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Construction of full-length cDNA clones of cucumber mosaic virus RNAs 1, 2 and 3: Generation of infectious RNA transcripts

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Summary. Full-length cDNA copies of cucumber mosaic virus (CMV) RNAs 1 and 2 of the Fny strain were constructed from partial cDNA clones and were cloned downstream of bacteriophage T7 promoters. In one pair of clones, transcription proceeded from an unaltered T7 promoter such that in vitro transcripts representing RNAs 1 and 2 contained an additional 17 nucleotides at their 5′ termini. In a second pair of clones, the T7 promoter/cDNA junction was altered by oligonucleotide-directed mutagenesis such that the in vitro transcripts contained only an additional G residue at their 5′ ends. In addition, a full-length cDNA copy of Fny-CMV RNA 3 was constructed from two overlapping cDNA clones and was cloned downstream of an altered T7 promoter such that the resultant in vitro transcripts also contained only an additional G residue at their 5′ ends. In vitro transcripts derived from all clones contained an additional C residue at their 3′ ends. In vitro transcripts representing RNAs 1, 2 and 3 which contained an additional residue at each terminus were shown to be infectious together in several hosts of CMV.

Key words: Recombinant DNA – Plant virus – T7 promoter – In vitro transcription

Introduction

Cucumber mosaic virus (CMV) is a positive-sense RNA plant virus that has a tripartite genome consisting of RNAs 1, 2 and 3 in decreasing order of M_r (Peden and Symons 1973). Virions of CMV also encapsidate RNA 4, the mRNA for coat protein synthesis which is a subgenomic RNA generated from the 3′ half of RNA 3 (Peden and Symons 1973; Schwinghamer and Symons 1975; Gould and Symons 1978).

Recently, the complete nucleotide sequences of RNA 1 (Rizzo and Palukaitis 1989) and RNA 2 (Rizzo and Palukaitis 1988) of the Fny strain of CMV have been determined. Fny-CMV RNA 1 is composed of 3357 nucleotides (nt) and encodes a 111404 M_r protein while Fny-CMV RNA 2 consists of 3050 nt and encodes a 96720 M_r protein. By analogy with studies involving brome mosaic bromovirus (BMV) (Kiberstis et al. 1981) and alfalfa mosaic virus (AlMV) (Nassuth and Bol 1983), both of which have tripartite genomes, the proteins encoded by CMV RNAs 1 and 2 are believed to be involved in the replication of the viral genome. Furthermore, Rezaian et al. (1984; 1985) have shown extensive nucleotide sequence homology between the RNAs 1 and between the RNAs 2 of AlMV, BMV and CMV. Other nucleotide sequence comparisons have suggested that RNA 1 may encode a nucleotide-binding protein while RNA 2 may encode the polymerase subunit of the replicase (Kamer and Argos 1984; Hodgman 1988). Finally, Nitta et al. (1988) have demonstrated that only CMV RNAs 1 and 2 are necessary to induce viral replicase activity in protoplasts, while all three CMV RNAs are necessary to infect whole plants (Peden and Symons 1973). Together, these studies strongly suggest that CMV RNAs 1 and 2 encode proteins of the viral replicase.

The development of in vitro transcription systems using bacteriophage promoters, and the advances in cDNA cloning technologies have made it possible to generate full-length viral RNAs from cDNA copies. Since a large number of RNA viruses have no natural DNA intermediates, manipulation of cloned cDNAs by site-specific mutagenesis allows one to obtain mutant RNA viruses that can be examined to elucidate the mechanisms of gene expression. Biologically active transcripts have been derived from cDNA clones of several plant and animal viruses (Ahlquist et al. 1984; Mizutani and Colonno 1985; Loesch-Fries et al. 1985; Dasmahapatra et al. 1986; Dawson et al. 1986; Langereis et al. 1986; Meshi et al. 1986; van der Werf et al. 1986; Rice et al. 1987; Vos et al. 1988; Ziegler-Graff et al. 1988; Hamilton and Baulcombe 1989; Domier et al. 1989; Weiland and Dreher 1989).

This paper describes the construction of transcription vectors containing full-length cDNA clones of Fny-CMV RNAs 1, 2 and 3 from which in vitro transcripts can be generated. Together, transcripts representing Fny-CMV RNAs 1, 2 and 3 were shown to be infectious in several host plants. These cloned cDNAs in transcription vectors, in conjunction with their nucleotide sequences, are a valuable tool for studying CMV gene expression at the molecular level.

Material and methods

DNA purification and manipulation. Alkaline lysis (Birnboim and Doly 1979) was used in the large scale isolation of plasmid DNA. DNA fragments were separated by electrophoresis and extracted from either agarose gels (Dretzen

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et al. 1981) or from low melting temperature agarose gels (Maniatis et al. 1982). *Escherichia coli* cells were made competent and transformed with plasmid DNA as described by Messing (1983). The procedure of Birnboim and Doly (1979) was used to screen recombinant clones rapidly. Single-stranded DNAs of pIBI76 (International Biotechnologies, 1986) derivatives were obtained with the aid of helper phage M13K07 (Vieira and Messing 1987) by the method of Dente and Cortese (1987).

Complementary DNA cloning. Fny-CMV was propagated and isolated, and the viral RNAs were extracted and purified as previously described (Palukaitis and Zaitlin 1984). Complementary DNA clones specific to the 5′ ends of RNAs 2 and 3 were prepared by the method of Ahlquist (1986) using total Fny-CMV RNA.

