Infectious cucumber mosaic virus RNA transcribed in vitro from clones obtained from cDNA amplified using the polymerase chain reaction

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Full-length cDNA to RNA 1, RNA 2 and RNA 3 of cucumber mosaic virus strain Q (CMV-Q) was amplified using the polymerase chain reaction (PCR). The first-strand primer contained a BamHI site and sequences complementary to the 3' terminus of the RNA. The second-strand primers contained a BamHI site, a T7 promoter and sequences corresponding to the 5' terminus of each RNA. After cleavage with BamHI, the PCR products were cloned into the BamHI site of the vector pEMBL9(+). Five clones of each RNA were selected and RNA transcripts were synthesized in vitro from each clone using T7 RNA polymerase. The constructs were designed to allow transcription to initiate precisely at the 5' terminus of each RNA. All the transcripts were found to be infectious when inoculated onto Nicotiana tabacum cv. Samsun plants in sets of three, corresponding to RNA 1, RNA 2 and RNA 3. Of the transcript sets, four induced symptoms indistinguishable from symptoms induced by CMV-Q RNAs. However a fifth transcript set induced much more severe symptoms. Plasmids were also constructed to allow synthesis of transcripts with one or two additional G residues at the 5' terminus of each RNA. Although the yields of such transcripts synthesized in vitro with T7 RNA polymerase were higher, their infectivity was lower than that of those with no additional residues at their 5' termini.

Introduction

The production of cDNA clones from which infectious RNA can be transcribed in vitro has become an essential tool for molecular genetic studies of positive-strand RNA viruses. This was first achieved for brome mosaic virus, for which full-length cDNA copies of each of the three genomic RNAs were linked to a modified phage lambda P<sub>M</sub> promoter (P<sub>M</sub>) in the vector pPM1 and transcribed in vitro using Escherichia coli RNA polymerase (Ahlquist & Janda, 1984; Ahlquist et al., 1984). A similar approach was used to obtain infectious in vitro transcripts from cDNA clones of tobacco mosaic virus (Dawson et al., 1986; Meshi et al., 1986), black beetle virus (Dasmahapatra et al., 1986) and poliovirus (Kaplan et al., 1985). A limitation on the production of infectious transcripts using E. coli RNA polymerase has been the variable efficiency of different commercial batches of the enzyme (Ahlquist et al., 1987; Janda et al., 1987). This has led to the use of more efficient and reliable RNA polymerases from bacteriophages T3, T7 and SP6 for the synthesis of infectious transcripts from cDNA clones of a range of plant viruses (Janda et al., 1987; Vos et al., 1988; Domier et al., 1989; Eggen et al., 1989a, b; Heaton et al., 1989; Petty et al., 1989; Quillet et al., 1989; Weiland & Dreher, 1989), animal viruses (van der Werf et al., 1986; Rice et al., 1987) and bacteriophage Qβ (Shaklee et al., 1988).

The most common procedure to produce cDNA clones from which infectious transcripts can be obtained has involved cloning full-length double-stranded cDNA into a vector containing the appropriate bacteriophage promoter. In vitro mutagenesis has been used to eliminate, or reduce the number of, nucleotides between the transcription initiation site and the 5' end of the viral RNA because the presence of more than one additional 5' nucleotide can greatly reduce or abolish the infectivity of the transcripts (Janda et al., 1987; Eggen et al., 1989). However, Weiland & Dreher (1989) have recently described a procedure in which an oligonucleotide containing a T7 promoter linked directly to the 5' viral sequence is used to prime second-strand cDNA synthesis, allowing the cDNA to be cloned into standard vectors and eliminating the need for in vitro mutagenesis. We now describe an adaptation of this procedure in which the production and cloning of full-length cDNAs are considerably facilitated by amplification using the polymerase chain reaction (PCR) (Saiki et al., 1988). The procedure has been used to obtain cDNA clones of cucumber mosaic virus (CMV), from which infectious transcripts have been synthesized in vitro.
Methods

Virus and viral RNA. *Nicotiana tabacum* cv. Samsun plants were grown at 20 °C under a mixture of fluorescent and tungsten lighting for 15 h per day. Seedlings with four to six leaves were inoculated with CMV Q-strain (CMV-Q) (Habili & Francki, 1974) obtained from the American Type Culture Collection. Plants were harvested 10 days after inoculation and virus was isolated and purified as described by Lot et al. (1974). Viral RNA was extracted from purified virus particles as in Peden & Symons (1973).

Oligonucleotides. These were synthesized using an Applied Biosystems Model 381A DNA synthesizer.

Cloning. General procedures were as in Sambrook et al. (1989), unless otherwise stated.

