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In vitro mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: Evidence that a domain of the 75-kDa readthrough protein is important for efficient virus assembly

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ABSTRACT **RNA 2 of the multipartite genome of beet necrotic yellow vein virus carries the cistron for 21-kDa viral coat protein at its 5′ extremity. The amber termination codon of the coat protein cistron undergoes suppression ≈10% of the time so that translation continues into an adjacent 54-kDa open reading frame, yielding a 75-kDa readthrough protein. The roles of coat protein and the readthrough protein in infection were investigated with biologically active transcripts of RNA 2. Much of the coat protein cistron of the RNA 2 transcript could be deleted without interfering with viral replication and local lesion formation on leaves, although formation of the rod-shaped virions did not occur. Mutants in which the amber coat protein termination codon was replaced with an ochre codon or a tyrosine codon were also viable. The ochre codon was suppressed both *in vitro* and *in planta*. The mutant containing the tyrosine substitution produced only the 75-kDa readthrough protein and was deficient in viral assembly. Deletions in the 54-kDa readthrough domain were also viable *in planta* but had different effects on virus assembly. A deletion in the C-terminal portion of the readthrough domain did not interfere with RNA packaging but, unexpectedly, deletions in the N-terminal portion were assembly deficient, although 21-kDa coat protein was produced *in planta*. Thus, the 75-kDa protein can apparently intervene in virion assembly even though it has not been detected in purified virions.**

Beet necrotic yellow vein virus (BNYVV), the agent of rhizomania disease of sugarbeet (1), has a multipartite (+)-sense RNA genome (2) consisting usually of four, but in some Japanese isolates of five (3), distinct RNA components (4–6). In BNYVV virions, the RNA components are packaged in long, rigid nucleoprotein helices [superficially resembling tobacco mosaic virus (TMV) particles] consisting of a single RNA molecule and multiple copies of the 21-kDa coat protein. The two longest viral RNAs, RNAs 1 and 2, encode basic functions involved in viral multiplication in all hosts (7–9). RNAs 3 and 4, on the other hand, feature specifically in the infection process under field conditions (8–11). Thus, BNYVV RNAs 1–4 are always present in naturally infected sugarbeet roots but infection of leaves of *Chenopodium quinoa* or *Tetragonia expansa* by mechanical inoculation requires only RNAs 1 and 2 (7, 10, 12–14).

RNA 1 (6.8 kilobases) is necessary and sufficient for viral RNA replication in protoplasts (unpublished observations) and thus encodes basic replicase functions. RNA 2 (4.7 kilobases) contains six open reading frames (ORFs), of which the first encodes the 21-kDa viral coat protein (5). This cistron terminates with an amber (UAG) termination codon followed by an in-phase ORF of 54 kDa (see Fig. 1) referred to as the readthrough domain. Suppression of this UAG occurs both *in vitro* (15) and *in vivo* (16) ≈10% of the time that ribosomes encounter it. The resulting fusion protein (P75) has a predicted mass of 75 kDa and an apparent mass of 85 kDa during PAGE (5, 15). Similar C-terminal extensions of viral coat proteins by readthrough into adjacent ORFs have been noted for soil-borne wheat mosaic virus (17) and luteoviruses (18–20). Bahner *et al.* (21) have detected the 56-kDa coat protein readthrough species of potato leafroll luteovirus in virions and speculate that the readthrough protein may be involved in interactions between the virus and its aphid vector. Extension of the putative viral RNA-dependent RNA polymerase by termination codon suppression has also been reported for a number of viruses, including TMV (see ref. 22 for review).

Biologically active transcripts corresponding to BNYVV RNAs 1 and 2 are available (14). In this paper, we have used mutated RNA 2 transcripts to investigate the effect of changes in the viral coat protein and P75 upon replication and encapsidation of viral RNA during leaf infections. An unexpected finding was that a short deletion in the coat protein proximal portion of the readthrough domain of P75 interfered with accumulation of BNYVV virions during infection.

