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Defective RNAs of Clover Yellow Mosaic Virus Encode Nonstructural/Coat Protein Fusion Products

K. ANDREW WHITE,* J. B. BANCROFT,† AND GEORGE A. MACKIE*.¹

Departments of *Biochemistry and †Plant Sciences, University of Western Ontario, London, Ontario, Canada N6A 5C1

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A small group of 1.2-kb RNAs present on polyribosomes from clover yellow mosaic virus (CYMV)-infected tissue contains sequences from the genomic RNA (gRNA) of CYMV and is encapsidated by CYMV coat protein. Some features of these RNAs suggest that they are similar to defective interfering (DI) RNAs, and would be the first to be reported for the potexvirus group. The prototype 1.2-kb RNA is 1172 nucleotides in length excluding a probable poly(A) tail and is composed of two noncontiguous regions corresponding to 757 nucleotides of the 5' and 415 nucleotides of the 3' termini of CYMV's gRNA. The sequence of the prototype 1.2-kb RNA reveals that the two terminal gRNA regions present in this RNA encode a single open reading frame (ORF) joining the N-terminus of the 191-kDa nonstructural product and the C-terminus of the coat protein to form a 35-kDa 191-kDa/coat protein fusion product. The coding properties of this prototype RNA have been confirmed by translation *in vitro* of native and synthetic transcripts of the 1.2-kb RNAs, both of which direct the synthesis of the anticipated 35-kDa product which reacts with anti-CYMV antiserum. Three additional 1.2-kb RNA species, each of which contains a unique junction site, have been characterized. In all cases, a fusion ORF encoding a 191-kDa/coat protein fusion product is encoded on the RNA. The presence of a fusion ORF in all members of the 1.2-kb RNA species analyzed suggests that maintenance of this ORF may be important for the survival of this class of RNA within the plant. This coding strategy represents a novel property of plant virus defective RNAs. © 1991 Academic Press, Inc.

INTRODUCTION

Small incomplete RNAs which are derived from viral genomic RNA (gRNA) are associated with several plant viruses (Hillman *et al.*, 1987; Burgyan *et al.*, 1989; Li *et al.*, 1989). These RNAs often interfere with viral replication and are incapable of independent replication. For these reasons they are referred to as defective interfering RNAs (DI RNAs). DI RNAs are distinguished from satellite RNAs by their high degree of homology to the gRNA of the virus with which they are associated. In contrast, satellite RNAs show little nucleotide sequence homology to the gRNA of the parent virus (Francki, 1985). Although DI RNAs are commonly found in association with animal viruses, they appear to be much less prevalent in viral infections of plants. The first definitive DI RNA identified in plants was found associated with the cherry strain of tomato bushy stunt virus (TBSV; Hillman *et al.*, 1987). DI RNAs are also associated with cymbidium ringspot virus (CyRSV), another tombusvirus (Burgyan *et al.*, 1989; Rubino *et al.*, 1990). Sequence analysis of these DI RNAs has re-

vealed several stretches of sequence derived from different regions of the gRNA of the parent virus. DI RNA G associated with an isolate of the carmovirus turnip crinkle virus (TCV-B) is comprised primarily of two regions corresponding to the 5'- and 3'-terminal regions of the gRNA of the parent virus, and contains additional nonviral sequences at its 5' end (Li *et al.*, 1989). The TCV-B DI RNA intensifies symptoms when present in infections, whereas DI RNAs of the tombus group attenuate them. Coinfection of protoplasts with TBSV and DI RNAs results in reduced synthesis of gRNA relative to infections lacking the DI RNAs (Jones *et al.*, 1990). This reduction of gRNA production may be responsible, at least in part, for symptom attenuation observed in whole plants (Jones *et al.*, 1990).

The potexviruses represent a group of flexuous, filamentous plant viruses which contain a single strand of messenger-sense RNA. Clover yellow mosaic virus (CYMV) possesses a 7015 nucleotide gRNA which is, to date, the largest RNA sequenced from this group (Sit *et al.*, 1990). A 191-kDa protein which contains putative consensus sequences for NTPase-helicases as well as for RNA polymerases (Skryabin *et al.*, 1988; Argos, 1988) is encoded by the most 5' open reading frame (ORF) in the viral RNA. During infections, CYMV produces at least two coterminal subgenomic RNAs (sgRNAs) with approximate lengths of 2.1 and 1.0 kb (Bendena *et al.*, 1987) encoding, respectively, a 25-

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. M63511–M63514.

¹ To whom reprint requests should be addressed. Fax: (519) 661-3175.

kDa protein of unknown function and coat protein (White and Mackie, 1990). A 1.2-kb RNA containing CYMV sequences was previously identified in polyribosomes extracted from infected plants, but was not extensively characterized (Bendena *et al.*, 1987). We report here that some of the properties of this RNA species are consistent with those of DI RNAs.