For the RNA 2-specific clone, first strand cDNA was primed with an oligonucleotide (5′-ACATCCTCGGGAGTGTCGAC-3′) which is complementary to nt 144–163 of Fny-CMV RNA 2. This sequence is located at the end of the cDNA insert of pFny200 which represents the 5′ terminal region of RNA 2 (see Results and discussion). The 3′ terminal six nt of this oligomer contain a *Sal*I site. Second strand cDNA was primed with an oligonucleotide (5′-GGATCCGCATGCGTTTATTTACAAGAGCG-3′), the 3′ terminal 17 nt of which correspond exactly to the 5′ end of Fny-CMV RNA 2. In addition, the 5′ terminal 12 nt of this oligomer contain a *Bam*HI site and an *Sph*I site.

A cDNA clone specific for the 5′ terminal 650 nt of RNA 3 was prepared as follows. First strand cDNA was primed with an oligonucleotide (5′-CACGCTAGCTGTGGTACCGG-3′) which is complementary to nt 631–650 of Fny-CMV RNA 3. This sequence is located near the end of the cDNA insert in pBS-Fny3 which corresponds to the 5′ terminal region of RNA 3 (see Results and discussion). This oligomer contains an *Nhe*I site. Second strand cDNA was primed with an oligonucleotide (5′-GGATCCTAATACGACTCACTATAGGTAATCTTAC-3′), the 3′ terminal ten nt of which correspond exactly to the 5′ end of Fny-CMV RNA 3 (J. Owen and P. Palukaitis, unpublished results). In addition, the 5′ terminal six nt contain a *Bam*HI site. Between the *Bam*HI site and the sequence corresponding to the 5′ end of RNA 3 lies an altered T7 promoter sequence. The cloning and nucleotide sequence of Fny-CMV RNA 3 (J. Owen, M. Shintaku and P. Palukaitis unpublished results) will be described elsewhere.

Double-stranded cDNA was blunt end-ligated to pUC18 linearized with *Hin*cII (Norrander et al. 1983). Cells of *Escherichia coli* strain DH5α (Jesse 1986) were made competent and transformed with the ligation mixtures. The resultant white colonies were blotted onto nitrocellulose and probed with randomly primed, ^{32}P-labeled cDNA made to total Fny-CMV RNA, and transformants carrying CMV-specific cDNA clones were detected. Plasmid DNAs from CMV-specific transformants were screened for the presence of inserts by restriction enzyme analysis.

Construction of synthetic DNA fragments. The 3′ terminal nucleotides absent from the cDNA inserts of pFny100, pFny200 and pBS-Fny3 (see Results and discussion) were added using DNA fragments prepared as follows. Three pairs of oligonucleotides (5′-GTGGGGGTCTCTAAAAGGAGACCACTGCAGTTTC-3′ and 5′-CTGCAGTGGTCTCCTTTTAGAGACCCCCACGAAA-3′ for pFny100; 5′-GTGGGGCCTCCAAAAGGAGACCACTGCAGTTTT-3′ and 5′-CTGCAGTGGTCTCCTTTTGGAGGCCCCACAAAA-3′ for pFny200; 5′-GTGGGGGCCTCCAAAAGGAGACCACTGCAGTTTC-3′ and 5′-CTGCAGTGGTCTCCTTTTGGAGGCCCCCACGAAA-3′ for pBS-Fny3) were synthesized; the members of each pair are complementary to each other. The oligonucleotides were phosphorylated at their 5′ ends in separate 10 µl reaction mixtures containing 50 mM TRIS-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1.0 mM ATP, 5.0 units of T4 polynucleotide kinase (United States Biochemicals) and 1.0 µg of oligonucleotide. Incubation was at 37° C for 30 min. Subsequently, each phosphorylated oligonucleotide in the above reaction mixture was combined with its complementary partner. One microliter of 1 M NaCl was added to each of the three resultant 20 µl reaction mixtures which then were held at 100° C for 2 min and slowly cooled to room temperature to anneal the complementary oligonucleotides.

DNA sequencing. Single-stranded plasmid DNAs obtained with the use of helper phage were sequenced by the dideoxynucleotide chain termination method (Sanger et al. 1977; 1980), except that 7-deaza-dGTP was used instead of dGTP to alleviate band compressions (Mizusawa et al. 1986).

Nucleic acid hybridizations. For colony hybridization, DNA from bacterial colonies was fixed to nitrocellulose as described by Maniatis et al. (1982). Nitrocellulose filters were probed by a method described by Palukaitis (1984) with randomly primed, ^{32}P-labeled cDNA made to total Fny-CMV RNA as described previously (Palukaitis 1986).

Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis was performed as described by Kunkel et al. (1987). Single-stranded plasmid DNA, purified from *E. coli* strain CJ236 (Kunkel et al. 1987) with the aid of helper phage, was used as template in the mutagenesis reactions. Deletion mutagenesis of pFny1 and pFny2 (see Results and discussion) was directed by the following oligonucleotide to give pFnyΔ1 and pFnyΔ2, respectively: 5′-ACTCACTATAGTTTATTTAC-3′. Insertion mutagenesis of pFnyΔ1 and pFnyΔ2 was directed by the following oligonucleotide to give pFnyΔ1::1 and pFnyΔ2::1, respectively: 5′-GACTCACTATAGGTTTATTTAC-3′. The mutagenesis reaction mixtures were used to transform cells of competent *E. coli* strain JM101 (Messing 1979).

