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INFECTIOUS RNA TRANSCRIPTS FROM CLONED cDNAS OF CUCUMBER MOSAIC VIRAL SATELLITES

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SUMMARY: Complete cDNA copies of two variants of CARNA 5, the satellite of cucumber mosaic virus, have been cloned in the transcription vector pPM1. These two naturally-occurring CARNA 5s are capable and incapable, respectively, of inducing a lethal necrotic disease of tomato upon coinoculation with the genomic RNAs 1, 2, and 3 of cucumber mosaic virus. Uncapped transcripts synthesized in vitro from the two linearized, recombinant plasmids are infectious and each induces the appropriate symptomatology upon coinfection with cucumber mosaic viral RNAs on tomato plants. Progeny CARNA 5s isolated from such infected plants correspond to their natural CARNA 5 counterparts.

INTRODUCTION: For several years this laboratory has been engaged in attempts to relate the disease-modulating properties of cucumber mosaic virus (CMV)-associated satellites (1), which we have termed CARNA 5[1] (for CMV-Associated RNA 5), to their molecular structures. We have been particularly interested in discovering the nucleotide sequence domain(s) responsible for the capability of some but not other CARNA 5 variants to elicit lethal necrosis in tomato upon coinfection with its helper virus CMV (2). Thus far we have conducted our search for the "necrogenic" domain(s) via a comparison of the nucleotide sequences of different naturally occurring CARNA 5 variants (3-7, and unpublished work). These efforts, although useful, have not been conclusive. The feasibility of an alternative approach for linear RNAs became apparent with the construction by Ahlquist and Janda of a novel

[1] Abbreviations: CMV, cucumber mosaic virus; CMV-1, cucumber mosaic virus strain 1; CARNA 5, cucumber mosaic virus-associated RNA 5; D-CARNA 5, S-CARNA 5, and 1-CARNA 5, CARNA 5s isolated from CMV strains D, S, and 1, respectively; ds, double-stranded; cDNA, complementary DNA; PAGE, polyacrylamide gel electrophoresis; BMV, brome mosaic virus; E. coli, Escherichia coli.

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transcription vector (8) from which biologically active transcripts of cloned cDNAs of brome mosaic virus RNAs were synthesized in vitro (9). The possibilities for further application of recombinant DNA technology in the study of RNA structure-function relationships induced us to use a similar approach toward finding CARNA 5's necrogenic sequence domain(s).

We report here the enzymatic synthesis of biologically active RNA transcripts from full-length cDNA clones of both a necrogenic and a non-necrogenic CARNA 5.

MATERIALS AND METHODS

Isolation of CARNA 5s and their double-stranded (ds) forms. Satellites were isolated from preparations of purified CMV strain D and strain S by methods described (10, 7). They were designated D-CARNA 5 and S-CARNA 5 and are, respectively, capable and incapable of inducing tomato necrosis in the presence of their helper viruses. Their ds forms were isolated from the corresponding infected plant tissues (11).

Synthesis, cloning, and nucleotide sequence determination of CARNA 5 cDNA. Full-length ds cDNA copies of D- and S-CARNA 5 were prepared using synthetic oligonucleotides to prime synthesis simultaneously from the 3' ends of both (+) and (-) strands of denatured dsCARNA 5. Primers were synthesized on an Applied Biosystems model 380A automatic DNA synthesizer[2]. While primers binding to the (-) strands of dsD- and dsS-CARNA 5 (dGTTTTGTTTGATGGAG and dGTTTTGTTTGTTAGAG, respectively) were directly complementary, the primer hybridizing to the (+) strands (dGAATTCGGGTCCTG) contained an added EcoRI recognition site at its 5' end to facilitate forced cloning into the plasmid vector pPM1 (8; a gift of P. Ahlquist). Fifty pmoles of dsCARNA 5 and 750 pmoles of each primer (previously phosphorylated) in 50 µl of sterile, deionized water were denatured (4 min, 100°), quick-cooled, and then incubated for 1.5 hr at 42° in reaction mixtures (final volume 300 µl) containing 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 140 mM KCl, 30 mM β-mercaptoethanol, 500 µM each of dGTP, dCTP, and dTTP, 100 µM of dATP including 30 µCi of [α-^{32}P]dATP (800 Ci/mmol, New England Nuclear), 150 units RNasin (Promega Biotec), and 105 units of avian myeloblastosis virus reverse transcriptase (Molecular Genetics Resources, Inc.). The cDNA was isolated, hybridized, and the incomplete 3' ends were filled in by incubation for 1 hr at 37° with the Klenow fragment of E. coli DNA polymerase I (Bethesda Research Laboratories). After restriction with EcoRI, the cDNA was ligated into gel-purified pPM1 previously cleaved with both SmaI and EcoRI, and then used to transform E. coli strain JM83 (12). After selection on ampicillin-containing medium, screening by colony hybridization, and restriction digestion analysis, the CARNA 5 cDNA inserts and flanking junction regions of selected clones were sequenced (13).

