be required for replication (Mohan et al., 1995). ORFs 3 and 5 encode the 22-kDa coat protein (CP) and readthrough protein, respectively. ORF4 encodes a 17-kDa protein that has been implicated in virus movement (Chay et al., 1996). The NCR downstream of ORF5 contains a *cis*-acting translational enhancer sequence (3'TE), which facilitates cap-independent translation of ORFs 1 and 2 (Allen et al., 1999; Wang et al., 1997). This region also contains a small ORF (ORF6), which is present in many members of the *Luteoviridae* (Chalhoub et al., 1994). This approximately 700 nt long region, including ORF6, has not been assigned a function in the life cycle of the virus.

BYDV-PAV produces three subgenomic RNAs (sgRNAs). The locations of the transcriptional start sites and promoters have been determined for all three sgRNAs (Koev and Miller, 2000). The largest of the sgRNAs, sgRNA1, encodes the coat, 17-kDa, and readthrough proteins from two initiation codons (Dinesh-Kumar et al., 1992). The two smaller sgRNAs are not known to encode proteins. Sequences in the 5' NCR of an mRNA influence initiation codon selection and overall translation efficiency (Kozak, 1989). The effects of changes in the sequence of nucleotides immediately surrounding the initiation codons of ORFs 3 and 4 have been studied in detail (Dinesh-Kumar and Miller, 1993). It also has been shown that duplication of the intergenic NCR and flanking regions does not interfere with either replication of virus RNA or the synthesis of sgRNA1 (Koev et al., 1999). The 5' NCRs of *Potato virus X* and *Tobacco mosaic virus* act as potent translational enhancers for genes located downstream (Gallie et al., 1987; Smirnyagina et al., 1991). However, the role of the nontranslated leader of sgRNA1, specifically the NCR between ORFs 2 and 3, in CP translation initiation has not been studied.

The goal of this study was to investigate the role of the 5' NCR of sgRNA1 in CP expression and the 3' NCR downstream of ORF5 in the replication of BYDV-PAV RNA. Because of the differences observed in the efficiency of ORF3 translation between *in vitro* and *in vivo* expression systems (Dinesh-Kumar and Miller, 1993), we chose to conduct these experiments *in vivo* using infectious full-length cDNA clones of an Illinois isolate of the PAV strain

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of BYDV (BYDV-PAV-IL) cloned downstream of a *Cauliflower mosaic virus* (CaMV)-35S promoter. By transfecting oat protoplasts with wild-type and mutant clones, we showed that the portion of the 5' NCR of sgRNA1 that is located between ORFs 2 and 3 was not required for the translation of ORF3, but that the 3' NCR contained sequences required for replication of BYDV-PAV genomic RNA (gRNA).

Materials and Methods

Virus and viral RNA purification. BYDV-PAV-IL was purified from infected plants as described previously (D'Arcy et al., 1983). BYDV-PAV-IL genomic RNA was purified from SDS-disrupted virions by sucrose gradient centrifugation (Fouly et al., 1992).

Plasmid constructions. A double-stranded cDNA fragment representing the 3'-terminal 5 kb of the BYDV-PAV-IL genome was synthesized using an oligonucleotide primer complementary to the 3' 20 nt of the viral genome (Fouly et al., 1992) and inserted into the *Xba*I and *Sst*I sites of pTZ19R (Pharmacia, Piscataway, NJ, USA) to produce p19RPAV514. DNA fragments containing the CaMV-35S promoter from pBI221 (Clontech, Palo Alto, CA) and a cDNA fragment representing the 5'-terminal 1 kb of the BYDV-PAV-IL genome were generated and combined by polymerase chain reaction (PCR) (Innis and Gelfand, 1990). pGP11 was made by inserting the 1.8 kb fragment containing the 35S promoter and the 5' region of the BYDV-PAV-IL genome into p19RPAV514 followed by *Pst*I digestion (Fig. 1A).

To construct a plasmid in which most of ORF5 was deleted, two *Apa*I sites were introduced into pGP11 at positions 3479 and 4804 using uracil-substituted ssDNA from pGP11 and a pair of mutant primers (Sambrook et al., *1989*). The mutated plasmid DNA was digested with *Apa*I, removing most of ORF5, and religated to produce pGP115 (Fig. 1A). This plasmid contained a unique *Apa*I site inserted just downstream of ORF3. Clones containing deletions in the NCR upstream of ORF3, pNCR2627, pNCR2744, pNCR2758, pNCR2773, and pNCR2811, were constructed by inverse PCR using a primer that started at position 2858 in conjunction with primers that ended at positions 2744, 2758, 2773, and 2811, respectively (Fig. 2A; Weiner and Costa, 1995). Clones containing deletions downstream of ORF5, pNCR4919, pNCR5111, and pNCR5308, were constructed by inverse PCR using primers that deleted positions 4919-5111, 5111-5308, and 5308-5476, respectively (Fig. 3A).

