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## **Cloning of Lettuce Necrotic Yellows Virus RNA and Identification of Virus-specific Polyadenylated RNAs in Infected *Nicotiana glutinosa* Leaves**

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### SUMMARY

The negative-stranded genomic RNA of lettuce necrotic yellows virus (LNyV) was isolated and estimated by denaturing agarose gel electrophoresis to consist of about 13000 nucleotides (nt). Hybridization of randomly labelled LNyV RNA to poly(A)<sup>+</sup> RNA from infected *Nicotiana glutinosa* plants revealed the presence of five distinct complementary RNA species (cRNAs) ranging in size from 900 to 6450 nt. Individual recombinant cDNA clones derived from LNyV RNA hybridized to four of these cRNA species. Each cRNA contained unique sequences which together represented about 95% of the viral genome. From their size range and sequence complexity, it is assumed that the cRNAs represent messenger RNAs encoding the five LNyV structural proteins. The mRNA for the nucleocapsid protein is cRNA 3 because a recombinant cDNA expression vector clone containing sequences which hybridize to cRNA 3 produced a polypeptide which was detected with a monoclonal antibody specific for the nucleocapsid protein.

### INTRODUCTION

Lettuce necrotic yellows virus (LNyV) is the type member of subgroup 1 of the plant rhabdoviruses (Matthews, 1982), viruses that are characterized by their cytoplasmic site of assembly. Their bacilliform virus particles share several structural properties with other plant and animal rhabdoviruses (Jackson *et al.*, 1987). LNyV has a non-segmented negative-sense ssRNA genome with a  $M_r$  of about  $4.4 \times 10^6$  (Francki & Randles, 1973). Purified preparations of LNyV contain three major structural proteins (Dietzgen & Francki, 1988) which, following the nomenclature of Wagner *et al.* (1972), have been identified as the envelope glycoprotein G (approx. 78K), the nucleocapsid protein N (approx. 57K) and the matrix protein M (approx. 19K). In electropherograms stained with silver, up to 17 minor bands were also detected (Dietzgen & Francki, 1988), but most of these were shown to be *in vitro* degradation products or aggregates of the G and N proteins. A large L protein (approx. 190K) and an NS protein (approx. 38K) have been tentatively identified (Dietzgen & Francki, 1988) as putative constituents of the viral RNA transcriptase. Although the biological, epidemiological and structural properties of LNyV have been extensively studied (Francki *et al.*, 1989), little is known about its genome organization or replication strategy. Only two plant rhabdovirus genomes, those of sonchus yellow net virus (SYNV) and sowthistle yellow vein virus (SYVV), have been studied in any detail (Rezaian *et al.*, 1983; Heaton *et al.*, 1987*b*; Zuidema *et al.*, 1986, 1987; Stenger *et al.*, 1988). Both of these viruses accumulate in perinuclear spaces and hence unlike LNyV, are members of plant rhabdovirus subgroup 2.

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This communication reports the first characterization of the genome of a subgroup 1 plant rhabdovirus and describes the molecular cloning of LNYV RNA and the use of recombinant plasmid clones to identify virus-specific RNAs induced in infected *Nicotiana glutinosa* plants. We have also identified the N protein gene mRNA by immunological analysis of fusion proteins derived from one of the cDNA clones.

#### METHODS

*Isolation of viral genomic RNA.* *Nicotiana glutinosa* plants were inoculated mechanically with an LNYV isolate from garlic with biological properties similar to those of the S.E.3 isolate (Stubbs & Grogan, 1963) and grown under glasshouse conditions for 8 to 12 days. The virus was purified by Celite filtration, differential centrifugation and calcium phosphate gel column chromatography (Francki *et al.*, 1989). LNYV RNA was extracted as described by Hunter *et al.* (1986) and the structural proteins were recovered from the phenol phase by precipitation with 5 volumes of 100 mM-ammonium acetate in methanol (Hurkman & Tanaka, 1986).