In vitro transcription. Transcription reactions (in 100 µl) were incubated for 1 hr at 37° and contained 5 µg of EcoRI-cut plasmid DNA, 5 U of E. coli RNA polymerase (Promega Biotec), 200 µM of each ribonucleoside triphosphate, 40 mM Tris-HCl (pH 8.0), 0.01 M $MgCl_2$, 0.15 M KCl, 0.05% BSA, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 100 U RNasin. For capped transcripts, the concentration of rGTP was decreased to 25 µM and 500 µM m^{7}GpppG (Pharmacia

[2]Mention of a commercial company or specific equipment does not constitute its endorsement by the U.S. Dept. of Agriculture over similar equipment or companies not named.

P-L Biochemicals, Inc.) was included. After 15 min and again after 30 min, a further 25 μM rGTP was added; incubation was for a total of 1 hr. After transcription, EDTA was added to 10 mM and 5 μl of the reaction mixture was analyzed by polyacrylamide gel electrophoresis (PAGE); the appropriate amount of the remainder was diluted into inoculation buffer.

Infectivity assay and analysis of progeny CARNA 5s. Twenty seven-day old tomato seedlings (Lycopersicon esculentum Mill., cv. Rutgers) were dusted with carborundum and inoculated with 0.4 ml of 0.03 M Na_2HPO_4 containing a mixture of CMV-1 RNAs 1, 2, and 3 at 8 μg/ml, with or without D-CARNA 5 at 1.2 μg/ml or 10-fold dilutions thereof (or reaction mixtures containing a similar amount of RNA transcripts from CARNA 5 cDNA). Plants were scored for tomato necrosis 12-21 days after inoculation. Tissue was harvested either 14 days after inoculation or just prior to the plant's death. Progeny CARNA 5s were characterized using PAGE under semidenaturing conditions (14) and their 3' terminal nucleotide sequences determined (4).

RESULTS AND DISCUSSION

Correct insertion of CARNA 5 cDNA into the transcription vector pPM1. Figure 1 shows that as a result of the forced cloning procedure used, the first nucleotide of the CARNA 5 cDNA inserts corresponds to the first nucleotide of CARNA 5. Thus, by analogy to the recombinant pPM1 plasmids containing cDNA inserts to BMV RNA (8), in vitro runoff transcription following cleavage at the EcoRI site immediately 3' to the CARNA 5 cDNA should result in transcripts whose 5' ends are coterminal with those of natural CARNA 5 and whose 3' ends have 4-5 extra nucleotides [-GAAU$(U)_{0-4}$] not present in natural CARNA 5. PAGE analysis of the transcription reaction mixtures did show clear evidence for the presence of RNA of the size expected for the transcripts (not shown).

Biological activity of RNA transcribed from CARNA 5 cDNA. The bioassays exploited the fact that natural D-CARNA 5, but not S-CARNA 5, induces a lethal necrotic disease (2,7) when inoculated on tomato plants together with