MATERIALS AND METHODS

Virus and RNA

Virions of CYMV were purified from broad bean plants by using the method of Bancroft *et al.* (1979) and genomic and subgenomic RNAs extracted as described by Erickson and Bancroft (1978). Polyribosomes were prepared from infected or uninfected plants following the method of Palukaitis (1984) and the RNAs were extracted from the polyribosomes as described by Bendena *et al.* (1987). RNAs were separated on agarose gels using the conditions of McMaster and Carmichael (1977). The transfer of RNA to nylon and hybridization of the blot with probe was as described by Mackie (1986).

Primer extension analysis

Primer extension experiments were carried out on 1.2-kb RNA purified by sucrose gradient centrifugation from total polyribosomal RNA or total RNA extracted from virions. Approximately 1 pmol of a 5' ³²P-labeled oligonucleotide (CY-2: 5'-ATGCGAATCTTTGCTGG, complementary to nucleotides 77 to 93) was mixed with either approximately 1 µg of purified 1.2-kb RNA from polyribosomes or 0.1 µg of purified 1.2-kb RNA from virions or 5 µg of total polyribosomal RNA. Annealing and reaction conditions have been described by White and Mackie (1990). Sequence markers were produced using phosphorylated CY-2 primer annealed to 5 µg of CYMV gRNA. Conditions for this reaction are those of Sit *et al.* (1990).

Cloning and sequencing of the 1.2-kb RNA

CYMV RNA preparations used for cloning the 1.2-kb RNAs were either polyribosomal RNA from CYMV-infected tissue or RNA extracted from CYMV virions. First-strand synthesis of cDNA was primed with oligonucleotide CY-11 complementary to the 3' terminus of CYMV RNA (5'-GAGAGTCGACTTTTTTTTTTTTTTTT-TTATACACCCAAAAGTCTACGGG). Approximately 100 pmol of this oligonucleotide was mixed with 3 µg of polyribosomal RNA from infected tissue or 1 µg of RNA purified from CYMV virions in 13 µl of H₂O. The mixture was heated at 90° for 2 min and then annealed sequentially at 45° for 10 min and at ambient tempera-

ture for 10 min. Extension of the primer was carried out at 50° for 40 min in a 25-µl volume containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.8 mM each of the four deoxyribonucleoside triphosphates, and 200 U of M-MLV reverse transcriptase (BRL, Inc.). The cDNA produced was then used as template for PCR amplification in the following mixture: approximately 100 pmol of a second primer (CY-12; 5'-GTGTGTCGACTAATACGACTCACTATAGAAAACA-AAACGAAAACAAAC), amplification buffer to a final concentration of 1× (Promega Biotec., Inc.), 2.5 U of *Taq* DNA polymerase (Promega Biotec., Inc.), and H₂O to a final volume of 100 µl. A total of 30 cycles of amplification was carried out. The products of the reaction were analyzed by agarose gel electrophoresis and a product with a relative mobility of 1.2 kb was eluted from the gel, digested with *Sa*II, ligated into the vector pSP64 (Melton *et al.*, 1984), and transformed into *Escherichia coli* MV1190 (Bio-Rad, Inc.).

Dideoxy sequencing reactions were performed with T7 DNA polymerase (Pharmacia) according to the manufacturer's instructions. Internal sequences of the cDNA which could not be deduced using vector-specific primers were determined using oligonucleotide primers complementary to CYMV sequences.

In vitro translation

In vitro transcription was carried out with approximately 2 µg of linearized plasmid DNA using T7 RNA polymerase. Subsequently, an aliquot of the unpurified transcription reaction was added directly to a rabbit reticulocyte lysate (Promega Biotec., Inc.) containing [³⁵S]methionine. The reaction was terminated by the addition of an equal volume of twice-concentrated SDS sample buffer followed by boiling. *In vitro* translation was also carried out on 0.25 µg of CYMV RNA extracted from virions and on 0.25 µg of brome mosaic virus (BMV) RNA. The products of translation were separated on an 11% polyacrylamide gel containing 0.1% SDS (Laemmli and Favre, 1975). Immunoprecipitation of translation products with antisera directed against CYMV particles was carried out as previously described (Bendena *et al.*, 1985).

RESULTS

Analysis of polyribosomal RNAs and ribonucleoprotein particles for CYMV related RNAs

Bendena *et al.* (1987) have previously identified four CYMV RNAs, with sizes of 7.0, 2.1, 1.2, and 1.0 kb, in polyribosomal preparations from CYMV-infected tissue. The 2.1- and 1.0-kb RNAs represent sgRNAs encoding a 25-kDa product and coat protein, respectively