Transformants putatively carrying pFnyΔ1 or pFnyΔ2 were screened by isolating mini-preparation plasmid DNA and assaying these plasmids for the loss of a *Hin*dIII site present in the DNA segment removed from pFny1 and pFny2. Transformants putatively carrying pFnyΔ1::1 or pFnyΔ2::1 were screened by purifying single-stranded plasmid DNAs and sequencing the T7 promoter. The cDNA inserts and the fused T7 promoters of pFnyΔ1, pFnyΔ2, pFnyΔ1::1 and pFnyΔ2::1 were subsequently sequenced.

In vitro transcription. Transcription reactions contained 40 mM TRIS-HCl (pH 8.0), 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine-$(HCl)_3$, 5 mM DTT, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.5 mM UTP, 2.0 units of T7 RNA polymerase (United States Biochemicals) per µl and 100 ng per µl of plasmid DNA linearized with *Pst*I. Transcription reaction volumes ranged from 100–250 µl. For

capped transcripts, 0.5 mM m^7GpppG (New England Biolabs) was included in the reaction mixture and the GTP concentration was reduced to 75 μM. Incubation was conducted at 37° C for 1 h. After completion of the transcription reactions, 0.2 units of RQ1 DNase (Promega) per μl of reaction mixture was added and incubation was continued at 37° C for an additional 10 min. Reaction mixtures were subsequently extracted sequentially with phenol and chloroform. RNA was ethanol-precipitated and resuspended in H_2O.

Infectivity assays. Capped in vitro transcripts generated from pFny109, pFny209 and pFny309 (see Results and discussion) were used to inoculate leaves of *Chenopodium quinoa*, tobacco (*Nicotiana tabacum* cv. Xanthi nc) and zucchini squash (*Cucurbita pepo* cv. Black Beauty). Ten six-inch-tall *C. quinoa* plants, five 4–6 leaf-stage tobacco plants, and three 10–12-day-old squash plants were held in the dark for 24 h prior to inoculation. The younger leaves of the *C. quinoa* and tobacco plants, and the cotyledons of the squash plants then were dusted lightly with carborundum and the entire surface of one or two leaves of each plant was rubbed with 5 μl of inoculum containing 1.0 μg of each transcript in 50 mM TRIS-HCl (pH 8.6), 50 mM Na_2HPO_4 using a glass paddle. For the *C. quinoa* plants, another leaf of each plant was mock-inoculated with buffer alone. After inoculation, leaves were rinsed of carborundum and the plants were held in an environmentally-controlled chamber with a 14 h photoperiod and a constant temperature of 24° C. Leaves of tobacco and squash plants that developed systemic symptoms were harvested, virions were purified and viral RNA was extracted and analyzed by agarose gel electrophoresis as previously described (Palukaitis and Zaitlin 1984).

Enzymes and chemicals. Restriction endonucleases were purchased from Amersham, Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs and United States Biochemicals. T4 DNA ligase and Klenow fragment of *E. coli* DNA polymerase I were obtained from United States Biochemicals. Avian myeloblastosis virus reverse transcriptase was purchased from Promega Biotec and United States Biochemicals. All enzymes were used as recommended by their manufacturers. Oligonucleotides were obtained from the Cornell Biotechnology Program. Amersham and New England Nuclear supplied α-[^{32}P]dATP (400–800 Ci/mmol).

Results and discussion

Construction of pFny106 and pFny206

The transcription vector pIBI76 was used in the construction of plasmids containing full-length cDNA clones specific for RNAs 1 and 2 to give pFny106 and pFny206, respectively. These plasmids were constructed such that in vitro transcription would proceed from the bacteriophage T7 promoter of pIBI76.

The full-length cDNA insert of pFny106 was constructed first by ligating cDNA fragments of pFny101 and pFny100 (Rizzo and Palukaitis 1989) to give pFny103 as shown in Fig. 1. Plasmid Fny101 contains a 0.55 kb cDNA insert which is 5′ coterminal with RNA 1; the authentic 5′ terminal viral sequence of this cDNA insert is flanked by an *Sph*I site. Plasmid Fny100 contains a 2.9 kb insert which is 3′ coterminal with RNA 1; however, pFny100 lacks the 3′ terminal seven nt of RNA 1.

The 3′ terminal nucleotides absent from the cDNA insert of pFny100 were added as follows. Two oligonucleotides were synthesized and annealed (see Materials and methods) to yield a DNA fragment illustrated in Fig. 1. This synthetic DNA fragment contained the seven nt (representing the 3′ terminus of RNA 1) absent from the cDNA insert of pFny100, a *Pst*I site flanking the 3′ end of these seven nt and *Bst*XI-compatible cohesive ends. This fragment was ligated to pFny103 that had been linearized at a unique *Bst*XI site very close to the end of the CMV cDNA insert corresponding to the 3′ end of RNA 1. The resulting full-length, RNA 1-specific cDNA insert flanked by unique *Sph*I and *Pst*I sites at its 5′ and 3′ ends, respectively, was cleaved from pFny104 and ligated to pIBI76 to give pFny106 as shown in Fig. 1.