MATERIALS AND METHODS

cDNA Clones. The BNYVV RNA 1 and RNA 2 transcription vectors pB15 and pB218 and the plasmid pBF14, which carries cDNA corresponding to the 3′-terminal half of RNA 2, have been described (14). Deletions were introduced into pB218 by conventional techniques (23) involving digestion with restriction enzymes or mild treatment of linearized DNA with BAL-31 or exonuclease III followed by recircularization with T4 DNA ligase. The linker inserted during construction of pB218-ΔM1 (see *Results and Discussion*) had the sequence 5′-AGATCTCCCGGGCTCGAG-3′. Oligonucleotide-directed mutagenesis (24) of the coat protein amber termination codon was carried out on a subclone containing residues 1–2077 of RNA 2. After mutagenesis, plasmids containing the desired substitutions were identified by sequence analysis and introduced on an *Nci* I/*Mlu* I restriction fragment (nucleotides 668–1138) back into pB218 to yield pB218-TAA and pB218-TAT. The accuracy of the various constructs was confirmed by restriction enzyme digestion and sequence analysis (25).

***In Vitro* Transcription.** Full-length cDNA of RNA 2 was obtained by ligating *Bst*XI/*Bam*HI-cut pB218 or its mutated derivatives to the 3′-terminal *Bst*XI/*Sal* I fragment derived

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Abbreviations: BNYVV, beet necrotic yellow vein virus; TMV, tobacco mosaic virus; ORF, open reading frame.

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from pBF14 (14). Transcripts were synthesized and inoculated to leaves of *C. quinoa* as described (14). Transcripts were translated in rabbit reticulocyte lysate (14) and [^{35}S]methionine-labeled translation products were visualized by autoradiography after PAGE.

Detection of Viral RNA and Coat Protein. Total RNA was isolated from inoculated leaves 8 days postinoculation with either protocol Pol or protocol TM (26). Viral RNAs were detected in the RNA extracts by Northern hybridization with ^{32}P-labeled antisense viral transcripts produced *in vitro* used as probes (8). Total soluble protein was extracted (14) from inoculated leaves 8 days postinoculation, denatured, and separated by PAGE in 10–12.5% polyacrylamide gels. After electrophoretic transfer to nitrocellulose, viral coat protein-related polypeptides were detected on Western blots by using one of two antisera: a rabbit polyclonal antiserum raised against purified virions (a gift of O. Lemaire, INRA, Colmar, France) or a rabbit polyclonal antiserum raised against a fusion protein containing the N-terminal portion of the bacteriophage λcI protein and the C-terminal portion of the 21-kDa viral coat protein (residues 121–189), expressed in the vector PEA305Δ*Hin*dIII-2 (27) and purified from bacteria as described (16). The same procedure was used to raise an antiserum against a fusion protein containing the C-terminal portion (nucleotides 1139–2217) of the 54-kDa readthrough domain.

RESULTS AND DISCUSSION

Mutations in the Coat Protein Cistron. In a previous study, we described an RNA 2 sequence variant (pB25) with a point mutation resulting in replacement of arginine by serine at position 119 of the coat protein (14). This substitution did not alter transcript infectivity as measured by the ability of transcript carrying the mutation to provoke local lesion formation when inoculated along with wild-type RNA 1 transcript to leaves of *C. quinoa*. Viral RNA and coat protein accumulated within the lesions but assembly of virions did not take place (14). This finding indicated that spread of infection to neighboring cells does not require packaging of viral RNA. It may be argued, however, that since the mutation in pB25 involved only a single base, other functions that might map to the coat protein cistron could have escaped detection.

In this paper, we have tested the effects of other mutations within the coat protein cistron. Plasmid pB218, which contains the 5′-terminal 2715 residues of RNA 2 cloned downstream of a bacteriophage T7 RNA polymerase promoter (14), was the starting material. Mutants (Fig. 1) were created by eliminating sequences between neighboring restriction sites in the coat protein cistron (mutants pB218-A, pB218-B, and pB218-ΔS) or by filling in an *Nco* I site (nucleotide 668) to create a +4-nucleotide frameshift (pB218-N) with a stop codon at nucleotide 687. Full-length RNA 2 transcripts carrying each of these mutations were prepared as described (14). Transcripts of pB218-A, -B, and -N directed synthesis of *in vitro* translation products of the expected size (data not shown). No effort was made to characterize the coat protein-related translation product of pB218-ΔS transcript (predicted size, ≈7 kDa) because of the difficulty of resolving polypeptides of this size by conventional PAGE.