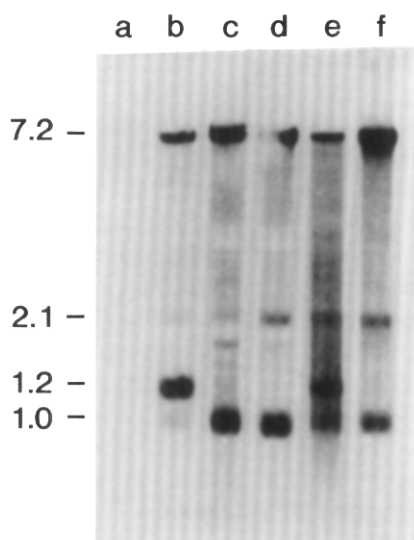


Fig. 1. CYMV RNAs from infected broad bean plants. Total polyribosomal RNA isolated from CYMV-infected tissue (5 μ g) or total RNA extracted from CYMV particles (0.5 μ g) was denatured, resolved by electrophoresis, and blotted to nylon. The blot was hybridized with a [32 P]RNA probe complementary to the coat protein coding region of CYMV's gRNA. The samples in lanes a–d are total polyribosomal RNA from: (a) uninfected plants; (b) plants infected with a CYMV stock containing 1.2-kb RNAs; (c) a first systemic passage using purified CYMV as the initial inoculum; (d) an eleventh systemic passage of a series using purified CYMV as the initial inoculum. Lanes e and f show total RNA extracted from CYMV particles purified from plants infected with CYMV stocks containing (e) or lacking (f) 1.2-kb RNAs.

(White and Mackie, 1990). Both of these sgRNAs are also encapsidated (White and Mackie, 1990). We have examined more closely the 1.2-kb RNA species which is present in viral infections produced by some stocks of CYMV. RNAs from polyribosomes isolated from infected or control tissue or RNA extracted from virions were denatured, separated by electrophoresis, and transferred to nylon membranes. Blots were incubated with a probe specific for the 3' portion of the CYMV gRNA. All four CYMV RNAs, including the 1.2-kb RNAs, can be detected in polyribosomal preparations from infected tissue (Fig. 1, lane b). Furthermore, the 1.2-kb RNAs can be detected in virions prepared from infected leaves indicating that they can be encapsidated (Fig. 1, lane e). Virions isolated from CYMV infections produced from viral stocks lacking the 1.2-kb RNAs did not contain this RNA species (Fig. 1, lane f). No additional RNA species beyond those listed above have been detected in total RNA preparations from infected leaves, regardless of the stock used for inoculation (data not shown).

Virions isolated from infected tissue which contained the 1.2-kb RNAs were subjected to a series of three successive local lesion passages at low multiplic-

ity in *Gomphrena globosa*. Four virus isolates of the third local-lesion passage were used separately as inocula for a series of 11 serial passages with sap (tissue ground 1:2 (w/v) in diluent) in the systemic host broad bean. The polyribosomal RNA from the first systemic passage no longer contained 1.2-kb RNAs (Fig. 1, lane c) nor did these RNAs arise during subsequent passages (Fig. 1, lane d). Therefore, under our conditions these RNAs are not readily generated *de novo* using purified CYMV as the initial inoculum and consequently are not obligatory sgRNAs.

To determine if the presence of the 1.2-kb RNAs interfered with virus production, we examined virus yields from infected tissue in which these RNAs were present or absent. When virions from equal amounts of tissue from each source were analyzed on sucrose gradients, both preparations were found to contain about the same quantity of nucleoprotein particles. However, the proportion of full-length virus particles in the preparation containing the 1.2-kb RNAs was half that of preparations lacking it and was accompanied by an increase in smaller particles (data not shown). This suggests that the presence of the 1.2-kb RNAs in viral infections results in a lower virus yield which may be due in part to the efficient encapsidation of the 1.2-kb RNAs (Fig. 1, lane e). We could not detect any difference between the symptoms induced in broad bean plants by CYMV infections produced from viral stocks lacking or containing the 1.2-kb RNAs. The latter, therefore, do not appear to attenuate symptoms drastically in the host tested.

Mapping of 1.2-kb RNAs in CYMV gRNA

In order to determine which regions of the CYMV gRNA are present in the 1.2-kb RNAs we analyzed CYMV RNAs present in polyribosomal preparations from CYMV-infected broad bean leaves with a set of consecutive cDNA probes spanning the CYMV genome (Fig. 2). Denatured polyribosomal RNAs were separated electrophoretically and transferred to nylon membranes. Figure 2 shows Northern blots of polyribosomal RNA extracted from CYMV-infected tissue containing the 1.2-kb RNAs. The 7.0-kb gRNA is identified by all probes (Fig. 2, lanes a, c, e, g, i, and k; probes 1 to 6), but the 1.0-kb sgRNA encoding coat protein hybridizes only to probes corresponding to the 3' region of the gRNA (Fig. 2, lanes i and k; probes 5 and 6). The 1.2-kb RNAs hybridize efficiently only with probes representing the 5' and 3' extremities of the CYMV genome (Fig. 2, lanes a, c, and k; probes 1, 2, and 6). Probes corresponding to the central region of CYMV gRNA do not hybridize with the 1.2-kb RNAs (Fig. 2, lanes e, g, and i) although they do anneal to gRNA. The results of this analysis are summarized in the lower