The virtually full-length RNA 2-specific cDNA insert of pFny200 (Rizzo and Palukaitis 1988) was used in the construction of pFny206 as shown in Fig. 2. The cDNA insert of pFny200 is 3.0 kb in length and lacks the 5′ terminal 19 nt and the 3′ terminal 4 nt of RNA 2. As with pFny100, the 3′ terminal 4 nt were added by ligating a synthetic DNA fragment to pFny200 linearized with *Bst*XI to give pFny204 (Fig. 2).

Oligonucleotide primers were used to generate cDNA clones specific to the 5′ terminal 150 nt of Fny-CMV RNA 2 (see Materials and methods). Subsequent nucleotide sequencing of the cDNA insert of one such clone (pFny203), and comparison with the nucleotide sequence of Fny-CMV RNA 2, revealed that this insert represented the authentic 5′ terminal 150 nt of RNA 2. Since the oligonucleotide used to prime first strand synthesis contained a *Sal*I site, the cDNA inserts of pFny200 and pFny203 both contained this common *Sal*I site. In addition, since the oligonucleotide used to prime second strand cDNA synthesis contained an *Sph*I site adjacent to the authentic 5′ end of the RNA 2 sequence, the cDNA insert of pFny203 could be cleaved from the vector by restriction with *Sal*I and *Sph*I. As illustrated in Fig. 2, this cDNA fragment was ligated together with the cDNA fragment of pFny204 to pIBI76 to give pFny206. As in pFny106, the full-length cDNA insert of pFny206 is flanked by unique *Sph*I and *Pst*I sites at its 5′ and 3′ ends, respectively.

Modifications of bacteriophage T7 promoter sequences

In vitro transcripts derived from the T7 promoter have vector residues at their 5′ termini (Fig. 3). Van der Werf et al. (1986) and Janda et al. (1987) have demonstrated that additional 5′ terminal residues severely reduce the infectivity of in vitro transcripts generated from full-length viral cDNAs in transcription vectors. Therefore, the T7 promoter of pIBI76 was fused to the cDNAs of CMV RNAs 1 and 2 by oligonucleotide-directed mutagenesis. This fusion was accomplished by subcloning small 5′ terminal cDNA fragments from pFny101 and pFny203 into pIBI76 to give pFnyl and pFny2, respectively (Fig. 4). Single-stranded pFny1 and pFny2 purified with the aid of helper phage served as templates in the oligonucleotide-directed mutagenesis reactions (see Materials and methods). Deletion mutagenesis was performed on pFny1 and pFny2 such that 17 nt of the vector were removed from the T7 promoter/