The RNA 2 transcripts were inoculated to leaves of *C. quinoa* along with wild-type RNA 1 transcript and the inoculated leaves were examined for the presence of local lesions 8 days later. Mutants pB218-A, pB218-B, pB218-N, and pB218-ΔS produced numerous local lesions (20–80 lesions per leaf). The lesions obtained with pB218-B were similar to the mild chlorotic lesions characteristic of an infection with wild-type RNAs 1 and 2, while the lesions produced by pB218-ΔS and pB218-N were of the same type but slightly smaller. Mutant pB218-A produced necrotic local lesions. Possibly, the altered coat protein produced by this mutant is cytotoxic.

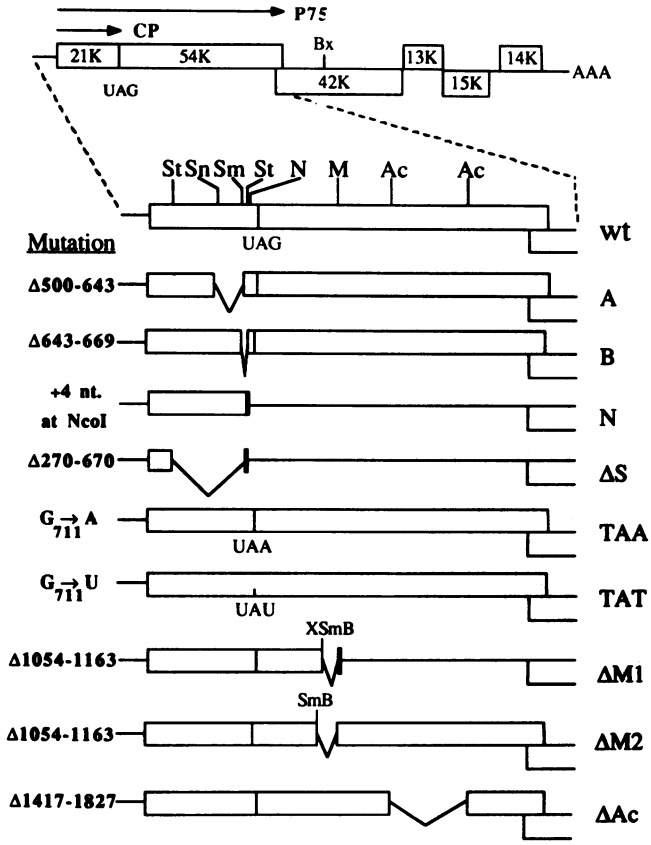

FIG. 1. Structure of wild-type (wt) BNYVV RNA 2 and mutants. Open rectangles represent ORFs and arrows represent coat protein (CP) and P75 translation products. Position of the suppressible UAG termination codon (nucleotides 709–711) of the coat protein cistron is indicated. Deletions are represented by broken lines with the coordinates of each deletion (in nucleotides) given on the left. C-terminal missense sequences resulting from frameshift are represented by solid rectangles. Restriction sites referred to in the text are *Bst*XI (Bx), *Sty* I (St), *Sna*BI (Sn), *Sma* I (Sm), *Nco* I (N), *Mlu* I (M), *Acc* I (Ac), *Xho* I (X), and *Bgl* II (B).

Coat protein-related polypeptides of approximately the predicted size were detected on immunoblots of total soluble protein for mutants pB218-A and pB218-B (≈16 kDa and ≈20 kDa, respectively; Fig. 2, lanes 4 and 5). In addition to the ≈20-kDa species, mutant pB218-B also produced a coat protein-related polypeptide slightly smaller than wild-type P75. This species was recognized specifically by antiserum raised against a fusion protein containing the C-terminal two-thirds of the 54-kDa readthrough domain (data not shown), providing concrete evidence that the band in question corresponds to the readthrough protein. In the case of pB218-A, discrete high molecular weight products were obscured by a background smear presumably caused by aggregation of coat protein-related polypeptides. This aggregation may be a consequence of the heavy necrosis produced by infection with this mutant. pB218-N, on the other hand, should have produced a readily visible polypeptide of 19 kDa, although no such species was detected (lane 6). Possibly, the pB218-N product is unstable *in vivo* because, unlike pB218-A and pB218-B, it does not retain the normal coat protein C terminus. Finally, it should be noted that the signal intensities of the coat protein-related polypeptides on the Western blot do not necessarily reflect the relative amounts of each peptide present because epitopes may have been eliminated by the mutations.