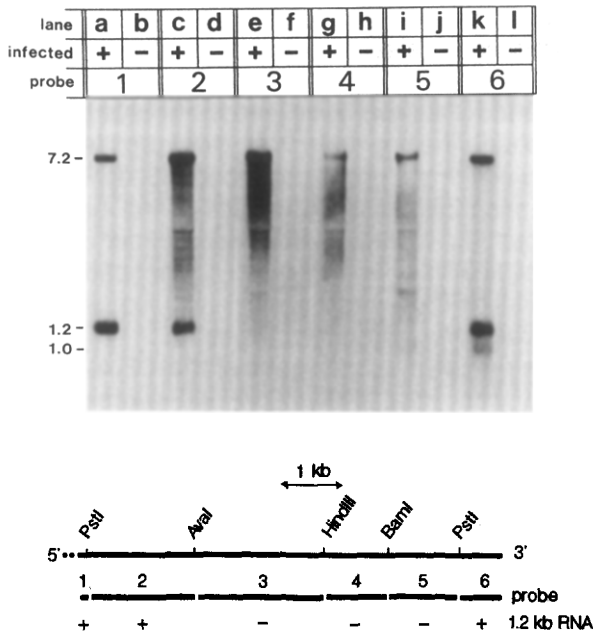


FIG. 2. Localization of CYMV gRNA sequences present in the 1.2-kb RNAs. Total polyribosomal RNA (5 μ g) isolated from healthy plants or from plants infected with a CYMV stock containing 1.2-kb RNAs was denatured, resolved by electrophoresis, and blotted to nylon. The blots were hybridized with a set of consecutive nick-translated cDNA probes (designated 1 through 6) corresponding to over 90% of CYMV's gRNA. Lanes a, c, e, g, i, and k contained identical samples of polyribosomal RNA from infected plants hybridized with probes 1, 2, 3, 4, 5, and 6, respectively. Lanes b, d, f, h, j, and l contained polyribosomal RNA from uninfected plants hybridized with probes 1, 2, 3, 4, 5, and 6, respectively. At the bottom are shown the general positions of the probes relative to gRNA.

panel of Fig. 2. Furthermore, oligonucleotides complementary to the 5' and 3' ends of CYMV gRNA each hybridized efficiently to the 1.2-kb RNAs under stringent conditions suggesting that these RNAs do contain the extreme termini of the gRNA (data not shown). The 1.2-kb RNAs, therefore, clearly constitute reasonably discrete entities containing the extremities of the CYMV gRNA, but not internal regions.

Primer extension analysis of the 5' termini of the 1.2-kb RNAs was carried out to determine if their structure was similar to that of the gRNA. The major extension products generated from purified 1.2-kb RNAs migrated to the same position as the major products generated when gRNA was extended with the same primer (Fig. 3, compare lane 3 with lanes 4 and 5). This demonstrates that the 1.2-kb RNAs and the gRNA contain the same number of residues between the primer and their termini. Primer extension also indicates that these RNAs, similar to the CYMV gRNA and sgRNAs, behave as if they were capped. We believe, therefore, that the extreme 5' terminus of the 1.2-kb RNAs is identical with that of the gRNA.

Cloning and sequencing of the 1.2-kb RNAs

We have cloned the 1.2-kb RNAs of CYMV by PCR amplification of cDNA produced from CYMV RNA preparations which contain it. Either total polyribosomal RNA or total RNA purified from virions was used as template for first-strand synthesis. CYMV RNAs were primed for reverse transcription with oligonucleotide CY-11 which is complementary to the first 23 residues upstream of the poly(A) tail of the CYMV gRNA and which also contains a poly(deoxythymidylate) tract and a *SalI* site 5' to the CYMV specific region. A second primer, CY-12, containing a sequence identical with the 5' end of the CYMV gRNA was used in conjunction with the first primer for the subsequent PCR reaction. The second primer also included a T7 RNA polymerase promoter and a *SalI* site 5' to the CYMV sequence. PCR products were analyzed by electrophoresis in agarose gels. Only preparations of CYMV RNA which contained the 1.2-kb RNAs (as determined by Northern blotting) yielded a product with electrophoretic mobility corresponding to approximately 1.2 kb. Following digestion with *SalI*, the 1.2-kb cDNA PCR product was ligated into the *SalI* site of the vector pSP64.