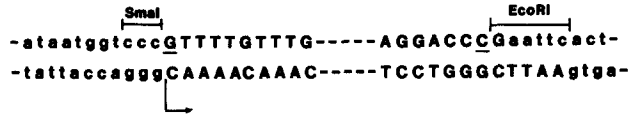

FIG. 1. Nucleotide sequences of the recombinant pPM1-CARNA 5 plasmids at the junctions between the plasmid DNA and the CARNA 5 cDNA. Lower case letters indicate DNA of plasmid origin; capital letters denote sequences derived from the CARNA 5 cDNA inserts; underlined letters correspond to the 5' and 3' terminal nucleotides of CARNA 5. Restriction enzyme sites indicated are those used in the construction and/or pre-transcriptional cleavage of the recombinant DNA. Arrow denotes the transcription initiation site.

the helper virus CMV. Thus, transcripts from D- and S-CARNA 5 cDNA inserts in the recombinant pPM1 plasmid should be necrogenic and nonnecrogenic, respectively, if replicated and expressed in tomato. This was tested in six separate bioassays, in which transcription mixtures and the genomic RNAs 1,2,3 of CMV strain 1 were used for combined inoculation on tomato. The choice of CMV-1 as helper virus was made deliberately because its satellite, 1-CARNA 5, if present as a contaminant in purified preparations of CMV RNAs 1,2,3, is not necrogenic and can be distinguished easily from D-CARNA 5 and S-CARNA 5 with semidenaturing PAGE (14; see also Fig. 2). Table 1 shows a representative bioassay. It demonstrates that only the D-CARNA 5 transcripts and natural D-CARNA 5 induce tomato necrosis, while the S-CARNA 5 transcripts do not. As expected, the helper CMV-1 alone does not cause any more than some background-level tomato necrosis. To quantitate the infectivity of the D-CARNA 5 transcripts, we compared them with natural D-CARNA 5 in a dilution-endpoint bioassay. Table 1 shows that in this assay

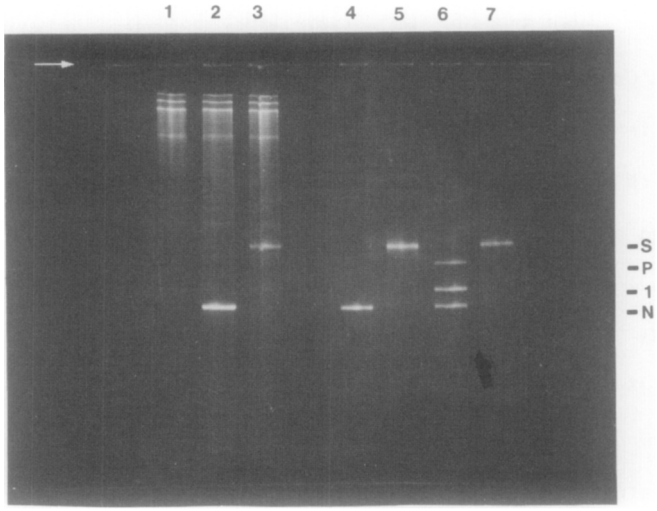

FIG. 2. PAGE characterization of progeny CARNA 5 accumulated in tomato plants infected with CMV-1 RNAs 1,2,3 plus in vitro transcripts of D-CARNA 5 and S-CARNA 5 cDNAs. PAGE was on 9% gels under semidenaturing conditions (14). Lanes 1-3 contain total viral progeny RNAs from inoculations with: Lane 1, RNAs 1,2,3 alone; Lane 2, RNAs 1,2,3 + D-CARNA 5 cDNA transcripts; Lane 3, RNAs 1,2,3 + S-CARNA 5 cDNA transcripts. Lanes 4 and 7 contain the purified CARNA 5s from the mixtures shown in Lanes 2 and 3, respectively. The marker RNAs are natural S-CARNA 5 (S) in Lane 5, and natural D-CARNA 5 (D), 1-CARNA 5 (1; ref. 14), and the satellite of peanut stunt virus (P; ref. 15) in Lane 6.

Table 1. DILUTION END-POINT ASSAY[1] FOR TOMATO NECROSIS INDUCED BY TRANSCRIPTS OF CARNA 5 cDNAS IN THE PRESENCE OF CMV-1 GENOMIC RNAS

	No. dead plants/Total no. plants inoculated with			
	CMV-RNA 1,2,3 (control)	CMV-RNA 1,2,3 plus		
Dilution of CARNA 5 or transcripts		D-CARNA 5[3]	Uncapped D-CARNA 5 transcripts	Uncapped S-CARNA 5 transcripts
Undiluted	1/20[2]	19/20	18/20	0/20
10^{-1}	n.t.	20/20	12/20	n.t.
10^{-2}	n.t.	19/20	10/20	n.t.
10^{-3}	n.t.	16/20	6/20	n.t.
10^{-4}	n.t.	3/20	1/20	n.t.
10^{-5}	n.t.	2/20	0/20	n.t.