Transfection of oat protoplasts. Oat protoplasts were prepared from oat suspension cultures as described (Dinesh-Kumar et al., 1992). Approximately one million protoplasts were electroporated with 0.1 µg of genomic BYDV-PAV-IL RNA or 20 µg of *Sma*I-digested cDNA clone. The transfected protoplasts were incubated at room temperature and harvested 48 hr after transfection.

Detection of 22-kDa CP and viral RNAs. Transfected and control oat protoplasts were vortexed briefly with 0.45-mm glass beads (Sigma, St. Louis, MO, USA) and subjected to Western-blot analysis for the detection of 22-kDa CP using a rabbit polyclonal antiserum raised to purified BYDV-PAV-IL (Cheng et al., 1994). Total RNA was extracted from protoplasts (Chomczynski

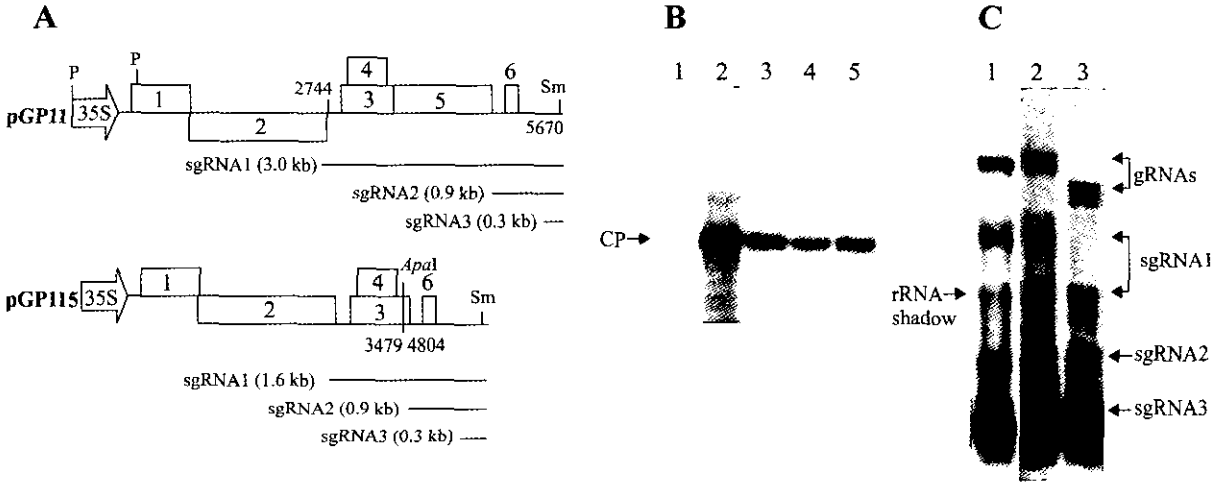

Fig. 1. Infectivity of BYDV-PAV-IL cDNA clones driven by the CaMV-35S promoter. (**A**) pGP11 represents a full-length cDNA of the BYDV-PAV-IL RNA genome joined to the CaMV-35S (35S). The positions of the *Pst*I (P) sites used during cloning and the *Sma*I (Sm) site used to linearize the plasmid prior to transfection are indicated. Clone pGP115 contains a deletion on most of ORF5 (positions 3480-4803) and a unique *Apa I site*. Numbers in open boxes indicate open reading frames. The predicted sizes of the sgRNAs are indicated below each clone. (**B**) Western blot analysis of total protein extracted from protoplasts transfected with BYDV-PAV-IL RNA (lane 3), pGP11 (lane 4), pGP115 (lane 5). Proteins from mock-inoculated protoplasts (lane 1) and purified virus (lane 2) were included as controls. The migration of the 22-kDa CP is indicated by an arrow. (**C**) Northern blot analysis of total RNA extracted from protoplasts transfected with BYDV-PAV-IL RNA, pGP11, and pGP115 (lanes 1-3, respectively). RNAs were separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized to a biotin-labeled probe corresponding to the 3' end of the BYDV-PAV-IL RNA genome. The migrations of the gRNA and sgRNAs are indicated. The position of a shadow caused by the migration of large amounts of ribosomal RNA (rRNA) is indicated to the left of the Northern blot.