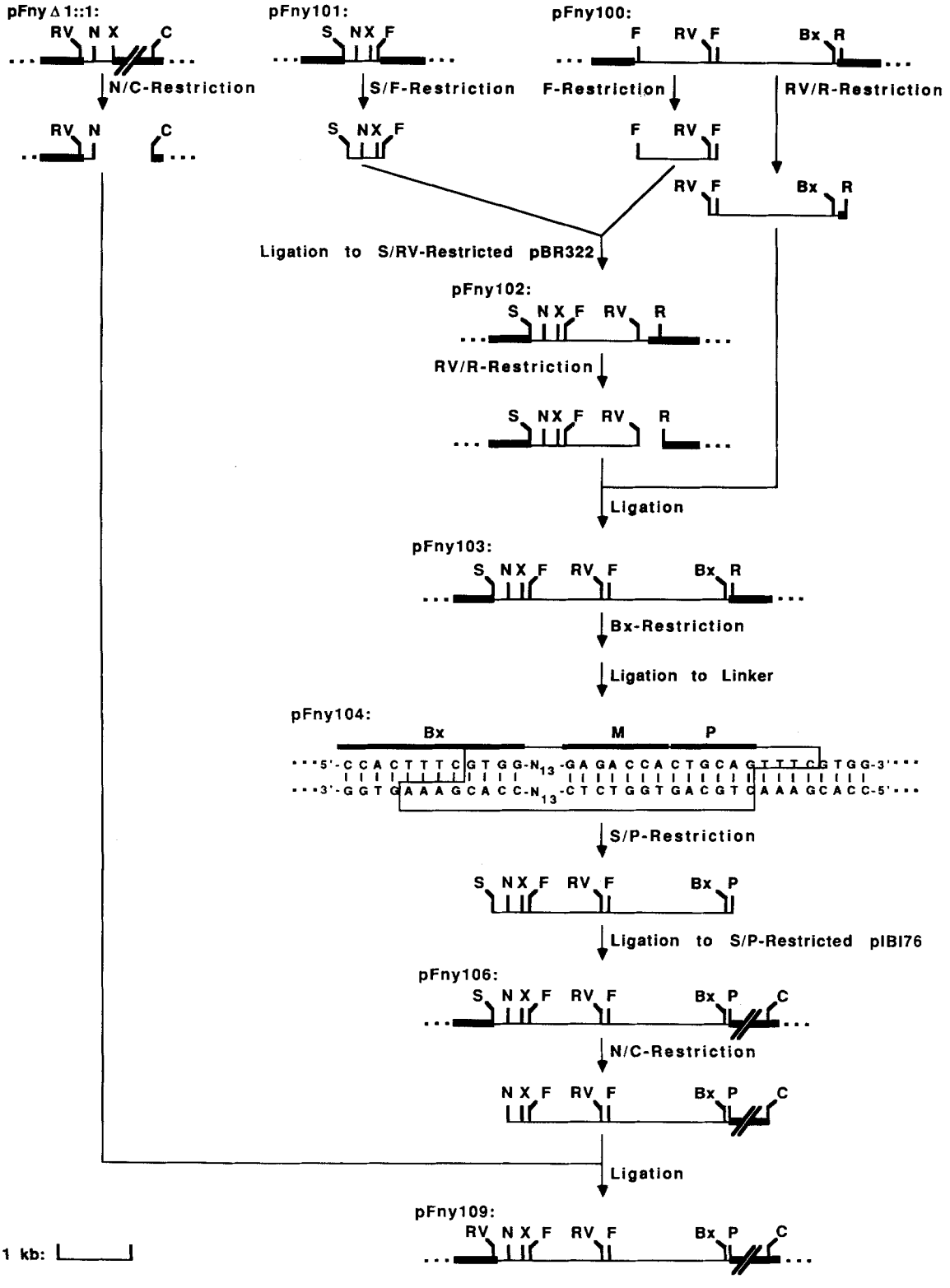

Fig. 1. Construction of transcription vectors containing full-length cDNA clones of CMV RNA 1: pFny106 and pFny109. These two plasmids are identical except that pFny106 contains an additional 16 nucleotides (nt) at its T7 promoter/cDNA junction. Within each construct, the *thin lines* represent cDNA inserts while the *thick lines* represent vector DNA. A DNA segment derived from a synthetic fragment is *boxed*. Bx, *Bst*XI; C, *Cla*I; F, *Fsp*I; M, nucleotides absent from the cDNA insert of pFny100; N, *Nru*I; N_{13}, a 13 nt-long segment; P, *Pst*I; R, *Eco*RI; RV, *Eco*RV; S, *Sph*I; X, *Xba*I

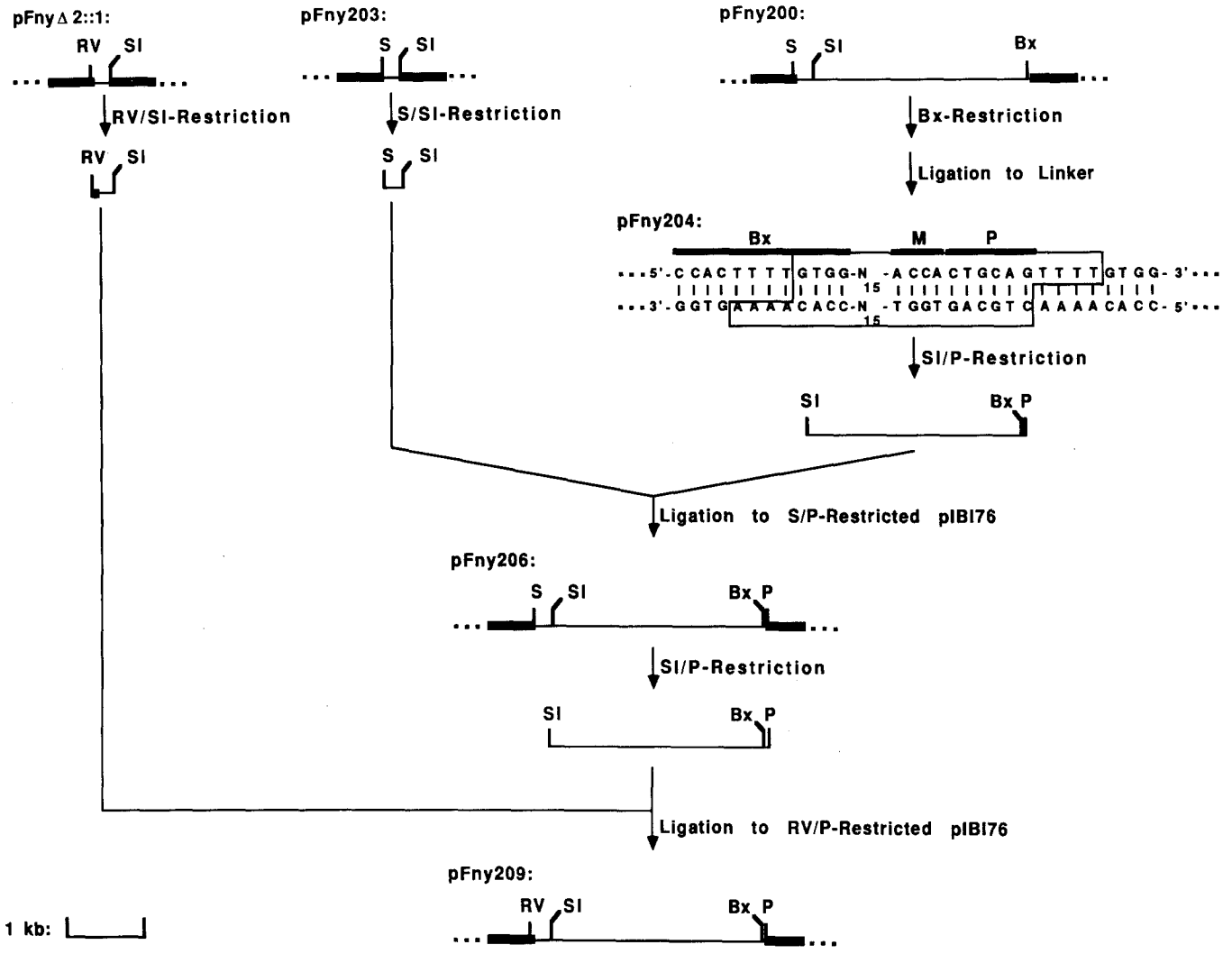

Fig. 2. Construction of transcription vectors containing full-length cDNA clones of CMV RNA 2: pFny206 and pFny209. These two plasmids are identical except that pFny206 contains an additional 16 nt at its T7 promoter/cDNA junction. Within each construct, the *thin lines* represent cDNA while the *thick lines* represent vector DNA. A DNA segment derived from a synthetic fragment is *boxed*. Bx, *Bst*XI; M, nucleotides absent from the cDNA insert of pFny200; N_{15}, a 15 nt-long segment; P, *Pst*I; RV, *Eco*RV; S, *Sph*I; Sl, *Sal*I

