Characterization of the Coat Protein Subgenomic RNA of White Clover Mosaic Virus

By R. L. S. FORSTER,* P. J. GUILFORD AND D. V. FAULDS

Plant Diseases Division, DSIR, Private Bag, Auckland, New Zealand

(Accepted 22 September 1986)

SUMMARY

The genomic RNA (6-2 kb) and a 0-9 kb RNA were detected by Northern hybridization in leaves infected with white clover mosaic virus (WCIMV). The 0-9 kb RNA was efficiently encapsidated by one, but not by eight other, isolates of WCIMV. Both RNA species were shown to possess 3’-terminal tracts of poly(A) 200 to 300 nucleotides long by end-labelling with [32P]pCp and digestion with ribonucleases A, Phy M, T1 and U2. The 0-9 kb RNA was mapped to the 3’-terminal region of the genomic RNA using cDNA probes. In rabbit reticulocyte lysates, the 6-2 kb RNA directed predominantly the synthesis of a protein of mol. wt. 160000 (160K) and several additional proteins. The 0-9 kb RNA directed synthesis of a 25K protein. This protein was shown to be the virus coat protein by its co-migration in gels with the WCIMV coat protein and by its specific immunoprecipitation with antiserum to virus particles. Both RNA species were found in specific association with polysomes indicating that they function as messenger RNAs in infected cells.

INTRODUCTION

White clover mosaic virus (WCIMV) is a member of the potexvirus group. This group comprises viruses with one single-stranded genomic RNA which is polyadenylated at the 3’ terminus and has a 7-methylguanosine cap at the 5’ terminus (Koenig, 1971; AbouHaidar & Bancroft, 1978; Sonenberg et al., 1978; AbouHaidar, 1983). The genomic RNA of potato virus X (PVX), the type member of the potexvirus group, directs the synthesis in vitro of proteins up to 180000 mol. wt. (180K), but not the virus coat protein (Wodnar-Filipowicz et al., 1980). The coat protein cistron of PVX is located at the 3’ end of the genomic RNA (Morozov et al., 1983) but the method by which this cistron is expressed has not been determined. However two methods have been proposed for the expression of the coat protein cistron of other potexviruses. The coat protein of narcissus mosaic virus (NMV) is translated from a 0.84 kb subgenomic RNA which is efficiently encapsidated (Short & Davies, 1983), while the coat protein of daphne virus X (DVX) is also translated from a polyadenylated 0.8 kb subgenomic RNA found in infected tissue but which is not efficiently encapsidated (Guilford & Forster, 1986). In contrast, degradation of the genomic RNA in cell-free translation systems was reported to provide the template for the synthesis in vitro of the coat protein of papaya mosaic virus (PMV) (Bendena et al., 1985).

In this paper we describe an isolate (M) of WCIMV from New Zealand which encapsidates, in addition to the genomic RNA, a polyadenylated subgenomic RNA coding for the coat protein. Both the genomic and subgenomic RNA species occur in specific association with polysomes indicating that they function as messenger RNAs in infected cells.

METHODS

Virus isolates. Isolates C, M, O and four other isolates of WCIMV were obtained from individual white clover plants from different areas of New Zealand. A virus isolate from Tasmania, Australia and an isolate from Japan were kindly supplied by Drs G. R. Johnstone and C. Hiruki respectively. All nine isolates were indistinguishable
by enzyme-linked immunosorbent assay (Clark & Adams, 1977) using antiserum to another isolate of WCIMV from New Zealand (results not shown).

**Purification and electron microscopy of virus particles.** Virus isolates were purified from systemically infected leaves of pea (*Pisum sativum*) cv. Victory Freezer using the method of Franki & McLean (1968). Virus particles were further purified by sedimentation in sucrose gradients as described by Morris-Krisinch et al. (1983). Virus particle lengths were determined from electron micrographs of sap from infected leaves of *Nicotiana clevelandii* negatively stained with 1% sodium phosphotungstate, pH 7.0. A cross grating replica (2160 lines/mm) was used as a size standard.

**Extraction, gel electrophoresis and Northern transfer of WCIMV RNA.** RNA was extracted from purified virus using phenol and SDS (Forster & Morris-Krisinch, 1985). RNA was extracted from leaves and fractionated on columns of oligo(dT)-cellulose by the methods of Guiford & Forster (1986). RNA was fractionated in 10 to 40% sucrose gradients by the method of Bendena et al. (1985). Methods for gel electrophoresis, denaturation of RNA with glyoxal-DMSO or formamide and formaldehyde, and for Northern transfer to nitrocellulose were as described by Guiford & Forster (1986). The RNAs of alfalfa mosaic virus (Brederode et al., 1980; Barker et al., 1983; Cornelissen et al., 1983a, b) and tobacco ringspot virus (Murant et al., 1981) were used as molecular size standards in denaturing gels.