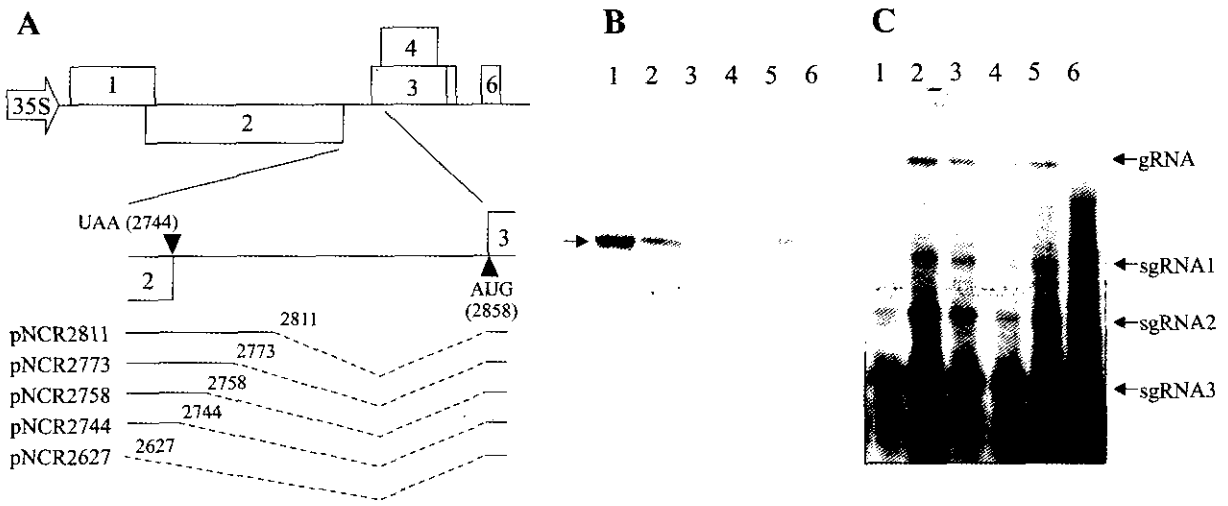

Fig. 2. Analysis of deletion mutants in the 5' NCR of sgRNA1. (**A**) Using pGP115 as a template, a set of deletions was generated in the portion of the 5' NCR of sgRNA1 that corresponded to the intergenic region between ORFs 2 and 3. Deletion clones pNCR2811, pNCR2773, pNCR2758, pNCR2744 and pNCR2627 contained deletions of sequences between positions 2811-2858, 2773-2858, 2758-2858, and 2744-2858, respectively. The 5' and 3' termini of the deletions are indicated in the figure for each clone. (**B**) Western-blot analysis of total protein extracted from the protoplasts transfected with pGP115, pNCR2811, pNCR2773, pNCR2758, pNCR2744 and pNCR2627 (lanes 1-6, respectively). Proteins were separated on 12% SDS-PAGE, transferred to a nylon membrane, and probed with polyclonal antisera to purified BYDV-PAV-IL. The arrow indicates the migration of the 22-kDa CP. (**C**) Northern-blot analysis of total RNA extracted from protoplasts transfected with pGP115, pNCR2811, pNCR2773, pNCR2758, pNCR2744 and pNCR2627 (lanes 1-6, respectively). Total RNAs extracted from transfected protoplasts were separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized to a biotin-labeled probe corresponding to 3' end of RNA genome. The migrations of the gRNA and sgRNAs are indicated.