T7 promoter:	TAATACGACTCACTATA⌈GGGAGA
pFny1, 2, 106, 206:	··· TAATACGACTCACTATA⌈GGGAGAAGCTTGCATGCGTTTAT ···
pFnyΔ1, Δ 2, 108, 208:	··· TAATACGACTCACTATA⌈GTTTAT ···
pFnyΔ1::1, Δ 2::1, 109, 209:	··· TAATACGACTCACTATA⌈GGTTTA ···
pFny309:	··· TAATACGACTCACTATA⌈GGTAAT···

Fig. 3. DNA sequences surrounding the T7 promoter/cDNA junctions of plasmids containing cDNA specific to CMV RNAs 1, 2 or 3. *Underlined letters* represent viral cDNA sequences. The cDNA sequences shown here correspond to the 5′ terminus of RNAs 1, 2 or 3. Each transcription initiation site is designated by an *arrow*

cDNA junction to give pFnyΔ1 and pFnyΔ2, respectively (Figs. 3 and 4). Plasmid FnyΔ1 and pFnyΔ2 were designed so that in vitro transcripts generated from them would have no vector residues at their 5′ termini. However, examination of Fig. 3 reveals that the region of the T7 promoter altered in pFnyΔ1 and pFnyΔ2 (i.e. the 3′ terminal six nt of the promoter) fits the T7 promoter consensus sequence poorly: only one of the six nucleotides matches.

To obtain a modified promoter that more closely resembled the T7 promoter consensus sequence, insertion mutagenesis was performed on pFnyΔ1 and pFnyΔ2 to give pFnyΔ1::1 and pFnyΔ2::1, respectively (Figs. 3 and 4). In this configuration, three of the six 3′ terminal nucleotides of the altered T7 promoter of pFnyΔ1::1 and pFnyΔ2::1 match the T7 promoter consensus sequence (Fig. 3). Although in vitro transcripts generated from pFnyΔ1::1 and pFnyΔ2::1 would have one additional G residue at their 5′ ends, Janda et al. (1987) have shown that an additional G residue at the 5′ ends of in vitro transcripts derived from full-length cDNAs of BMV reduces the specific infectivity only threefold.

Transcriptional activities of the T7 promoters of pFny1,

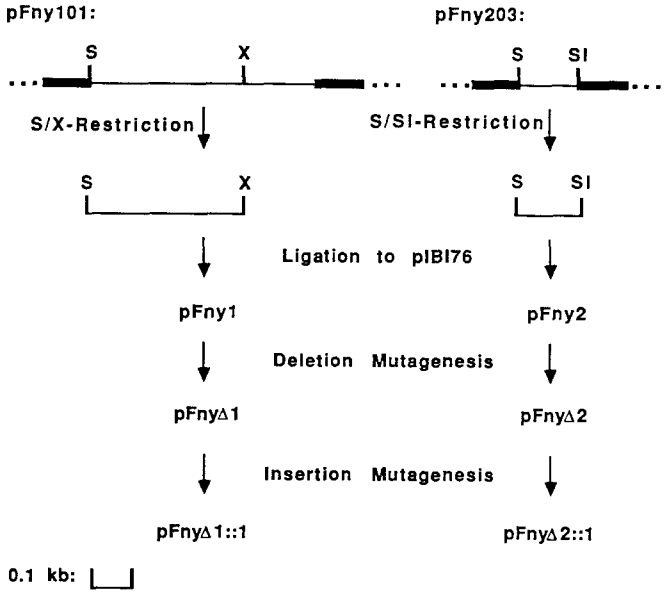

Fig. 4. Construction of transcription vectors containing 5′ terminal cDNA fragments derived from an RNA 1-specific plasmid (pFny101) and an RNA 2-specific plasmid (pFny203). The resultant DNA sequences surrounding the T7 promoter/cDNA junctions are shown in Fig. 3

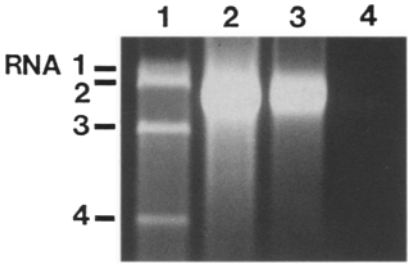

Fig. 5. Transcriptional activities of an unmodified and modified T7 promoters. In each case, 5.0 μg of plasmid linearized with *ScaI* was transcribed and the reaction mixture was treated as described in Materials and methods. Total RNAs derived from pFny 1 (lane 2), from pFnyΔ1::1 (lane 3) and from pFnyΔ1 (lane 4) were electrophoresed in 1.5% agarose containing ethidium bromide. Lane 1 contains Fny-CMV virion RNAs which are numbered at the left. The nucleotide sequences of the T7 promoters of pFny1, pFnyΔ1::1 and pFnyΔ1 are given in Fig. 3

pFnyΔ1 and pFnyΔ1::1 were compared. Plasmid DNAs linearized at a *ScaI* site within the vector DNA were used as templates for run-off transcription. Resultant RNAs were predicted to be 2.3 kb long. Figure 5 shows the total amounts of RNA produced from 5.0 μg of each template. While the T7 promoter of pFnyΔ1::1 showed reduced transcriptional activity relative to the unaltered T7 promoter of pFnyl, the T7 promoter of pFnyΔ1 was virtually inactive.