*Isolation of RNA from healthy and LNYV-infected N. glutinosa.* RNA was isolated as described by Rezaian *et al.* (1983) and fractionated by oligo(dT)-cellulose (type 7, Pharmacia) column chromatography into poly(A)<sup>+</sup> and poly(A)<sup>-</sup> RNA fractions. The procedure described by Jacobson (1987) was followed, but NaCl was replaced by LiCl. The RNA concentration was estimated spectrophotometrically assuming  $A_{260} 25 = 1$  mg/ml.

*Construction of cDNA clones.* First-strand cDNA was synthesized from LNYV genomic RNA using Moloney murine leukaemia virus reverse transcriptase. Enzymes were purchased from Bethesda Research Laboratories (BRL). The second-strand cDNA was synthesized using *Escherichia coli* DNA polymerase I alone, followed by *E. coli* RNase H (D'Alessio & Gerard, 1988). Double-stranded cDNA was poly(C)-tailed with dCTP using terminal deoxynucleotide transferase. The homopolymer-tailed ds cDNA was then annealed to an equimolar amount of dGTP-tailed pUC9 plasmid (Pharmacia) and used to transform *E. coli* MAX Efficiency DH5 $\alpha$  competent cells (BRL) by the standard heat-shock method (Maniatis *et al.*, 1982). Ampicillin-resistant, white colonies were selected and screened for LNYV-specific inserts by colony hybridization (Grunstein & Hogness, 1975) to end-labelled (Rezaian *et al.*, 1983) LNYV RNA random fragments. Plasmid DNA suitable for <sup>32</sup>P labelling or restriction endonuclease analysis was isolated by the rapid boiling method of Holmes & Quigley (1981) as described by Maniatis *et al.* (1982). The insert sizes were determined by agarose gel electrophoresis of *Pst*I-cut plasmid and analysed by Southern hybridization (Maniatis *et al.*, 1982) using end-labelled random fragments of LNYV RNA as probe.

*Northern blot hybridization.* RNA samples were denatured with glyoxal (Cover *et al.*, 1981), separated by 1.5% agarose (BRL; Ultrapure) gel electrophoresis and transferred to nitrocellulose membranes as described by Thomas (1980). Hybridization reactions were carried out at 42 °C essentially as described by Wahl *et al.* (1979), except that dextran sulphate was omitted from the solutions. Tobacco mosaic virus (TMV) and brome mosaic virus (BMV) RNAs used as size markers were hybridized with <sup>32</sup>P-labelled cDNA probes prepared as described by Gustafson *et al.* (1982). RNA size standards (BRL) were probed with nick-translated lambda DNA as recommended by the supplier.

*Subcloning and expression of cloned cDNA.* LNYV cDNA sequences were fused in phase with the  $\beta$ -galactosidase gene in the expression vector series pUR290 to 292 (Ruther & Muller-Hill, 1983) and transformants were identified by screening with LNYV-specific polyclonal antibodies (Helfman & Hughes, 1987). Bacterial colonies were grown in the presence of 500  $\mu$ M-IPTG to induce the expression of fusion proteins. Sedimented bacteria were resuspended in electrophoresis sample buffer (Laemmli, 1970), boiled, vortexed to shear DNA, and clarified by centrifugation. Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Polypeptides to be analysed by immunoblotting were electrophoretically transferred to nitrocellulose membranes (Towbin *et al.*, 1979). Residual binding sites on the blot were blocked with TBS (50 mM-Tris-HCl pH 7.4, 200 mM-NaCl) containing 12.5% (w/v) powdered skim milk and 5% (v/v) newborn calf serum (blocking buffer). Polyclonal antiserum to LNYV (McLean *et al.*, 1971) was used at a 1:1000 dilution and monoclonal antibody MAb-N (Dietzgen & Francki, 1988) at 1  $\mu$ g/ml. Goat anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad) were used at a 1:1000 dilution. Blots were developed with 4-chloro-1-naphthol. Antibodies and conjugate were diluted in blocking buffer containing 5 units/ml heparin (Dietzgen & Francki, 1986). All steps were incubated at room temperature for 1 h. The membranes were washed four times for 10 min each in TBS between each step.