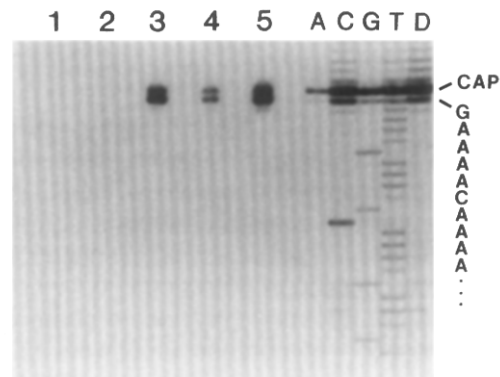


FIG. 3. Characterization of the 5' terminus of 1.2-kb CYMV RNAs. 1.2-kb RNAs were partially purified from total polyribosomal RNA from CYMV-infected plants or from total RNA extracted from virions by sucrose gradient centrifugation. These RNA preparations along with total polyribosomal RNA from uninfected plants and total polyribosomal RNA from CYMV-infected plants lacking 1.2-kb RNAs were analyzed by primer extension with oligonucleotide CY-2. The RNAs examined were: lane 1, no RNA; lane 2, polyribosomal RNA from uninfected leaves; lane 3, polyribosomal RNA from CYMV-infected leaves lacking 1.2-kb RNAs; lane 4, 1.2-kb RNAs from polyribosomes; lane 5, 1.2-kb RNAs from virions. A, C, G, and T contained the products of a dideoxynucleotide sequencing reaction carried out on purified CYMV gRNA using phosphorylated oligonucleotide CY-2 as primer (D, no dideoxynucleotide was added to the sequencing reaction). CAP indicates sequencing products corresponding to the 7 mG cap present at the 5' terminus of CYMV gRNA (Sit *et al.*, 1990). The partial sequence shown is the complement of the sequencing ladder.

The sequence of the prototype 1.2-kb RNA was determined by dideoxy nucleotide sequencing of pAW1 obtained from polyribosomal RNA (Fig. 4). The sequence of this RNA is 1172 nucleotides in length excluding a probable poly(A) tail. It contains 757 nucleotides from the 5' terminus of the CYMV gRNA and 415 nucleotides from the 3' terminus. The predicted size correlates well with the size of authentic 1.2-kb RNAs determined by electrophoresis. The sequence also confirms the analysis of the 1.2-kb RNAs by Northern blotting (Fig. 2) which suggested that only the 5' and 3' extremities of the gRNA are present. Twelve cDNA clones, six derived from polyribosomes and six from CYMV virions obtained from the same viral stock, were sequenced across the junction region (Fig. 4, arrow) and all yielded junctions identical with that in Fig. 4. Interestingly, this junction maintains the reading frame of the large 191-kDa nonstructural protein encoded at the 5' end of CYMV gRNA (ORF 1) into the coat protein reading frame situated at the 3' end of the gRNA. The

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1  GAAACAAAACGAAAACAAAACAAAUCUUCGUAAACACCCUCCACACACCAUUCUAAUCC 60
                               35 K M A R V R A S L S
61  CGAAUCGCCCGCAAACAGCAAAAGAUUCGCAUAAAUGGCCCGUGUACGAGCAUCUCUCUC
    Q F T D P S Q K I L I Q S D Q Y E N V K
121  CCAAUUCACCGCCACCAAAAUAUUUCUACAARAGCGACCAAUUGAAAAGUAAAA
    K T L G T Y K L T N P Y A H S E S T A D
181  GAAAACACUUGGCACAUACAACUAACCAACCCUAUCGCCACUCCGAGUCCACCGCUGA
    L L E D L G I A T N P F A A E P H T H G
241  CUUUAUAGAGAUUAGGUUAAGCAACUAAACCAUUCGUGCAGCAACACACACACAUUG
    A A K A I E N D L Y Y I A S T R M T K E
301  AGCUGCUAAAGCUAUAGAAAACGCAUUAUUAUACUAGCUUACACAGAAUGACUAAGGA
    E P V T F M F M K R A K L Q Y F R R G P
361  GGAACCGAUAAACUUCUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUA
    Q Q N D T F I N O I V E P K D V A R Y D
421  CCAACAAAAGACACAUCAUAAAUAUUAUUGUGGAGCCAAAGACGUGGCGAGAUAACGA
    E D T L H S T I P T I E T K T V F I G D
481  CGAAGACACCCUUCUACUCCACCAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUA
    T L H F L P P S F L T K L F A R N P K I
541  CACUCUCCACUCCUCCACCAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUA
    Q T V L A T M V L P T E A L Y G L T S L
601  CCAACAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUA
    Y P N V Y S L S Y H K P S K F R R K A L
661  AUUCCAAAAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAG
    F S Y A P G G H K G A E N W A S Q N Y K
721  CUUCUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAGUUAUAG
    E A D R F A A F D F F E G V S S S A A L
781  AGAAGCAGAGGUUUCGAGCAUUCGACUUCUUCGAGGAGUUCUUAUCCUCCGUGCCCU
    S P P G G L I R E P S P N E R M A N E T
841  AAGCCCCCAGGAGGUUAUUCGAGAAACCAAGCCAAUUAUUAUUAUUAUUAUUAUUAU
    N K N V H L Y Q T A S R G S N L A T T S
901  UAACAAGAACGUCCACCUUACCAACAGCAUCCGAGGCGAGCAUUCUUGCUAACACAG
    T V A T K G A Y S T N A S N A G F L I T
961  UACGGUAGCCACCAAGGAGCUUAUCUACCAACCGGUCCAAACCGUGGUUUCUUAUAC
    G P E *
1021  AGGCCCGGAGUAAACCAACCAACCAACCAUCUUAUUAUUAUUAUUAUUAUUAUUAUUA
1081  CGGCGUCCACUGGUUUUAGUUGCGGUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAU
1141  GGACUGCGAGACCCGAGACUUUUGGGUGUUAU
    
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Fig. 4. Sequence of the prototype 1.2-kb RNA of CYMV. A probable poly(A) tail is indicated by An. The predicted amino acid sequence of an encoded 35-kDa product is shown above the corresponding nucleotide sequence. The arrow indicates the position of the internal junction.