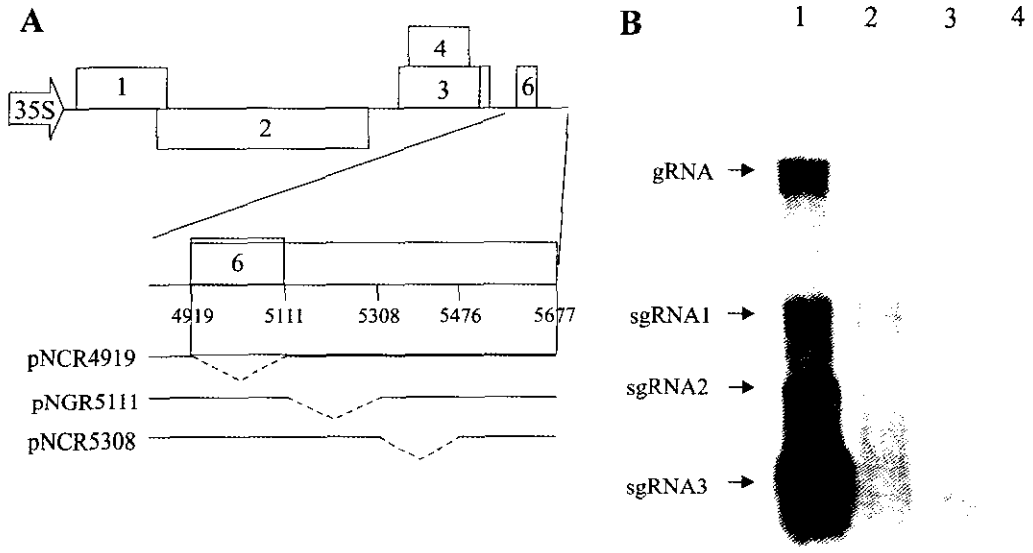

Fig. 3. Analysis of deletion mutants in the 3' NCR downstream of ORF5. (**A**) Using pGP115 as a template, a set of deletions was constructed downstream of ORF5. Clones pNCR4919, pNCR5111, and pNCR5308 contained deletions of sequences between positions 4919-5111, 5111-5308, and 5308-5476, respectively. The end points of the deletions and positions of neighboring ORFs are indicated above the clones. (**B**) Northern-blot analysis of total RNA extracted from protoplasts transfected with pGP115, pNCR4919, pNCR5111, and pNCR5308 (lanes 1-4, respectively). BYDV-PAV-IL RNAs were separated on a formaldehyde-agarose gel, blotted to a nylon membrane, and hybridized to a biotin-labeled probe corresponding to 3' end of RNA genome. The migrations of gRNA and sgRNAs are indicated.

and Sacchi, 1987) and analyzed by Northern-blot hybridization with biotin-labeled probes corresponding to positions 5295-5677 of BYDV-PAV RNA (Chomczynski, 1992; Mertz and Rashtchian, 1994).

Results

Replication of BYDV-PAV RNA synthesized *in vivo* from a CaMV-35S promoter. The infectivity in oat protoplasts of BYDV-PAV-IL cDNA clones driven by the CaMV-35S promoter was tested initially by Western-blot analysis. Because BYDV-PAV CP is expressed from sgRNA1, its expression is dependent upon virus replication (Miller and Rasochova, 1997). The 22-kDa CP was detected in protoplast extracts 48 hr after transfection with linearized plasmid templates. Protoplasts transfected with 20 μg of pGP11 or pGP115 (Fig. 1A) accumulated about the same amount of CP as protoplasts transfected with 0.1 μg of purified BYDV-PAV-IL RNA (Fig. 1B lanes 3-5). Even though pGP115 contained a deletion of most of ORF5, protoplasts transfected with pGP115 accumulated the 22-kDa CP as well as the wild-type clone pGP11. These results are similar to those of Mohan et al. (1995) who showed that partial deletion of ORF5 did not affect the replication of BYDV-PAV in oat protoplasts transfected with *in vitro* transcripts. Using a probe corresponding to the 3' end of BYDV-PAV-IL RNA, Northern-blot analysis of total RNA extracted from oat protoplasts transfected with purified BYDV-PAV-IL RNA detected both genomic and subgenomic viral RNAs (Fig. 1C, lane 1). Protoplasts transfected with pGP11 or pGP115 accumulated BYDV-PAV RNAs in proportions similar to those observed in protoplasts transfected by purified BYDV-PAV-IL RNA (Fig. 1C, lanes 2 and 3). pGP115 produced sgRNAs 2 and 3 of the same size as the wild-type clone, but produced shorter gRNA and sgRNA1 due to the deletion of 1.4 kb in ORF5.