*Production of M13 hybridization probes.* LNYV cDNA sequences were subcloned in opposite orientations into phage M13mp19 and ssDNA was purified (Dale *et al.*, 1985). Single-strand-specific probes were synthesized as described by Hu & Messing (1982) using an M13 hybridization probe primer (New England Biolabs).

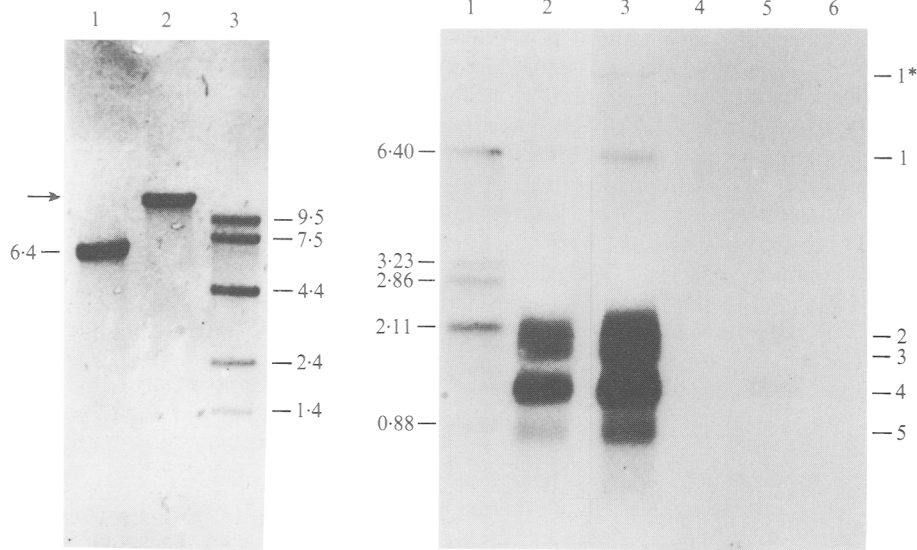


Fig. 1

Fig. 2

Fig. 1. Electrophoresis of glyoxal-formamide-denatured LNYV genomic RNA (lane 2) and RNA standards in 1% agarose in TAE buffer. RNA species were stained in ethidium bromide, visualized with ultraviolet light, photographed and printed in reverse. TMV RNA (600 ng) and a 9.5 to 0.24 kb RNA ladder (BRL) (1  $\mu$ g) were applied as size markers (lanes 1 and 3, respectively). Sizes of RNAs are indicated in kb.

Fig. 2. Presence and size of LNYV cRNAs in LNYV-infected *N. glutinosa*. Glyoxylated RNA samples (2  $\mu$ g) were separated by electrophoresis in 1% agarose gels in TAE buffer, transferred to nitrocellulose and hybridized to  $^{32}$ P-labelled LNYV genomic RNA random fragments. The positions of individual cRNAs are indicated on the right. Lane 1, size markers (kb, indicated at left) containing a mixture of TMV and BMV RNAs (250 ng each) were visualized by hybridizing to  $^{32}$ P-labelled cDNA. Lanes 2 and 3 contain poly(A)<sup>+</sup> RNA fractionated from total RNA extracted from LNYV-infected *N. glutinosa* leaves 8 days after infection (lane 5). The lanes were exposed for 2 (lane 2) or 5 (lane 3) days without an intensifying screen to resolve cRNAs 2 and 3 and to expose cRNA 1\*. Lane 4 represents poly(A)<sup>+</sup> RNA fractionated from total RNA extracted from healthy *N. glutinosa* leaves (lane 6).