**Determination of the 3' terminal sequence.** RNA molecules from purified particles of a New Zealand isolate (isolate M) were labelled at their 3' termini with cytidine-3',5'(32P)bisphosphate and T4 RNA ligase as described by England & Uhlenbeck (1978). Labelled RNAs were fractionated in 5% polyacrylamide, 7 M-urea gels containing 90 mM-Tris, 90 mM-boric acid and 1 mM-EDTA, visualized by autoradiography and eluted as described by Winter & Brownlee (1978). End-labelled RNA was digested for 15 min at 55 °C with RNases T1, A, Phy M and U2 in 25 mM-sodium citrate, either at pH 5.0 (T1, A, Phy M) or at pH 3.5 (U2), containing 7 M-urea and 1 mM-EDTA. Partial alkaline digestions for size markers were carried out in 50 mM-NaHCO3/NA2/C03, pH 9.2, and 1 mM-EDTA at 90 °C for 15 min. Samples were electrophoresed in polyacrylamide, 7 M-urea gels and autoradiographed.

**cDNA synthesis and cloning.** Unless otherwise stated, oligo(dT)12-18 was used as the primer for cDNA synthesis. cDNA for 3'-terminal probes was synthesized from the genomic RNA of isolate M fractionated on a sucrose gradient, using 1/4000th the normal level of dCTP (Cann et al., 1983). Using alkaline agarose gel electrophoresis (Maniatis et al., 1982), cDNA synthesized under these conditions was found to contain molecules up to 600 nucleotides long. In contrast, cDNA synthesized from RNA of isolate M by the method of Maniatis et al. (1982) was found to contain molecules approaching 6 kb in length. For cDNA cloning, the first DNA strand was synthesized by the method of Maniatis et al. (1982) from RNA of isolate M polyadenylated in vitro (Barker et al., 1983) and the second DNA strand was synthesized using RNase H and DNA polymerase I (Gubler & Hoffman, 1983). The dsDNA was tailed with deoxyeytidine using terminal deoxynucleotidyl transferase (Bethesda Research Laboratories) according to the supplier's recommendations and annealed with PstI-cut, dG-tailed pBR322 plasmid (Amersham). The annealed DNA was used to transform *Escherichia coli* strain RR1 treated with CaCl2 (Maniatis et al., 1982). Two recombinant plasmids, designated p9A and p11A, were identified by colony hybridization (Maniatis et al., 1982) with cDNA prepared by the random primer method of Maniatis et al. (1982). Clone p11A had an insert of approx. 1500 bp (determined by agarose gel electrophoresis) which hybridized with 3'-terminal cDNA probes. Clone p9A had an insert of approx. 500 bp which did not hybridize with clone p11A nor with 3'-terminal cDNA probes. Plasmid DNA of these clones purified using CsCl was 32P-labelled for use as probes by nick translation (Rigby et al., 1977).

**In vitro translation.** Translations in messenger-dependent rabbit reticulocyte lysates (Pelham & Jackson, 1976) were done in the presence of [35S]methionine. Optimal ionic conditions for translation of WCIMV RNA were 100 mM-KCl and 0-25 mM-MgCl2. Following translation at 30 °C for 50 min, products were separated on 12-5% polyacrylamide gels and then fluorographed. The methods of Forster & Morris-Krisinch (1985) were used for immunoprecipitations with either pre-immune serum or antiserum to WCIMV which had a dilution endpoint of 1:2048 in microprecipitin tests.

**Isolation of polysomal RNA.** Polyribosomes were isolated from leaf tissue by the method of Jackson & Larkins (1976) as modified by Palukaitis et al. (1983). Polyribosomes were disrupted with EDTA and the polysomal RNA was separated from WCIMV virus particles by high speed centrifugation as described by Vance & Beachy (1984). RNA in the supernatant fraction after the final cycle of high speed centrifugation was extracted twice with phenol and chloroform in the presence of 1% SDS and 100 mM-NaCl, and precipitated with ethanol at -20 °C.