Deletions in the 5' NCR of sgRNA1. To assess the role of the 5' NCR of sgRNA1 in translation initiation of ORF3, a series of clones were generated using pGP115 as a template that contained deletions extending upstream from the initiation codon (Fig. 2A). Protoplasts individually transfected with deletion clones pNCR2811, pNCR2773, pNCR2758, and pNCR2744 accumulated sgRNA1 and 22-kDa CP (Fig. 2B and C, lanes 1-5). As a result of the deletions, the sequence upstream of the initiation codon of ORF3 was different in each clone (Fig. 4). The wild-type clone pGP115 accumulated the most CP. Of the deletion clones, pNCR2811, which contained the smallest deletion, accumulated the most CP. pNCR2744, which contained the largest deletion, produced the least amount of CP (Fig. 2B). Even though the transcription start site for sgRNA1 is at position 2658, it was not possible to produce deletions upstream of position 2744, the position of the termination codon of ORF2, which encodes the viral replicase. For example, protoplasts transfected with clone pNCR2627, which contained a deletion of 120 nucleotides from the 3' end of ORF2, accumulated neither CP nor genomic length RNAs (Fig. 2B and C, lane 6). A purine at position -3 relative to the AUG is sometimes associated with enhanced expression in monocotyledonous plants (Cavener and Ray, 1991). However, the level of CP expression was correlated with the length of the 5' NCR of sgRNA1, but not the sequence upstream of the CP AUG codon. Clone pNCR2773 contained a purine (G) at −3 (Fig. 4), but did not translate as well as either pGP115 or pNCR2811, which contained no or smaller deletions.

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                    -3
                    ↓
pGP115     CACCACTAGAGAGGTGGTGAATGAATTCAG
pNCR2811   CCCTGTACATTAGCTCTCGGATGAATTCAG
pNCR2773   ATCTTAGCTGGGTTTGGGATATGAATTCAG
pNCR2758   TATTAATTACCAAATCTTAGATGAATTCAG
pNCR2744   CACTCACAAAACGAATATTAATGAATTCAG
pNCR2627   ACAGCAAATCGTCGAGGGGAATGAATTCAG
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Fig. 4. *Sequences upstream of the AUG codon of ORF3 in wild-type and deletion clones. The nucleotide sequences of the region upstream of the AUG codon of ORF3 were determined for pGP115, pNCR2811, pNCR2773, pNCR2758, pNCR2744, and pNCR2627. The AUG codon for ORF3 is bold and underlined.* The 'G' residue at position −3 relative to the AUG codon in pNCR2773 (bold and underlined) agrees with the consensus for monocotyledonous plants.

Deletions in the 3' NCR of BYDV-PAV-IL RNA. To determine whether sequences downstream of ORF5 are required for the transcription, translation, and/or replication of BYDV-PAV-IL RNAs, three deletion clones were constructed that contained deletions of nt 4919-5111, 5111-5308, and 5308-5476 (Fig. 3A). None of the resulting mutant clones, pNCR4919, pNCR5111, and pNCR5308, accumulated detectable levels of genome-sized RNAs in protoplasts (Fig. 3B), suggesting that nucleotide sequences downstream of the 3 TE are required for replication and/or translation of BYDV-PAV-IL RNA.

Discussion

In this study, we examined the effects of deletions in the 5' NCR of sgRNA1 on initiation of translation of ORF3 and deletions downstream of ORF5 on the replication of BYDV-PAV-IL gRNA using template RNAs synthesized *in vivo* from linearized plasmids. The full-length cDNA clones driven by the CaMV-35S promoter replicated and expressed viral sequences as shown on Western and Northern-blots of oat protoplast extracts (Fig. 1). Infectious *in vitro* transcripts

synthesized from full-length cDNA clones by bacterial RNA polymerases have been used to study gene functions, virion formation, movement, transcription and translation of members of the *Luteoviridae* and other plant viruses (Ahlquist et al., 1984; Brugidou et al., 1995; Petty et al., 1989; Young et al., 1991; Veidt et al., 1992). However, using the CaMV-35S promoter to synthesize infectious viral RNAs *in vivo*, there is no need to produce *in vitro* transcripts. Similar results with infectious viral RNAs synthesized *in vivo* from the CaMV-35S promoter have been reported by others (Ding et al., 1995; Gal-On et al., 1995; Meyer and Dessens, 1997; Mori et al., 1991).

The infectivity of the initial dose of cDNA was about 1% that of purified BYDV-PAV-IL RNA. This apparently low infectivity of the cDNAs may have been due to the fact that CaMV usually infects dicots. Hence, the CaMV-35S promoter may function less well in oat protoplasts. Schledzewski and Mendel (1994) reported that the expression levels of reporter genes from monocot-specific promoters were up to 15-fold higher than those of the CaMV-35S promoter in maize and barley. However, the CaMV-35S promoter is well characterized and its transcription start site is known (Yamaya et al., 1988). Meyer and Dessens (1997) constructed infectious clones of *Barley mild mosaic bymovirus* using a CaMV-35S promoter coupled with two transcriptional enhancers. The CaMV-35S promoter used in our clones did not contain these enhancer sequences. The amount of BYDV-PAV-IL RNA synthesized from the cDNAs must have been very low and/or short-lived, since genomic length RNAs were never detected from protoplasts transfected with non-replicating clones (Fig. 3). In addition, there may have been processing of nuclear-export barriers that reduced the infectivity of RNAs transcribed by host RNA polymerase. Linear cDNA clones infected protoplasts and produced molar ratios of viral RNAs similar to those observed in plants and protoplasts infected with viruses or RNA, respectively. These results suggest that the infection by DNA clones is biologically equivalent to that by BYDV-PAV-IL RNA in oat protoplasts. Linear DNA molecules were about ten-fold more infectious than circular DNA clones, even when the circular clones contained the NOS terminator at the 3' end of cDNA (data not shown). It is likely that the transcripts from circular DNAs included extra nonviral nucleotides on the 3' end, which greatly reduced the infectivity of the RNA.