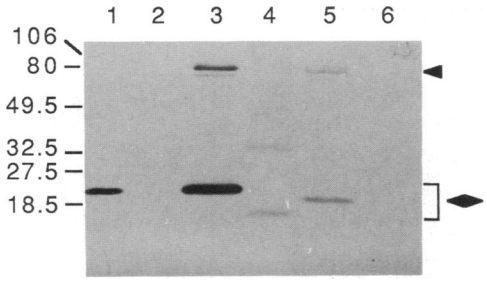

FIG. 2. Immunodetection of BNYVV coat protein-related polypeptides in *C. quinoa* leaves infected with RNA 1 transcript plus the following RNA 2 transcripts: wild-type (lane 3), mutant pB218-A (lane 4), mutant pB218-B (lane 5), mutant pB218-N (lane 6). Lane 1, 10 ng of purified BNYVV; lane 2, protein extracted from healthy tissue. After PAGE, proteins were electroblotted to nitrocellulose and detected by Western hybridization with antiserum raised against the coat protein–cI fusion protein. Mobility of molecular mass (in kDa) markers is given on the left. Diamond indicates position of 21-kDa coat protein and related polypeptides and triangle indicates position of P75. Band in lane 4 indicated by the small circle has the size expected for a dimer of the deleted coat protein species.

In a second experiment, *C. quinoa* leaves infected with the RNA 2 mutants described above were homogenized either in Pol buffer followed by immediate phenol extraction or in TM buffer followed by incubation at 37°C for 30 min before phenol extraction (14, 26). It has been shown previously that the former procedure (protocol Pol) yields both encapsidated and free viral RNA, while protocol TM yields only encapsidated viral RNAs, with nonencapsidated RNA being degraded during incubation of the homogenate at 37°C (14, 26). Viral RNA was abundantly present in the RNA extracted by protocol Pol from the leaves inoculated with mutants pB218-A, pB218-B, pB218-N, and pB218-ΔS (Fig. 3*A*, lanes 2–5) but was not detected when the tissue was carried through the TM extraction protocol (Fig. 3*B*, lanes 2–5). We conclude that replication of BNYVV RNAs in leaves and local lesion formation can occur even when substantial segments of the coat protein cistron are missing. Evidently BNYVV, like TMV (28, 29) and tobacco rattle virus (30) but unlike the bromoviruses (31, 32), does not require a functional coat protein and/or virion formation for cell-to-cell movement.

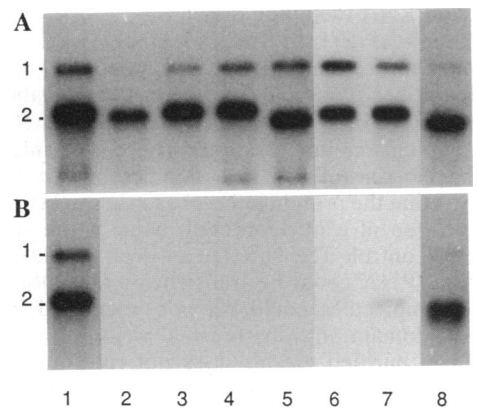

FIG. 3. Detection of BNYVV RNAs 1 and 2 by Northern hybridization of total RNA from *C. quinoa* leaves infected with RNA 1 transcript plus transcript of wild-type or mutant RNA 2. (*A*) RNA was extracted by protocol Pol (both encapsidated and nonencapsidated viral RNAs isolated). (*B*) RNA was extracted by protocol TM (only encapsidated viral RNA isolated). RNA 2 transcript included in the inoculum was wild type (lane 1), mutant pB218-A (lane 2), mutant pB218-B (lane 3), mutant pB218-N (lane 4), mutant pB218-ΔS (lane 5), mutant pB218-ΔM1 (lane 6), mutant pB218-ΔM2 (lane 7), and mutant pB218-ΔAc (lane 8). Positions of wild-type RNAs 1 and 2 are indicated on the left.