## RESULTS AND DISCUSSION

### Analysis of LNYV genomic RNA

RNA extracted from sucrose gradient-purified LNYV (Dietzgen & Francki, 1988) contained a single high  $M_r$  species of about 13000 nucleotides (nt) (Fig. 1). The size estimated by denaturing agarose gel electrophoresis and the calculated  $M_r$  of LNYV RNA of about  $4.4 \times 10^6$  are similar to those of SYN and SYVV RNAs, analysed under the same conditions (Stenger *et al.*, 1988). In addition, the sedimentation coefficients in sucrose density gradients of both LNYV and SYN RNAs were previously determined to be 44S (Francki & Randles, 1973; Jackson & Christie, 1977). The association of an RNA transcriptase with LNYV preparations (Francki & Randles, 1972) and the presence of viral cRNAs in plants infected with SYN (Rezaian *et al.*, 1983; Milner & Jackson, 1983), SYVV (Stenger *et al.*, 1988) and LNYV (this report) indicate that all these viruses are negative-stranded and have replication strategies similar to those of the animal rhabdoviruses (Banerjee, 1987).

### Detection of RNAs complementary to LNYV RNA in infected *N. glutinosa*

To investigate the size and complexity of LNYV cRNAs, RNAs from healthy and LNYV-infected *N. glutinosa* leaves were denatured with glyoxal-formamide and analysed by electrophoresis in 1% agarose gels. The electrophoretic patterns of total RNA and poly(A)<sup>+</sup> RNAs from healthy and infected plants appeared similar to those of poly(A)<sup>+</sup> RNA from

SYNV-infected and healthy tobacco (Rezaian *et al.*, 1983). The poly(A)<sup>+</sup> fractions which represented 0.5 to 1.0% of the total RNA, contained minor amounts of discrete rRNA bands which indicated that little degradation occurred during chromatography, but no unique virus-specific RNAs were detected by ethidium bromide staining (data not shown).

The number and size of poly(A)<sup>+</sup> RNAs complementary to LNYV RNA were determined by Northern blot analysis with random <sup>32</sup>P-labelled LNYV RNA fragments of less than 300 nt in length. The probes failed to hybridize to RNA from uninfected *N. glutinosa*, but five hybridizing components complementary to LNYV RNA (cRNAs) were detected in poly(A)<sup>+</sup> RNA from systemically infected *N. glutinosa* leaves harvested 8 days after inoculation (Fig. 2), about 3 days before the maximum virus yield (Wolanski & Chambers, 1971). The cRNAs were estimated to be about 6450, 2000, 1650, 1200 and 900 nt by comparison with TMV and BMV RNAs (Fig. 2). The LNYV cRNAs differ in size from those in SYNV- and SYVV-infected plants (Rezaian *et al.*, 1983; Stenger *et al.*, 1988). In particular, cRNAs 1, 2 and 5 of LNYV are about 150, 200 and 400 nt shorter, respectively, than the corresponding SYNV and SYVV cRNAs.

The relative intensity of hybridization suggested that sequences in cRNAs 2, 3 and 4 were more abundant than cRNAs 1 and 5. Faintly hybridizing bands representing the former cRNAs could be detected even in total RNA preparations from LNYV-infected *N. glutinosa*. In addition to cRNAs 1 and 5, small amounts of another component (cRNA 1\*) were detected (Fig. 2). As discussed below, cRNA 1\* probably represents a positive-sense, genomic-length replicative RNA species.

#### *Construction of recombinant DNA clones derived from LNYV RNA*

Random synthetic oligonucleotides were used to prime reverse transcription of LNYV genomic RNA. Double-stranded cDNA ranging in size from 300 to 4500 nt was inserted into pUC9. Six-hundred ampicillin-resistant colonies were screened for LNYV-specific sequences by colony hybridization. Of these, 168 hybridized strongly to <sup>32</sup>P-labelled LNYV RNA fragments and 40 had inserts ranging from 500 to 1400 bp. These 40 clones were selected for further characterization. Confirmation that the plasmids harbour LNYV sequences, but not host plant sequences, was achieved by Southern hybridization with <sup>32</sup>P-labelled LNYV genomic RNA and <sup>32</sup>P-labelled RNA from healthy *N. glutinosa* (data not shown).