BYDV-PAV-IL cDNA clones differed in their accumulation of viral RNAs. pGP115, which contained a nearly complete deletion of ORF5, accumulated higher levels of CPs and BYDV-PAV-IL RNAs than the wild-type clone, pGP11 (Fig. 2B). It is possible that the shorter clone is replicated more rapidly than the full-length genome or that the deletion brought the 3'TE closer to the 5' end of the genome, resulting in an increased level of translation of viral replicase.

The portion of the 5' NCR of sgRNA1 between ORFs 2 and 3 was not required for translation of ORF3 (Fig. 2B and 2C). The wild-type clone pGP115 produced the most CP, but pNCR2744, which retained only the first 77 nt of the 201 nt-long 5' NCR of sgRNA1, accumulated significant levels of the 22-kDa CP (Fig. 2C lane 5). *Potato leafroll virus*, a member of the *Polerovirus* genus, has a 212-nt NCR on sgRNA1. Similar to BYDV-PAV-IL, the deletion of most of its NCR had little effect on the translation of ORF3, but changed the ratios of the products of ORFs 3 and 4 (Juszczuk et al., 2000). It has been suggested that nucleotide sequences flanking the start codon for ORF3 provide a relatively poor context that permits translation of ORF4, which is nested within ORF3 (Dinesh-Kumar and Miller, 1993). Their *in vitro* translation studies suggested that a predicted secondary structure near the AUG of ORF3 was responsible for the differential translation initiation of ORFs 3 and 4. However, *in vivo* translation analysis in this study showed that the efficient translation of ORF3 is largely independent of both sequence context and upstream secondary structure. The 120 nt deletion at the 3' end of ORF2 in pNCR2627, which failed to replicate in protoplasts, likely inactivated the ORF2 product, which has been shown to be required for viral replication (Mohan et al., 1995).

One feature that distinguishes the *Polerovirus* and *Luteovirus* genera of the *Luteoviridae* is the length of the 3' NCR downstream of ORF5 (D'Arcy and Mayo, 1997). Luteoviruses and members of the *Luteoviridae* with luteovirus-like ORFs 1 and 2, e.g., *Soybean dwarf virus* (Rathjen et al., 1994), have long 3' NCRs, often over 800 nt in length. In contrast, poleroviruses have relatively short 3' NCR, usually less than 200 nt in length. None of the mutants in the 3' NCR of BYDV-PAV-IL (pNCR4919, pNCR5111, or pNCR5308) produced detectable amounts of genomic-length RNAs indicating that this region is required for replication and/or expression of viral RNA. Because pNCR5308 contained a deletion of 260 nt near the 3' terminus of the gRNA, it may have failed to replicate due to a defect in synthesis of (−) stranded RNA. The clone, pNCR4919, contained a deletion of the entire ORF6 including 6 nt of 3'TE (Fig. 3B, lane 2), which has been shown to be lethal. Based on mutagenesis experiments (AUG → AUC for ORF6), Mohan et al. (1995) suggested that ORF6 does not encode a protein, but it is likely that the nucleotide sequences of ORF6 may function as *cis*-acting elements in the replication of BYDV-PAV. This hypothesis is supported by the fact that the predicted size and sequence of the protein product from ORF6 varies largely among different BYDV-PAV isolates (Chalhoub et al., 1994). However, most of the

coding region of ORF6 was not included in 3' TE, suggesting that it may function as cis-acting sequences that are required for replication in addition to the 3' TE. 136-nt deletion of downstream ORF6 in pNCR5111 abolished the replication of the virus in the protoplasts (Fig. 3B lane 3). Taken together, these deletions likely removed previously unidentified *cis*-acting sequences necessary for replication or translation.

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