Furthermore, because of frameshift, pB218-N and pB218-ΔS terminate translation prematurely and should not produce a readthrough product; indeed, no product related to the 54-kDa ORF could be detected in Western blots of protein from the infected tissue (data not shown). Thus, expression of the readthrough domain is not required for infection either.

Several RNA 2 mutants with more extended deletions in the coat protein cistron were also created by mild exonuclease III digestion of *Sty* I-cut pB218 followed by recircularization. The smallest such deletion characterized (nucleotides 182–794 eliminated) removed all but the first 37 nucleotides of the coat protein cistron as well as 83 nucleotides following the coat protein termination codon. This mutant, as well as mutants with more extensive deletions in the same region, was not viable (data not shown). The failure of such mutants to replicate is probably due to elimination of 5′ proximal cis essential sequences required for interaction of RNA 2 with viral replicase or for postreplicational RNA stability. In BNYVV RNA 3, such cis essential sequences occupy a domain of ≈300 nucleotides at the 5′ terminus (26).

Modification of the Coat Protein Cistron Termination Codon. Two constructs were designed to test the effect of modification of the coat protein amber termination codon on infectivity. In pB218-TAA (Fig. 1), the UAG was replaced by an ochre (UAA) stop codon, a codon that, so far, has not been found to be naturally suppressible (22). In a second construct, pB218-TAT (Fig. 1), the UAG was replaced by the tyrosine codon UAU. This codon was chosen by analogy with the situation in TMV, where misreading of the amber termination codon of the 126-kDa putative viral replicase by the host $tRNA^{Tyr}$ is responsible for formation of a 183-kDa readthrough product (33).

Upon translation in rabbit reticulocyte lysate, transcripts of pB218-TAT produced only P75 (Fig. 4*A*, lane 1). The pB218-TAA transcript directed synthesis of both coat protein and P75, although the amount of P75 synthesized relative to coat protein was somewhat lower than that observed with the wild-type pB218 transcript (compare lanes 2 and 3). When inoculated to leaves along with RNA 1 transcript, RNA 2 transcripts containing each of the two mutations were infectious, as judged by the appearance of local lesions on the inoculated leaves and the presence of viral RNA on Northern blots of extracted leaf RNA (data not shown). Furthermore,

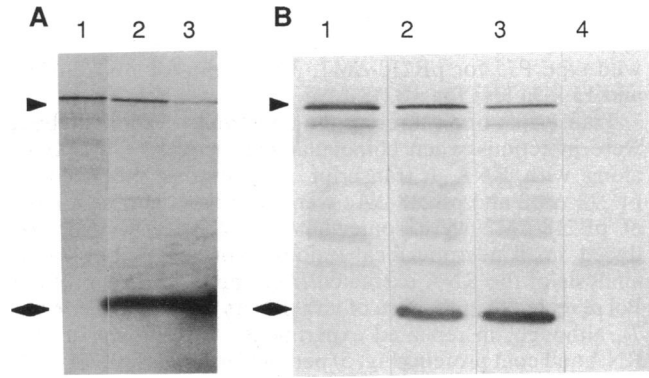

FIG. 4. Effect of mutations in the coat protein termination codon. (*A*) PAGE of [^{35}S]methionine-labeled translation products obtained in rabbit reticulocyte lysate programed with transcript of mutant pB218-TAT (lane 1), wild-type RNA 2 (lane 2), and mutant pB218-TAA (lane 3). (*B*) Immunodetection of coat protein and P75 in *C. quinoa* leaves infected with RNA 1 transcript plus transcript of mutant pB218-TAT (lane 1), wild-type RNA 2 (lane 2), and mutant pB218-TAA (lane 3). Lane 4 contains protein extracted from healthy tissue. Western hybridization was performed as described in Fig. 2 except that antiserum raised against purified BNYVV virions was used. Diamonds and triangles indicate positions of coat protein and P75, respectively.

for mutant pB218-TAA, the progeny viral RNA was correctly packaged into virions as judged by its resistance to nucleolytic degradation in crude extracts. The progeny RNA of mutant pB218-TAT, on the other hand, was degraded under the same circumstances (data not shown). Thus, apparently, the coat protein sequence is not competent for virion assembly when it is present *in planta* uniquely as part of P75. Western blot analysis of total soluble protein from the lesions confirmed that only P75 was produced for mutant pB218-TAT (Fig. 4*B*, lane 1), while in the case of pB218-TAA, in which assembly proceeded normally, both P75 and coat protein accumulated in proportions similar to those observed with wild-type RNA 2 (compare lanes 2 and 3).