Construction of pFny109 and pFny209

The 5′ terminal cDNA fragments of pFnyΔ1::1 and pFnyΔ2::1 containing the modified but functional T7 promoter were used in the construction of transcription vectors containing full-length cDNAs of RNAs 1 and 2 (pFny109 and pFny209, respectively) as illustrated in Figs. 1 and 2. Plasmid Fny106 and pFny109, and pFny206 and pFny209, respectively, are identical except in the number of vector residues at their T7 promoter/cDNA junctions (Fig. 3).

Construction of pFny309

A full-length, RNA 3-specific cDNA clone under the control of a T7 promoter was constructed in pUC18 to give pFny309. The RNA 3-specific cDNA insert of pBS-Fny3 (Owen and Palukaitis 1988) was used in this construction as shown in Fig. 6. Nucleotide sequencing of the cDNA insert of pBS-Fny3 (J. Owen, unpublished results), and comparison with the nucleotide sequence of O-CMV RNA 3 (Hayakawa et al. 1989), indicated that this plasmid contains 1.7 kb of cDNA representing the 3′ terminal nucleotides of Fny-CMV RNA 3. However, pBS-Fny3 does not contain cDNA representing the 5′ terminal 0.51 kb of RNA 3 and also lacks the 3′ terminal four nt of RNA 3. As with pFny100 and pFny200, the 3′ terminal nucleotides absent from the cDNA insert of pBS-Fny3 were added by ligating a synthetic DNA fragment to pBS-Fny3 linearized with *BstXI* to give pFny301 (Fig. 6).

Oligonucleotide primers were used to generate cDNA clones specific to the 5′ terminal 0.65 kb of Fny-CMV RNA 3 (see Materials and methods). One clone (pFny302) contained the expected 0.65 kb cDNA insert (M. Shintaku, unpublished results). The oligonucleotide used to prime first strand cDNA synthesis in pFny302 contained an *NheI* site and the oligonucleotide used to prime second strand synthesis in pFny302 contained an altered T7 promoter sequence adjacent to the authentic 5′ end viral sequence. Thus, the cDNA insert of pFny302 is flanked at the terminus representing the 5′ end of RNA 3 by a T7 promoter and at the other terminus by an *NheI* site common to pFny301. A cDNA fragment from pFny301 representing the 3′ end of RNA 3 was ligated to pFny302 as shown in Fig. 6 to give pFny309. The full-length cDNA insert of pFny309, therefore, is flanked by the altered T7 promoter (Fig. 3) and a *PstI* site at its 5′ and 3′ ends, respectively.

Infectivity of in vitro transcripts representing CMV RNAs 1, 2 and 3

Capped transcripts representing RNAs 1, 2 and 3 were generated from pFny106, pFny206, and pFny309, respectively. These transcripts were translated in a rabbit reticulocyte lysate and the resultant products were examined by gel electrophoresis. In all three cases, a protein comigrated with the observed translation product of virion Fny-CMV RNAs 1, 2 or 3 (data not shown), indicating that the open reading frames were intact.

Capped transcripts representing RNAs 1, 2 and 3 also were generated from pFny109, pFny209 and pFny309, respectively. Together, these transcripts were used to inoculate one leaf from each of ten *C. quinoa* plants. As a control, one leaf from each of the ten plants was mock-inoculated. Inoculation of *C. quinoa* with total Fny-CMV virion RNAs results in the development of chlorotic local lesions. Within 3 days, all leaves inoculated with the in vitro transcripts showed numerous chlorotic local lesions while the mock-inoculated leaves showed none (data not shown). Moreover, sap from the chlorotic local lesions was highly infectious on both tobacco and squash (data not shown).

Initially, only one in five tobacco plants and one in three squash plants inoculated with the in vitro transcripts became infected; virus purified from these two plants con-