**RESULTS**

**Analysis of virus particles and their RNAs**

WCIMV has been reported to consist of one distinct population of virus particles (modal length 480 nm) which contain the genomic RNA (Brandes & Bercks, 1965; Koenig, 1971). For eight isolates of WCIMW we examined, sap extracts of leaves of *N. clevelandii* also contained only one distinct population of virus particles of approx. 480 nm (Fig. 1a shows one typical
Coat protein subgenomic RNA of WCIMV

Fig. 1. Analysis of WCIMV particles and their RNAs. (a, b) Histograms of particle length distributions of WCIMV from N. clevelandii infected with isolates O (a) and M (b). (c) Autoradiograms of Northern transfers of WCIMV RNA: lanes 1 and 2, RNA from particles of isolates O and C respectively; lanes 3 and 4, RNA of isolate M; lanes 5 and 6, RNA from 85 nm particles of isolate M (lane 5) fractionated by one cycle of sucrose gradient centrifugation, and from particles in the corresponding region of a gradient containing isolate O (lane 6). Lanes 1, 2, 3, 5 and 6 were hybridized with the cloned cDNA probe p11A (corresponding to the 3'-terminal region of the genomic RNA); lane 4 was hybridized with the cloned cDNA probe p9A (corresponding to an internal position of the genomic RNA). Numbers are kb.
isolate). In contrast, one New Zealand isolate (isolate M) contained, in addition to particles of approx. 480 nm, a second population of particles approx. 85 nm long (Fig. 1b). In sucrose gradients the smaller particles were enriched in a minor component visible as a more slowly moving shoulder adjacent to the major peak containing the 480 nm particles.

RNA from unfractionated particles of each isolate was electrophoresed in agarose gels and either transferred to nitrocellulose and hybridized with cloned cDNA probes, or stained with ethidium bromide. Only one RNA species, the genomic RNA, was readily resolved in gels containing RNA from the isolates with one distinct population of virus particles (Fig. 1, lanes 1 and 2 show two typical isolates) whereas the genomic RNA and a second RNA species were detected in RNA of isolate M (Fig. 1, lane 3). The molecular sizes of the two RNAs were estimated under denaturing conditions (glyoxal-DMSO) to be 6.2 kb (genomic RNA) and 0.9 kb. The 0.9 kb RNA of isolate M hybridized with the cloned probe corresponding to the 3'-terminal region of the genomic RNA, pl1A (Fig. 1, lane 3), but not with the cloned probe p9A (Fig. 1, lane 4) which corresponded to an internal or 5' position of the genomic RNA, indicating that it is a subgenomic RNA which is derived from the 3'-terminal region of the genomic RNA.

The 0.9 kb RNA of isolate M was enriched in RNA from particles sedimenting in the minor
Coat protein subgenomic RNA of WCIMV

Fig. 3. Northern hybridization of poly(A)+ RNA from leaf tissues. Lanes 1 and 2, RNA from leaves of *N. clevelandii* infected with isolates C and M respectively; lanes 3 and 4, two exposures of polysomal RNA from leaves of pea systemically infected with isolate M; lane 5, polysomal RNA from uninfected leaves of pea. Lanes 1 and 2 were hybridized with cDNA prepared from the 3'-terminal region of the genomic RNA by the method of Cann *et al.* (1983); lanes 3 to 5 were hybridized with oligo(dT)-primed cDNA prepared according to Maniatis *et al.* (1982).

A u.v.-absorbing band in sucrose gradients (Fig. 1, lane 5). RNA from particles of the other eight isolates that was sedimented in the corresponding fraction of sucrose gradients contained 0.9 kb RNA (Fig. 1, lane 6 shows one typical isolate) indicating that these isolates do encapsidate a 0.9 kb RNA, but only at a relatively low level.