#### *Individual cRNAs contain unique nucleotide sequences*

The presence of unique nucleotide sequences among the six cRNAs detected by hybridization of poly(A)<sup>+</sup> RNA from LNYV-infected *N. glutinosa* with <sup>32</sup>P-labelled LNYV RNA (Fig. 2 and 3) was determined by hybridization with recombinant DNA clones. Plasmids with inserts larger than 800 nt exhibited six different classes of hybridization. Plasmids designated pLNYV 1, 2, 3 and 4 hybridized specifically only to cRNAs 1, 2, 3 or 4 as shown in Fig. 3. These results show that each of the cRNAs contains at least some unique sequence, despite the fact that none of the clones tested so far hybridized to cRNA 5.

Presumably, the synthesis of LNYV proteins, like that of other rhabdoviruses (Jackson *et al.*, 1987), is directed by discrete mRNAs complementary to the viral genome. Based on the relative sizes of the individual cRNAs and their coding capacities, the cRNAs were given provisional coding assignments. The 6450 nt cRNA 1 is of sufficient size to encode the 190K L protein (Dietzgen & Francki, 1988); the 78K G and the 57K N proteins are probably encoded by cRNAs 2 (2000 nt) and 3 (1650 nt), respectively. Synthesis of the 38K NS and the 19K M proteins is probably directed by cRNAs 4 (1200 nt) and 5 (900 nt), respectively. The L and NS proteins have not yet been unambiguously identified in purified virions (Dietzgen & Francki, 1988), but the presence of the 6450 nt and 1200 nt transcripts provides indirect evidence that these proteins are encoded by LNYV RNA. The combined size of the LNYV-specific transcripts represented about 95% of the viral genome sequence, the same range observed for SYNV (Milner & Jackson, 1983; Rezaian *et al.*, 1983). From these results, we conclude that the LNYV genome is transcribed *in vivo* into at least five individual mRNAs, designated cRNAs 1 to 5 and that these RNAs are comparable to the putative SYNV mRNAs (Rezaian *et al.*, 1983).