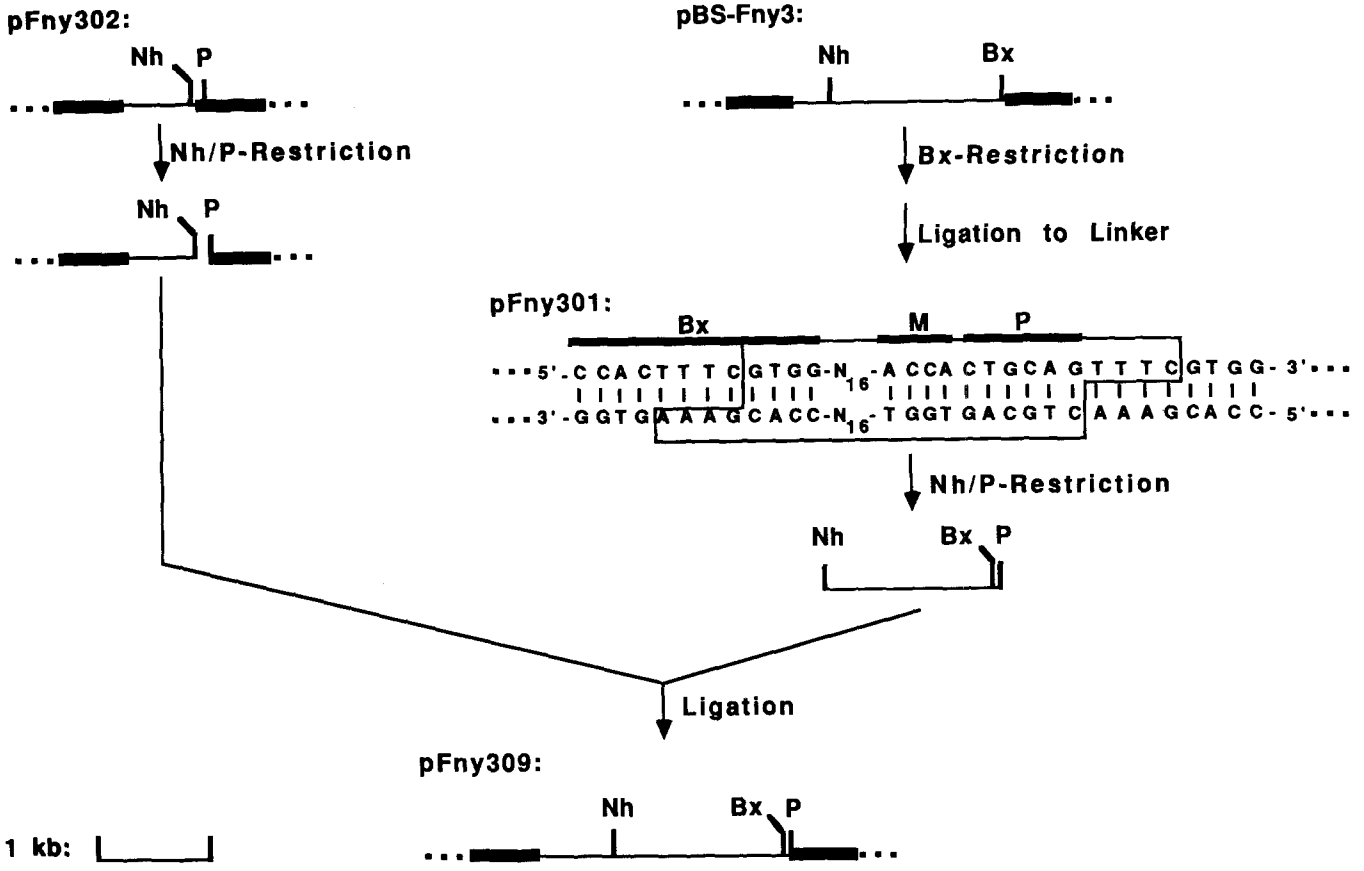

Fig. 6. Construction of a transcription vector containing a full-length cDNA clone of CMV RNA 3: pFny309. The *thin lines* represent cDNA inserts while the *thick lines* represent vector DNA. A DNA segment derived from a synthetic fragment is *boxed*. Bx, *Bst*XI; M, nucleotides absent from the cDNA insert of pBS-Fny3; N_{16}, a 16 nt-long segment; Nh, *Nhe*I; P, *Pst*I

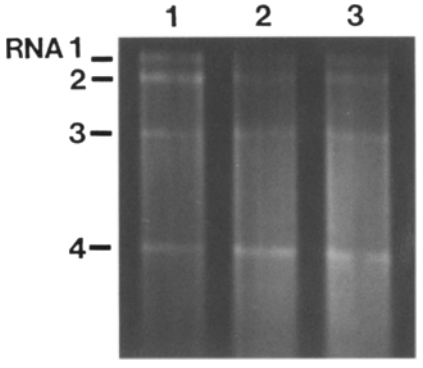

Fig. 7. Photograph of a gel containing virion RNAs purified from plants infected with capped in vitro transcripts generated from plasmids carrying full-length cDNA clones of CMV RNAs 1, 2 and 3 (pFny109, pFny209 and pFny309, respectively). Virion RNAs were derived from a tobacco plant infected with total Fny-CMV virion RNA (lane 1) and from a tobacco plant (lane 2) and a squash plant (lane 3) infected with the in vitro transcripts. RNAs were electrophoresed in 1.5% agarose containing ethidium bromide. Numbers along the left indicate the positions of the three viral genomic RNAs and the subgenomic RNA (4)

tained all four CMV RNAs as determined by gel electrophoresis (Fig. 7). In subsequent experiments, a higher percentage of plants became infected with the in vitro transcripts. Moreover, sap from such plants was highly infectious to other tobacco or squash plants and virus purified from any of these plants also contained all four CMV RNAs (data now shown). Since the transcripts inoculated at a concentration of 600 μg/ml were approximately as infectious as natural CMV RNAs inoculated at concentrations of 0.5–1 μg/ml, this suggests that the RNA transcripts are about 1000-fold less infectious than the natural CMV RNAs. Nevertheless, this conclusively demonstrates that together, the transcripts derived from full-length cDNA clones of Fny-CMV RNAs 1, 2 and 3 are infectious.

The ability to generate these infectious transcripts and to alter them by site-directed mutagenesis provides a method to obtain viral RNAs that can be used to investigate the gene expression of CMV.

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