Direct sequence analysis of pB218-TAA virion RNA in the vicinity of the mutation revealed that the ochre termination codon was still present in the progeny (data not shown). The mechanism by which this ochre codon is suppressed remains to be discovered. In particular, it is not known whether the 5′-GΨA-3′ anticodon of the $tRNA^{Tyr}$ responsible for suppression of the UAG of TMV (33) can also misread UAA. Partial inhibition of translational readthrough *in vitro* was also observed when the UAG termination codon of the TMV 126-kDa protein was replaced with UAA (34). This mutant was also viable *in planta* but the relative amounts of the 126-kDa species and the 183-kDa readthrough product in the infected tissue were not measured.

Deletions in the Readthrough Domain. A short deletion was introduced into the coat protein proximal portion of the readthrough domain by BAL-31 digestion of *Mlu* I-linearized pB218. After BAL-31 treatment, the plasmid DNA was circularized with ligase in the presence of an 18-nucleotide synthetic linker containing *Bgl* II, *Sma* I, and *Xho* I sites. A clone (pB218-ΔM1) was selected with a deletion of 109 nucleotides of the RNA 2 sequence and that had incorporated a linker at the site of deletion (Fig. 1). Because of frameshift, the ORF of the readthrough domain distal to the deletion is lost in pB218-ΔM1. A second construct, pB218-ΔM2, was created by filling in the unique *Xho* I site in the linker. The resulting addition of 4 nucleotides restores the ORF (Fig. 1). Finally, a third mutant, pB218-ΔAc (Fig. 1), with a 411-nucleotide deletion in the C-terminal portion of the readthrough domain, was created by eliminating the sequence between the two cDNA insert *Acc* I sites. Cell-free translation (data not shown) of transcripts of the three mutants produced coat protein plus shortened readthrough proteins of approximately the predicted size—i.e., 38 kDa less than wild-type P75 for pB218-ΔM1, 3 kDa less for pB218-ΔM2, and 15 kDa less for pB218-ΔAc.

Transcripts of all three of the constructs described above were infectious when coinoculated to leaves of *C. quinoa* along with RNA 1 transcript. The lesions produced by pB218-ΔM2 and pB218-ΔAc were wild type, although those of pB218-ΔM2 were somewhat smaller. pB218-ΔM1 produced small necrotic local lesions. Northern hybridization analysis of the RNA extracted from the lesions by protocol Pol revealed the presence of viral RNAs (Fig. 3*A*, lanes 6 and 7), although, in repeated experiments, the amount of viral RNA and coat protein (Fig. 5) per local lesion for pB218-ΔM1 and -ΔM2 was 3–5 times lower than in controls with wild-type transcript. This difference may be due at least in part to the smaller local lesion size for these mutants.

The progeny viral RNA of mutant pB218-ΔAc was resistant to nuclease degradation in crude extracts (Fig. 3*B*, lane 8). Unexpectedly, however, the progeny RNA of mutant pB218-ΔM1 was sensitive to nuclease degradation when subjected to the TM extraction protocol (lane 6), and no virions were detected in leaf dips prepared from infected tissue, indicating that viral RNA is not properly packaged. Similar results were obtained with the progeny RNA of pB218-ΔM2, although in some experiments a weak signal of encapsidated RNA was