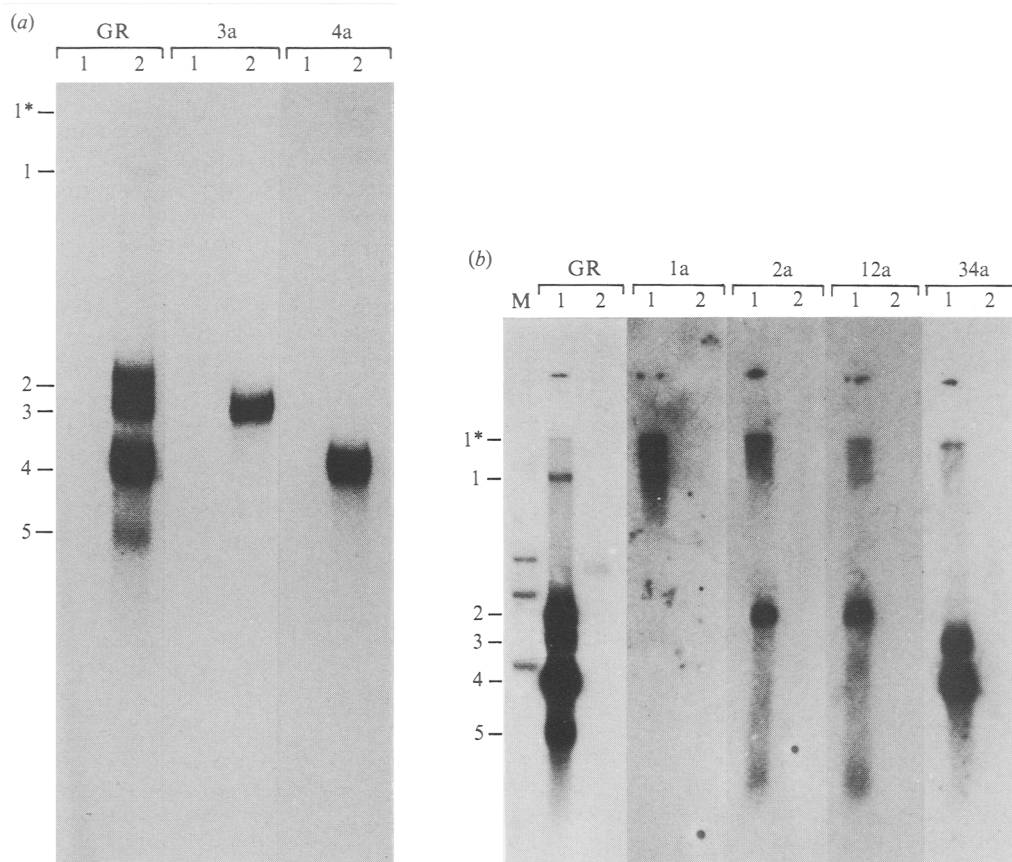


Fig. 3. Identification of pLNYV clones with sequence homology to individual cRNAs from LNYV-infected *N. glutinosa*. Poly(A)<sup>+</sup> RNA (2 µg) extracted from healthy *N. glutinosa* leaves (*a*, lanes 1; *b*, lanes 2) or from leaves 8 days after inoculation (*a*, lanes 2; *b*, lanes 1) was denatured with glyoxal-formamide, separated on 1.5% vertical (*a*) or horizontal (*b*) agarose gels, transferred to nitrocellulose and hybridized to nick-translated <sup>32</sup>P-labelled pLNYV clones 3a, 4a, 1a, 2a, 12a or 34a, as shown. The cRNAs were visualized by hybridizing with a random <sup>32</sup>P-labelled LNYV genomic RNA probe (GR). The numbers 1 to 5 at left indicate individual cRNAs. RNA markers (M) in (*b*) were visualized by hybridizing with nick-translated lambda DNA.

In addition to plasmids hybridizing only to a single cRNA, two intergenic clones (pLNYV 12 and 34) hybridized to cRNAs 1 and 2 or cRNAs 3 and 4, respectively (Fig. 3*b*). The hybridization patterns of these two clones indicate that sequences complementary to cRNAs 1 and 2 as well as cRNAs 3 and 4 are adjacent on the LNYV genome. We therefore assume that the genes encoding the L and G proteins are adjacent as are those encoding the N and NS protein, as is also the case with other rhabdoviruses (Rose & Schubert, 1987). The detection of further overlapping gene junction clones should enable us to establish a physical map of the LNYV genome, to identify proteins encoded by the genes and to sequence the genome.

The cRNA 1\* component present in poly(A)<sup>+</sup> RNA from LNYV-infected *N. glutinosa* contained sequences present in all clones tested by Northern hybridization (Fig. 3*a* and *b*). This RNA comigrated with genomic LNYV RNA in denaturing agarose gels (Fig. 4) and hybridized to kinase-labelled negative-sense LNYV RNA (Fig. 3). Moreover, a negative-sense ss M13 DNA probe prepared from clone pLNYV 2 hybridized to cRNA 1\*. However, a positive-sense probe from the same clone hybridized to LNYV RNA but not to cRNA 1\* (Fig. 4). These results indicate that cRNA 1\* represents a positive-sense LNYV RNA that may be an intermediate in replication of the viral genome, as has been proposed for a similar RNA associated with