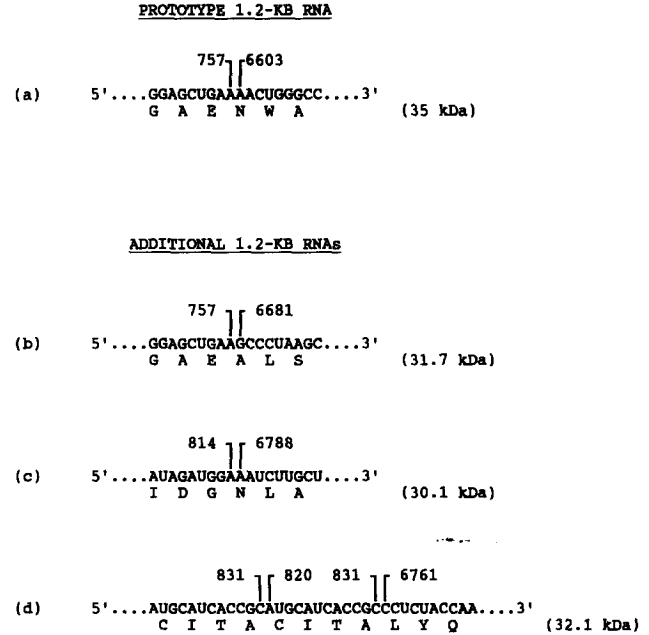


Fig. 5. Junction sites of various CYMV 1.2-kb RNA species. The partial RNA sequences corresponding to the junction region of the prototype 1.2-kb RNA (a) and several additional 1.2-kb RNAs containing different junction sites are shown (b-d). The nucleotides at the junction sites are numbered according to the scheme used for the gRNA of CYMV (Sit *et al.*, 1990). The predicted amino acid sequences of the fusion proteins are shown beneath the nucleotide sequences and the predicted sizes of the encoded fusion product are in parentheses to the right.

sequence predicts a 191-kDa/CP fusion protein of approximately 35 kDa (Fig. 4) which would contain 221 amino acids of the N-terminus of the 191-kDa non-structural protein and 91 amino acids of the C-terminus of coat protein.

In an effort to determine whether the 1.2-kb RNAs are internally homogeneous, we determined the sequences in their entirety of six other cloned 1.2-kb RNAs obtained from a polyribosomal RNA source different from that of the prototype. Three junction sequences, each differing from that of the prototype, were found among the six cloned 1.2-kb RNAs examined (Fig. 5, cf. a with b, c and d). As in the prototype, the juxtaposition of 5' and 3' sequences at the junction created an in-frame fusion between ORF 1 and the coat protein ORF in each case. The sequence of one of the additional 1.2-kb RNAs revealed the presence of a 12-base direct repeat at the junction site (Fig. 5,d). The insertion, however, maintained the open reading frame into the coat protein sequence. The authenticity of the predicted open reading frames in the additional 1.2-kb RNAs was verified by *in vitro* translation of synthetic transcripts from these cloned cDNAs (data not shown).

The majority of the sequence of the prototype 1.2-kb RNA (Fig. 4) and of additional 1.2-kb RNAs is identical

with the corresponding region of CYMV gRNA reported by Sit *et al.* (1990). There are five single-base alterations present in all 1.2-kb RNAs examined, two of which map in the 3' end of the coat protein coding region. These changes result in a conversion of the amino acid sequence of the C-terminus of the coat protein from PYHRPE (Sit *et al.*, 1990) to LITGPE. The other three single-base changes occur in the 3' non-coding region. These five single-base alterations detected in all 1.2-kb RNAs were also present in the gRNA from our viral stock (data not shown). This indicates that the 3' ends of the 1.2-kb RNAs are in fact faithful copies of the gRNA from which they were derived and that the sequence of the gRNA of our strain of CYMV has diverged from that of Sit *et al.* (1990). Our virus stock containing these differences is infectious and is efficiently replicated and encapsidated. Thus, these alterations in the 3' noncoding region and in the extreme C-terminus of coat protein confer no detectable phenotype. The prototype 1.2-kb RNA also contains two additional single-base substitutions at positions 193 (U→C) and 574 (C→U) which would not alter the amino acid sequence of the polypeptide relative to that of ORF 1 of CYMV. These two single-base substitutions are present only in the prototype 1.2-kb RNA and are not found in the gRNA of our stock.