[1]Conditions as described in Methods.
[2]A background level of necrosis-induction in the CMV RNAs 1,2,3 alone is due to a very low level of contaminating necrotic CARNA 5. Over five separate experiments, numbers of necrotic plants induced by CMV RNAs 1,2,3 alone were 0/20, 0/20, 0/20, 1/20, 1/20. n.t. = not tested.
[3]D-CARNA 5 is natural CARNA 5 isolated from virions.

the 50% lethal dose of the D-CARNA 5 transcripts is at a concentration level about 10 times higher than that of natural D-CARNA 5. This 10-fold difference in infectivity is quite comparable to that found with the infectious RNA transcripts of BMV cDNA versus natural BMV RNA (9). Since our transcripts lacked 5' caps, we attempted to increase their infectivity by including m^7GpppG in the *in vitro* transcription reaction (8, 16). Although no attempt has been made at this point to prove that the resulting transcripts were actually capped, they were of the same order of infectivity when bioassayed as their uncapped counterparts (not shown). Thus, the discrepancy in infectivities of the D-CARNA 5 transcripts versus that of natural D-CARNA 5 remains to be explained, but may well relate to the 4-5 extra nucleotides present at the 3' terminus of the transcripts. However, the fact that a cap is not required for infectivity of CARNA 5 transcripts seems unique, since transcripts of cDNAs of three plant viruses, whose RNAs (like CARNA 5) are naturally capped at their 5' ends, do require caps for biological activity (9, 17, 18). Whether this reflects a biologically significant difference between the satellite RNA and viral RNAs or merely relates to the relatively greater stability of a smaller RNA molecule remains to be determined.

In addition to the infections with CMV RNAs 1,2,3 with or without the S-CARNA 5 transcripts, the following additional control mixtures were also found to be nonnecrogenic when tested in the presence of helper virus: RNA transcripts of linearized plasmid pPM1 lacking an insert; linearized recombinant plasmid pPM1 (5 µg or 20 µg) containing D-CARNA 5 cDNA and incubated under transcription conditions but without E. coli RNA polymerase; RNA transcripts of linearized recombinant pPM1 plasmids containing cDNA to D- and S-CARNA 5 in the reverse (-) orientation, which were constructed using a different procedure (to be published). None of these additional control mixtures produced progeny CARNA 5s (see below).

Characterization of progeny CARNA 5 from infectious CARNA 5 cDNA transcripts and helper CMV. CMV produced in tomato plants from bioassays of the transcripts and control preparations was isolated and analyzed for the presence and type of progeny CARNA 5. From results shown in Lanes 1-3 of Figure 2 and controls mentioned above (not shown), it is clear that only inoculations with transcripts of D- and S-CARNA 5 cDNA produced progeny D- and S-CARNA 5, respectively. These and the bioassay results allow us to conclude unequivocally that: 1) the progeny CARNA 5s relate correctly to the particular transcripts included in the inoculum, and 2) the tomato necrosis disease relates exclusively to the D-CARNA 5 cDNA transcripts.

The progeny CARNA 5s produced from the D- and S-CARNA 5 transcripts were isolated from the RNA mixtures for further characterization. Lanes 4 and 7 show their respective PAGE patterns in comparison with appropriate markers (Lanes 5 and 6). Their 3' terminal nucleotide sequences were found to be identical to those of natural D- and S-CARNA 5, respectively (data not shown). Thus, as with the infectious transcripts of BMV cDNAs (9), the extra nucleotides at the 3' terminus of the CARNA 5 transcripts, presumably present during initiation of replication, were lost during the infection.

The availability of infectious transcripts from cloned CARNA 5 cDNAs should allow direct tests of structure-function relationships in the induction of the tomato necrosis disease.

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