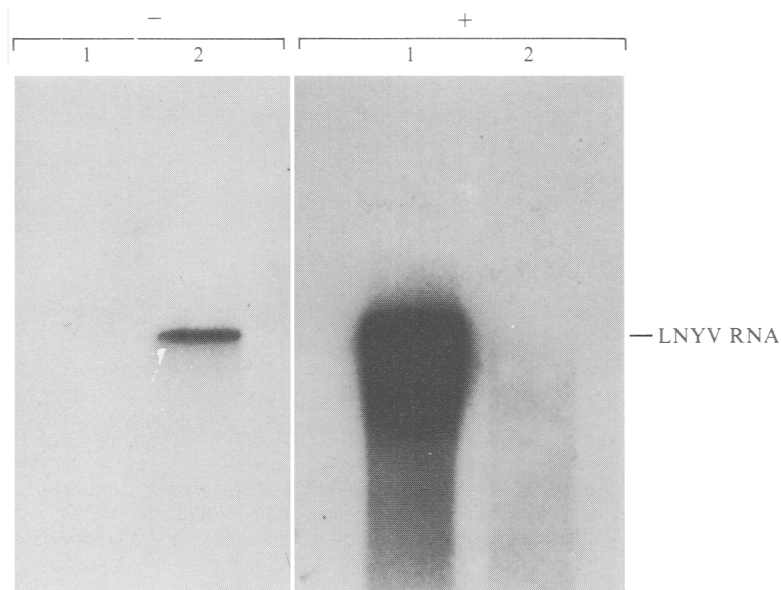


Fig. 4. Analysis of cRNA 1\*. Glyoxylated RNA samples were separated by electrophoresis in 1.5% agarose gels in TAE buffer, transferred to nitrocellulose and hybridized to  $^{32}\text{P}$ -labelled ss M13 probes which contained the pLNYV 2 insert in either the negative (-) or the positive (+) sense. Lane 1 is genomic LNYV RNA; lane 2 is poly(A)<sup>+</sup> RNA from LNYV-infected *N. glutinosa* leaves (2  $\mu\text{g}$ ).

polyribosomes from SYN $\nu$ -infected tobacco (Milner & Jackson, 1983). A genome-size RNA is also a minor constituent of extracts from SYVV-infected tobacco (Stenger *et al.*, 1988). Genomic length, positive-sense poly(A)<sup>+</sup> RNA transcripts, termed RNA 0, in vesicular stomatitis virus (VSV)-infected cells were reported by Freeman *et al.* (1977).

#### *Identification of cRNA 3 as the nucleocapsid protein mRNA*

Clone pLNYV 3 hybridized specifically with cRNA 3 (1650 nt) (Fig. 3a) which was provisionally identified as the nucleocapsid protein mRNA. When the 1100 bp insert was ligated in the correct orientation into the expression vector pUR291, transformed colonies (pUR-LNYV 3) grown in the presence of IPTG reacted with LNYV-specific antibodies. Fig. 5 illustrates the immunological analysis of protein preparations from two subclones derived from the pLNYV 3 insert which were grown under induced and uninduced conditions. Using polyclonal antibodies as probes, a doublet of bands corresponding to proteins of about 160K was detected, which presumably represents the  $\beta$ -galactosidase fusion protein. Surprisingly, a strongly reacting band of about 40K was also present in induced, but not in uninduced colonies. Neither of these proteins reacted with preimmune serum (data not shown) and neither was detected in induced controls harbouring the pUR291 plasmid without an insert. This information suggests that the 40K polypeptide is derived from pUR-LNYV 3 and that it is probably generated by proteolytic cleavage from the 160K fusion protein. The cause of the apparently specific cleavage of the fusion protein from the  $\beta$ -galactosidase moiety is not known, but it is likely that an adventitious proteolytic cleavage site has been introduced within the N protein sequence near the  $\beta$ -galactosidase junction. This apparently accounts for the large amount of 116K protein in the induced lanes (Fig. 5), since the host bacterial strain MV 1193 has a *lac* deletion. The fact that correspondingly high amounts of the 40K protein do not appear in Coomassie Brilliant Blue-stained samples may simply reflect further degradation of the LNYV-specific peptide. Such degradation could cause potential difficulties in analyses of fusion proteins. To increase the stability of  $\beta$ -galactosidase fusion proteins, host strains deficient in protein degradation might be employed. The successful use of such mutants lacking certain proteases, like *lon*<sup>-</sup> or *ompT*<sup>-</sup> has been reported (Young & Davis, 1983; Hellebust *et al.*, 1989).