Coding properties of the 1.2-kb RNAs

We have confirmed the potential coding properties of both native and *in vitro* transcripts of the prototype 1.2-kb RNA by *in vitro* translation and immunological techniques. Synthetic transcripts of the prototype 1.2-kb RNA were generated by runoff transcription of cloned cDNAs using T7 RNA polymerase. Cloned cDNAs pAW1 (prototype) and pAW2 used to generate the synthetic 1.2-kb transcripts were obtained from polyribosomal RNA or from virions, respectively. The RNA purified from virions used to clone pAW2 was also the source of the native 1.2-kb RNAs which were translated in this experiment. Aliquots of the *in vitro* translation reactions carried out in a rabbit reticulocyte lysate were separated electrophoretically. The major products synthesized from synthetic transcripts of pAW1 and pAW2 display identical mobilities and an estimated molecular weight of 35 kDa (Fig. 6, lanes d and e, respectively) as does an abundant polypeptide translated from preparations of CYMV RNA containing native 1.2-kb RNAs (Fig. 6, lane g). Templates lacking the 1.2-kb RNAs were unable to direct the synthesis of the 35-kDa product (Fig. 6, lane f). For comparison, the translation products synthesized from synthetic transcripts of a cloned cDNA encoding coat protein (pBALΔ6174; White and Mackie, 1990) were sepa-

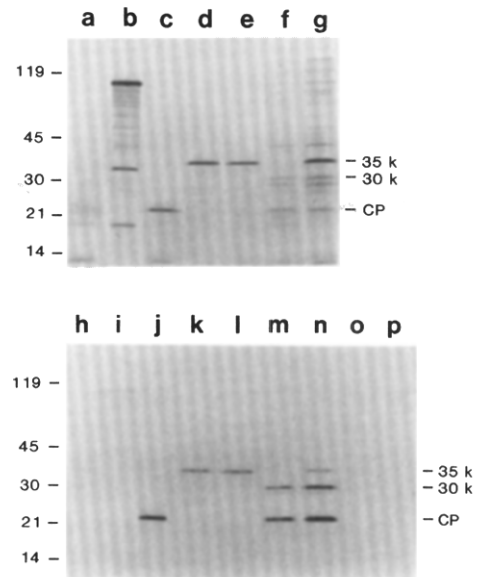


Fig. 6. Translation *in vitro* of native and synthetic transcripts of the CYMV 1.2-kb RNAs. Sequential transcription and translation of cloned cDNAs was carried out as described under Materials and Methods. In lanes a–g a portion of each translation reaction mixture was separated electrophoretically without further processing. In lanes h–p the *in vitro* translation products were immunoprecipitated with antisera to CYMV particles prior to electrophoresis. The templates for the *in vitro* translation reaction were: lane a, no added RNA; lane b, 0.25 μ g BMV RNA; lane c, a synthetic coat protein transcript; lanes d and e, synthetic 1.2-kb transcripts from pAW1 and pAW2; lane f, native RNA from CYMV virions free of 1.2-kb RNAs; lane g, native RNA from CYMV virions containing 1.2-kb RNAs. Lanes h–n contain immunoprecipitates of *in vitro* translation products shown in lanes a–g, respectively. Due to the high reactivity of coat protein with the anti-CYMV antisera only $\frac{1}{12}$ of the immunoprecipitate was applied to lane j compared to other lanes on the gel. In lane o immunoprecipitation of the *in vitro* translation product from lane e (i.e., 35-kDa product) was performed in the presence of unlabeled coat protein while in lane p immunoprecipitation of the *in vitro* translation product from lane e was performed with non-immune serum. The numbers in the left margin give the molecular weights (in thousands) of marker proteins separated in the same gels. The positions of CYMV coat protein (CP), the 35-kDa fusion product, and a 30-kDa coat protein readthrough product are shown on the right.

rated in lane c of Fig. 6. The size of the product generated from this RNA is consistent with its coding potential (White and Mackie, 1990). Since the translation of the RNA transcripts of pAW1 and pAW2 yields a product of the same relative molecular weight as that of native 1.2-kb RNAs, this further supports the authenticity of the cloned 1.2-kb RNA. Furthermore, the size (35 kDa) of these *in vitro* translation products is fully consistent with the predicted coding properties of the prototype 1.2-kb RNA.

To show that the 35-kDa polypeptides produced by *in vitro* translation of the 1.2-kb RNAs contained coat protein sequences, aliquots from the translation reac-

tions were immunoprecipitated with an anti-CYMV antiserum and separated by electrophoresis (Fig. 6, bottom). Coat protein is efficiently immunoprecipitated by this serum (Fig. 6, lanes j, m, and n). The 35-kDa product is also immunoprecipitated by this serum but less efficiently (Fig. 6, lanes k, l, and n). The lower affinity of the fusion protein for coat protein antibody may be due to the absence of N-terminal epitopes of coat protein on the 35-kDa protein since the fusion protein is predicted to contain only 91 of 212 amino acids of coat protein. Unlabeled coat protein is an effective competitor of precipitation of the 35-kDa product indicating that it is coat protein-specific antibodies which are reacting with this polypeptide (Fig. 6, lane o). Non-immune serum is unable to immunoprecipitate the 35-kDa protein (Fig. 6, lane p). Bands corresponding to approximately 30 kDa are visible in Fig. 6, lanes m and n. These represent a previously described coat protein readthrough product whose initiating codon lies 135 nucleotides upstream from the coat protein ORF (Bendena *et al.*, 1987; Sit *et al.*, 1990; White and Mackie, 1990).