**Analysis of the 3' terminus**

Approximately 85% of the RNA extracted from purified particles of isolate M was retained on oligo(dT)-cellulose columns in high salt conditions, indicating the presence of a poly(A)-containing tract [poly(A)+]. To determine the size and position of this tract, the 6.2 and 0.9 kb RNAs of isolate M were labelled at their 3' termini and digested with RNases A, T₁, U₂ and Phy M. Labelled fragments ranging in size from 200 to 300 nucleotides were produced following digestion of each RNA with RNases A and T₁. Labelled fragments ranging in size from approximately 300 nucleotides to mononucleotides were produced following digestion of each RNA with RNases U₂ and Phy M. Fig. 2 shows the effects of RNases A, T₁, and U₂ on the 0.9 kb RNA. A similar result was obtained with the 6.2 kb RNA. These results indicate that each RNA has a tract of poly(A) 200 to 300 nucleotides long at its 3' terminus.
Fig. 4. Autoradiograms of translation products made in rabbit reticulocyte lysates of RNA from purified particles of WCIMV isolates. Lanes 1 and 2, two exposures of the translation products of isolate M; lanes 3 and 4, isolates C and O respectively; lanes 5 and 6, translation products of RNA extracted from 85 nm particles of isolate M separated on a sucrose gradient (lane 5) and from particles in the corresponding fraction of gradients containing isolate O (lane 6) respectively; lanes 7 and 8, translation products of RNA from purified particles of isolate M immunoprecipitated with antiserum to WCIMV particles or pre-immune serum respectively. Numbers are mol. wt. × 10^-3. Open arrowhead indicates the position of the virus coat protein. Closed arrowhead indicates bands seen in gel lanes containing lysates incubated in the absence of added RNA.

Analysis of RNA from infected leaves

Poly(A)+ RNA preparations extracted from inoculated leaves of *N. clevelandii* 3 days after inoculation with either isolate M or isolate C were analysed by agarose gel electrophoresis (0.2 μg/lane) and Northern hybridization using cDNA probes. RNA from leaves infected with either isolate contained two major hybridizing bands (Fig. 3). Of these, the larger band co-migrated with the genomic RNA and the smaller broader band co-migrated with, and was presumably identical to, the encapsidated 0.9 kb RNA. The relative amounts of the two RNAs were similar for both isolates. Additional bands were detected in some autoradiograms of poly(A)+ RNA (and of polysomal RNA) from infected leaves, but were poorly resolved following photographic reproduction.

Translation of WCIMV RNAs in reticulocyte lysates

A product of apparent mol. wt. 160K was synthesized from encapsidated RNA of isolates M, C and O of WCIMV (Fig. 4) and the other six isolates. A product of 175K was resolved in some gels. A range of smaller products, including a product of 25K, was also synthesized from RNA of each isolate. The electrophoretic mobilities of the 160K and 25K products varied slightly among isolates. The 25K product was shown to be similar or identical to the virus coat protein by its immunoprecipitation with antiserum to WCIMV particles but not with pre-immune serum (Fig. 4, lanes 7 and 8) and by its co-migration with the virus coat protein in polyacrylamide gels. Relatively more 25K protein was synthesized during translation of RNA of isolate M than translation of RNA of other isolates, and more from the RNA of isolates M and O enriched for the 0.9 kb RNA than from unfractionated RNA (Fig. 4). Furthermore, the 25K protein was the major product translated from the 0.9 kb RNA of isolate M that had been purified by
fractionation in a sucrose gradient (Fig. 5). These results indicate that the 0.9 kb RNA is the messenger RNA for the coat protein.

The 160K product and several additional products were translated from purified genomic RNA of isolate M (Fig. 5). Time course experiments gave no evidence that the 160K product was a precursor of any of the additional products (result not shown). Several of the additional products, including products of 110K and 90K, were synthesized relatively more efficiently from RNA sedimenting between the 6.2 and 0.9 kb RNAs (Fig. 5).

**Analysis of polysomal RNAs**

RNA in the polysomal fraction of infected tissues was examined to determine whether the RNAs detected in leaf tissues infected with isolate M are translated in vivo. Polysomes were isolated from systemically infected leaves of peas cv. Victory Freezer within 24 h of the first appearance of systemic symptoms. Both the 6.2 and 0.9 kb RNAs were detected in the poly(A)+ fraction of polysomal RNA (Fig. 3). No bands hybridizing with WCIMV cDNA were detected in polysomal RNA from uninfected leaves (Fig. 3). In reticulocyte lysates, the major translation products of polysomal RNA from infected leaves were products of 160K and 25K (Fig. 6). As neither of these products was translated from polysomal RNA from uninfected leaves (Fig. 6), it would appear that they are the same as the translation products of encapsidated WCIMV RNA. Another product of 65K was detected after longer exposure of autoradiograms (result not shown) of the translation products of polysomal RNA from infected, but not uninfected, leaves. The origin of this protein has not been determined.

Reconstruction experiments were carried out to determine whether the presence of WCIMV RNAs was a result of contamination of the polysomal RNA fraction with WCIMV particles or RNA. Polysomal RNA extracted from uninfected leaves, to which purified virus particles (1 mg/5 g leaves) had been added immediately prior to extraction, did not direct synthesis in vitro of
detectable levels of the 160K and 25K products (Fig. 6b). However, polysomal RNA from 
uninfected leaves to which WC1MV RNA (50 μg/5 g leaves) had been added immediately prior 
to extraction directed synthesis of small amounts of the 160K and 25K products (Fig. 6; compare 
lanes 1 in a and 3 in b).