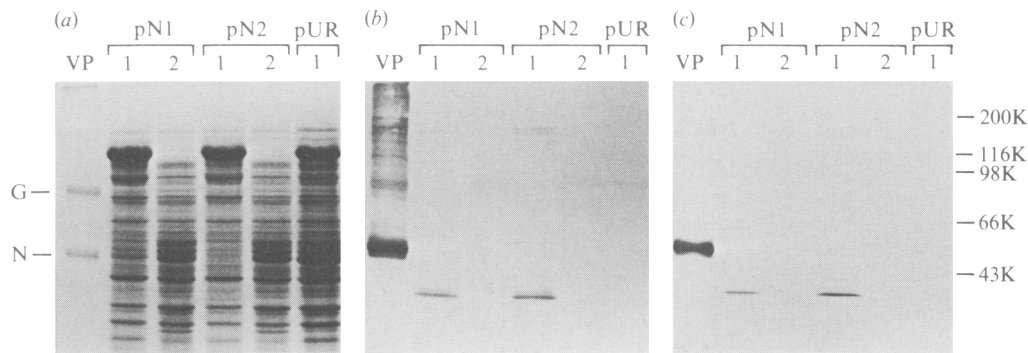


Fig. 5. Immunological detection of LNYV nucleocapsid fusion protein. Competent *E. coli* MV 1193 were transformed with pUR-LNYV 3 and screened with anti-LNYV serum. Antibody-reactive subclones harbouring the same LNYV insert (pN1 and pN2) and controls without insert (pUR) were grown in the presence (lane 1) or absence (lanes 2) of IPTG. Proteins were analysed on 10% polyacrylamide-SDS gels. Proteins in gels run in parallel were either stained (a) or transferred to nitrocellulose and reacted with polyclonal antiserum to LNYV (b) or monoclonal antibody to LNYV N protein (c). LNYV proteins (VP) were included as controls; the positions of the viral G and N proteins are indicated on the left. *M*, standards myosin,  $\beta$ -galactosidase, phosphorylase b, bovine serum albumin and ovalbumin are indicated at the right.

As judged from its size, the pLNYV 3 insert is capable of encoding a LNYV-specific polypeptide of about 40K which corresponds to the band of the same size detected by the LNYV-specific antibodies. Using a monoclonal antibody specific for the N protein (MAb-N) (Dietzgen & Francki, 1988) this polypeptide was shown to contain N protein sequences. MAb-N reacted exclusively with the 40K polypeptide (Fig. 5). In addition, it was demonstrated that a  $^{32}$ P-labelled DNA probe from pUR-LNYV 3 hybridized strongly and specifically to cRNA 3 in Northern blots (data not shown). These results therefore provide direct evidence that cRNA 3 is the nucleocapsid protein mRNA.

#### CONCLUSIONS

The physical properties of LNYV RNA and its gene expression strategy appear to be similar to those of other plant and animal rhabdoviruses (Jackson *et al.*, 1987). The LNYV genome consists of at least five genes that are transcribed during infection into distinctly sized mRNAs which probably correspond to the five structural proteins described (Dietzgen & Francki, 1988). So far, we have no evidence for a sixth gene encoding a non-structural protein as was recently found in the SYNIV genome (Heaton, 1986; Heaton *et al.*, 1987a) and in the fish rhabdovirus, haematopoietic necrosis virus (Kurath & Leong, 1985). However, since additional clones need to be characterized to map the genome completely, we cannot exclude the presence of additional cRNA species that comigrated with one of the cRNA bands.

LNYV differs from SYNIV and SYVV and is similar to VSV in having an easily demonstrable transcriptase activity (Francki & Randles, 1972, 1973). Yet the products of the *in vitro* enzyme activity were heterodisperse and failed to yield distinct RNAs (Francki & Randles, 1973; Toriyama & Peters, 1981), possibly because of nuclease activity in the virus preparations. However, the data presented here demonstrate the presence of discrete mRNAs in infected plant tissues corresponding in size to the LNYV structural proteins.

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