DISCUSSION

We have used several approaches to characterize a group of 1.2-kb RNAs found in some CYMV infections of broad bean plants. The structure of these RNAs suggest that they represent DI-like RNAs and establish them as the first such RNAs to be found associated with a member of the potexvirus group. A number of features of the 1.2-kb RNAs of CYMV distinguish them from previously characterized plant virus DI RNAs. First, the presence of the 1.2-kb RNAs in CYMV infections of broad bean plants produces no apparent alteration of symptoms whereas the DI RNAs associated with several other plant viruses can enhance or attenuate symptoms (Hillman *et al.*, 1987; Burgyan *et al.*, 1989; Li *et al.*, 1989). Since the mechanism of symptom development is not understood, this is not necessarily problematic. Knorr *et al.* (1991) found that the appearance of some classes of TBSV DI RNAs is not immediately accompanied by symptom attenuation, suggesting that different species of DI RNAs may differ in their ability to interfere with symptoms. The 1.2-kb RNAs of CYMV may, therefore, represent an example of the inability of a defective RNA to attenuate virus-induced plant pathogenesis.

A second distinguishing feature of the 1.2-kb RNAs is that they are derived entirely from the 5'- and 3'-terminal regions of the CYMV gRNA and encode two partial viral ORFs which are fused in-frame to create a single ORF (Fig. 7). This fused ORF is present in all four 1.2-kb RNA species characterized, despite the fact that each

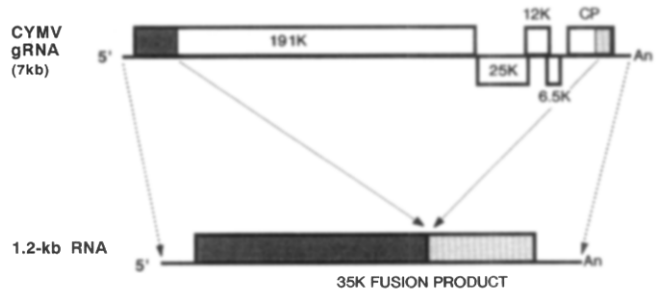


FIG. 7. Diagram of the general structure of the CYMV prototype 1.2-kb defective RNA. The organization of coding regions on the 7-kb gRNA of CYMV are shown with approximate M_r values of the encoded proteins (Sit *et al.*, 1990). Coding regions from which the 1.2-kb RNA (shown below) is derived are shaded.

of these RNAs possesses a unique junction site. The presence of such ORFs in these RNAs has been verified by translation *in vitro* of native and synthetic 1.2-kb RNAs. Moreover, these RNAs are likely translated *in vivo* since they are present in polyribosomes. The poor reactivity of the fusion protein with coat protein antibody, however, deterred any attempt to identify this protein in extracts from infected tissue. Interestingly, DIssE RNA associated with mouse hepatitis virus exhibits a structure and coding strategy similar to the 1.2-kb RNAs of CYMV (Makino *et al.*, 1988). In this case, the DI-encoded products have been detected in infected cells.

A third feature of the 1.2-kb RNAs of CYMV is that their coding capacity is much larger than that observed for other characterized plant virus DI RNAs. About 80% of the CYMV prototype 1.2-kb RNA forms a continuous coding sequence (Fig. 7). Since all four sequenced members of the 1.2-kb RNA family encode a 191-kDa/coat protein fusion protein, maintenance of this fusion ORF may be advantageous, if not essential, for these RNAs. One possibility is that translation of the 1.2-kb RNAs may stabilize them. The influence of translation on the stability of a plant mRNA has been described by Vancanneyt *et al.* (1990) who found that reducing the size of the ORF on the patatin mRNA led to a decrease in its steady-state level. Alternatively, the fused product may contain an RNA binding domain and could accordingly play a direct role in the replication or survival of the 1.2 kb RNAs.

The 1.2-kb RNAs of CYMV are clearly defective as they lack up to 5.8 kb (approx 80%) of the CYMV genome. Consequently, three entire ORFs and most of the "replicase" and coat protein coding regions are deleted (Fig. 7). This would abolish the ability of these RNAs to replicate autonomously. Indeed, synthetic transcripts of the prototype 1.2-kb RNA replicate only when coinoculated with CYMV gRNA (White, Bancroft, and Mackie, unpublished results).

We cannot identify the initial events which led to the contamination of some of our stocks of CYMV with defective 1.2-kb RNAs. Our attempts to regenerate these RNAs from purified virus by serial passages from sap were not successful even though a similar approach ultimately succeeded for TBSV (Morris *et al.*, 1988; Knorr *et al.*, 1991). While we cannot rule out the possibility that the conditions under which our passages were performed were suboptimal for generation of this type of RNA, it seems more likely that the successful formation of defective CYMV RNAs may be a rare event. We believe that to a large extent the initial deletion generating a defective RNA may be random. Subsequent propagation of the CYMV defective RNA seems to require an exact in-frame fusion of two protein coding sequences within limited regions of the genome, maintenance of encapsidation signals, and conservation of terminal nucleotide sequences which we presume to be necessary for RNA replication.

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