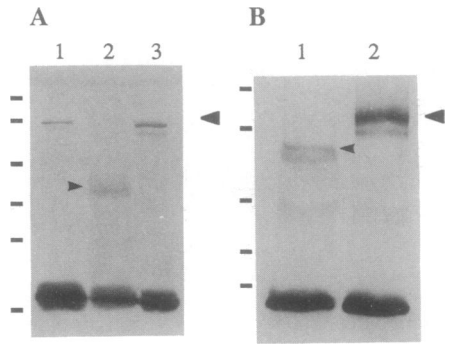

FIG. 5. Immunodetection of coat protein and P75-related species in protein extracted from *C. quinoa* leaves infected with RNA 1 plus RNA 2 transcripts carrying deletions in the P75 readthrough domain. (*A*) RNA 2 transcript in the inoculum was wild type (lane 1), pB218-ΔM1 (lane 2), or pB218-ΔM2 (lane 3). Four times more material was loaded in lanes 2 and 3 as in lane 1. (*B*) RNA 2 transcript in the inoculum was pB218-ΔAc (lane 1) or wild type (lane 2). Western hybridization was performed as described in Fig. 2. Diamond indicates position of coat protein and triangles indicate position of P75 or related species (for the mutants). Mobilities of molecular mass markers (as in Fig. 2) are shown on the left.

detected (lane 7). The inefficient packaging of the viral RNAs in pB218-ΔM1 and -ΔM2 is not due to failure to synthesize viral coat protein, as coat protein was readily detected on Western blots of soluble protein extracted from the local lesions (Fig. 5).

The foregoing observations suggest that P75, and in particular the coat protein proximal portion of the readthrough domain, is required for efficient assembly of BNYVV virions. An alternative possibility, that the debilitated packaging associated with pB218-ΔM1 and -ΔM2 is a consequence of interference of the altered readthrough proteins with assembly rather than a positive role for wild-type P75, is ruled out by experiments with a mutant (pB218-TS) in which the coat protein amber termination codon was converted to UAA and reinforced by two additional termination codons (UAA UAA UAG). No detectable readthrough of the triple stop codon could be detected during *in vitro* translation experiments with pB218-TS transcript or in leaves infected with the mutant but, during such infections, pB218-TS displayed impaired packaging of viral RNA (data not shown).

Participation of P75 in virus assembly was somewhat unexpected because the protein was not detected in purified virus preparations by serological techniques capable of detecting one copy of P75 per virus particle (U. Niesbach-Klösgen, personal communication). There is no information concerning the role of P75 in assembly, but an attractive possibility is that the protein intervenes in assembly initiation since nucleation of a nucleoprotein helix is the step most amenable to control. The putative association between P75 and the viral RNA could be transient or the initiating P75 molecule or molecules could become integrated, via their coat protein domain, into the finished virion. The failure to detect P75 in purified virions does not rule out the latter possibility, because the readthrough domain would almost certainly protrude from the nucleoprotein helix and would probably be sensitive to proteolytic attack in the course of virus purification.

Recent evidence indicates that P75 plays a role in virus–vector interactions. Tamada and Kusume (35) have described two BNYVV RNA 2 deletion mutants that appeared spontaneously during serial passage of virus on leaves. The mutant RNA 2s contained deletions of ≈500 and ≈600 nucleotides and, in *in vitro* translation experiments, directed synthesis of shortened readthrough proteins, of ≈67 and ≈58 kDa, respectively (35). The translated coat protein was of

normal size. The deletions in these mutants have been shown to fall within the C-terminal portion of the readthrough domain (T. Tamada, personal communication). Virus isolates containing the aforementioned deleted RNA 2 species are not transmissible by the natural vector of BNYVV, the soil-borne fungus *Polymyxa betae* (35). We predict that pB218-ΔAc will also prove to be nontransmissible. Evidently, a role for P75 in the viral assembly process represents an elegant means of ensuring that at least a portion of the virions in infected tissue will contain or be associated with the vector transmission factor.

The foregoing observations have established that BNYVV RNA 2 can tolerate extensive deletions within the viral coat protein and P75, including mutations that interfere with virus assembly, without inhibiting the ability of the virus to replicate and form local lesions on leaves. Preliminary experiments indicate, however, that mutants that do not package viral RNA do not spread efficiently from inoculated to upper leaves of spinach, a systemic host for BNYVV. We will show elsewhere that mutations in other ORFs of RNA 2 are lethal for whole leaf infections, probably because they do not allow cell-to-cell movement of the virus (unpublished data).

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