DISCUSSION

Our results show that in leaves infected with WCIMV, a 0.9 kb RNA is synthesized in 
addition to the 6.2 kb genomic RNA. Although the 0.9 kb RNA appears in autoradiograms as a 
relatively broad band, it hybridizes only with 3'-terminal cDNA probes indicating that it is a 
discrete subgenomic RNA rather than a population of heterogeneous RNA molecules like that 
detected in tissue infected with tobacco mosaic virus (Palukaitis et al., 1983). The presence of a 
poly(A) tract of variable length may explain the apparent size heterogeneity of this RNA 
(Vournakis et al., 1975). The presence of 3'-terminal poly(A) tracts and the hybridization with 3'- 
terminal cDNA probes indicate that the 6.2 and 0.9 kb RNAs are co-terminal. This has been 
confirmed by RNA sequencing of the first 80 nucleotides after the poly(A) tract (result not 
shown).

One of the WCIMV isolates (M) resembles NMV in that it encapsidates substantial amounts 
of the 0.9 kb RNA. Our other eight isolates resemble the two WCIMV isolates used in earlier 
studies by Koenig (1971) and Szybiak & Legocki (1981) (and also other potexviruses) in 
efficiently encapsidating only the genomic RNA. Inefficient encapsidation of the 0.9 kb RNA 
by the isolates we studied was apparently not due to less efficient synthesis of this RNA in vivo, 
because the level of accumulation in tissues was similar to that found with isolate M.

Szybiak & Legocki (1981) reported that the genomic RNA of WCIMV directed synthesis in 
vitro of a protein of 170K, but not the coat protein. We have obtained a similar result. Evidence 
from our translation experiments using RNA of isolate M fractionated on sucrose gradients 
indicates that the coat protein is translated instead from the 0.9 kb subgenomic RNA. As a 
similar mechanism has been demonstrated for the expression of the coat protein cistron of NMV 
and DVX (Short & Davies, 1983; Guilford & Forster, 1986) it would appear that the use of
subgenomic RNAs for expression of internal cistrons is a strategy employed by several potexviruses.

The virus coat protein is synthesized in vitro from encapsidated RNA of DVX (Guilford & Forster, 1986), PMV (Bendena et al., 1985) and our eight isolates of WCIMV which efficiently encapsidate only the genomic RNA. However, whereas the coat protein of PMV is reported to be synthesized from RNA in a heterogeneous population derived from molecules of genomic RNA degraded in reticulocyte lysates (Bendena et al., 1985), the coat protein of WCIMV is synthesized from a discrete 0.9 kb RNA which occurs at in RNA preparations from purified particles.

The presence of the 6.2 and 0.9 kb RNAs of WCIMV in specific association with polysomes indicates that both these RNAs function as messenger RNAs in infected cells. The major translation products of polysomal RNA from infected leaves were the 160K and 25K proteins. In contrast, only minor amounts of these two proteins were synthesized from polysomal RNA extracted from uninfected leaves to which WCIMV RNA had been added immediately prior to extraction. This result indicates that contamination of polysomes with WCIMV RNA is not significant. Neeleman et al. (1985) have reported that polysomes from tobacco leaves are contaminated with alfalfa mosaic virus RNA from virus particles. However polysomes from uninfected pea leaves to which purified WCIMV particles had been added immediately prior to extraction were not contaminated with detectable levels of WCIMV RNA. This indicates that the high speed centrifugation step following disruption of the polysomes with EDTA, used by Vance & Beachy (1984) to separate soybean mosaic virus particles from polysomes, is also effective for separating WCIMV particles from polysomal RNAs.

Leaves infected with DVX have been reported to contain four further RNAs in addition to the coat protein subgenomic RNA (Guilford & Forster, 1986). Several RNA species, possibly subgenomic RNAs, have also been reported to accumulate in protoplasts following infection with potato virus X (Adams et al., 1985). In poly(A)+ RNA from tissues infected with WCIMV we detected low levels of several hybridizing bands in addition to the 6.2 and 0.9 kb RNAs, but we have been unable to show that these RNAs have messenger RNA activity.

We thank T. J. Gardiner for assistance with enzyme-linked immunosorbent assays.

REFERENCES


(Received 16 June 1986)