Construction of pCMV1-(A to E), pCMV2-(A to E) and pCMV3-(A to E). First-strand cDNA was synthesized using RNA isolated from purified CMV particles as a template, an oligonucleotide primer (Fig. 1) and reverse transcriptase and other reagents from an Amersham cDNA Synthesis System Kit with conditions as described by the manufacturer. The product was electrophoresed through an alkaline 1·2% low melting temperature agarose gel. After neutralization of the gel and staining with ethidium bromide, distinct bands corresponding to full-length cDNA could be detected. These cDNAs were purified from the gel by melting the agarose at 65 °C, two rounds of phenol:chloroform extraction and precipitation with ethanol. Each cDNA product was then used as a template for a PCR. Reaction mixtures (100 μl) contained 67 mM-Tris-HCl pH 8·8 at 25 °C, 17 mM-ammonium sulphate, 2 mM-magnesium chloride, 10 mM-2-mercaptoethanol, 0·2 μg/ml bovine serum albumin, 6·5 mM-EDTA, 0·2 mM-dNTPs (dTTP, dCTP, dGTP, dTTP), 1 μg of each primer and 2 units AmpliTaq (Cetus), and were subject to a 30 cycle programme of 92 °C for 1 min, 40 °C for 1 min and 72 °C for 3 min. The oligonucleotide described above was used as the first-strand primer and three separate oligonucleotides were used for second-strand priming of RNA 1, RNA 2 and RNA 3 respectively (Fig. 1). The PCR products were extracted with phenol, precipitated with ethanol, resuspended and digested with *Bam*HI overnight. The products were then electrophoresed through a 13% low melting temperature agarose gel and the full-length double-stranded cDNA products were purified, ligated into *Bam*HI-cleaved pEMBL9 (+) (Dente et al., 1985) and transformed into *E. coli* DH5α F' cells (Gibco-BRL) using a Bio-Rad Gene Pulser according to the manufacturer’s instructions. Recombinant plasmids were purified by the method of Birnboim & Doly (1979) and those containing full-length cDNA corresponding to RNA 1, RNA 2 and RNA 3 were identified by restriction mapping and plasmid sequencing (Chen & Seeberg, 1985).

Construction of pCMV1GG. pCMV1G, pCMV2GG. pCMV2G, pCMV3GG and pCMV3G. Plasmid pT718U (Mead et al., 1986) contains a T7 promoter adjacent to a polylinker sequence from pUC18. The sequence AGGGAA which contains the T7 transcriptional start site at its first G was converted by in vitro mutagenesis (Kunkel et al., 1987) to the sequence AGGCCT which is a *Stu*I site. The resultant plasmid was designated pT718S. Double-stranded cDNA to CMV RNA 1, RNA 2 and RNA 3 was produced by the PCR as described above using the same first-strand primer (Fig. 1). The second-strand primers were: RNA 1, GTTTATTATCAAGA (pCMV1GG) or TTTATTATCAAGA (pCMV2GG) or TTTATTTACCAAGA (pCMV3GG) or TTTATTTACCAAGA (pCMV3GG) or TTAATCTTACCACTTT (pCMV3GG) or TTAATCTTACCACTTT (pCMV3GG). The PCR products were cleaved with *Bam*HI and cloned into *Stu/I* and *Bam*HI-cleaved pT718S. Clones containing full-length cDNAs of RNA 1, RNA 2 and RNA 3 were identified by restriction mapping and sequences adjacent to the T7 transcriptional start site were confirmed by plasmid sequencing (Chen & Seeberg, 1985).

In vitro transcription. Reaction mixtures (100 μl) contained 40 mM-Tris–HCl pH 8·8, 15 mM-magnesium chloride, 5 mM-dithiothreitol, 0·5 μg/ml bovine serum albumin, 1 mM-ATP, 1 mM-UTP, 1 mM-CTP, 0·5 mM-GTP, 2 mM-m2GpppG (Pharmacia), 100 units Inhibit-Ace (Northumbria Biologicals), 150 units T7 RNA polymerase (Gibco-BRL) and 10 μg each of *Bam*HI-linearized cDNA clones of RNAs 1, 2 and 3. The transcribed RNA was purified by treatment with DNase I to remove the template, followed by phenol extraction and ethanol precipitation.

Inoculation of plants with in vitro synthesized transcripts. *N. tabacum* cv. Samsun seedlings with four to six leaves were inoculated with in vitro transcripts (50 μg/ml of each transcript) as described by Osman & Buck (1989) and cultivated as described above. Plants were harvested 10 days after inoculation.

Dot-blot hybridization. RNA was extracted from leaf tissue, spotted onto Hybond-N membranes (Amersham) and hybridized with 32P-labelled probes as described by Osman & Buck (1989).

Gel electrophoresis of proteins. Samples of purified virus were adjusted to 1% SDS and 2% 2-mercaptoethanol and heated at 100 °C for 3 min. Aliquots were fractionated by electrophoresis and stained using Coomassie Blue stain. Gels were run using the discontinuous buffer system described by Laemmli (1970).

Immunodiffusion. Gel immunodiffusion analysis was as described by Osman et al. (1986).

Electron microscopy. Samples were stained in 1% phosphotungstic acid and examined in an electron microscope.

Results

Use of PCR for production of cDNA clones

CMV has a genome of three positive-strand RNA components, RNA 1 (3·4 kb), RNA 2 (3·0 kb) and RNA 3 (2·1 kb), which encode non-structural proteins 1a, 2a
Infectious transcripts of CMV

Infectious transcripts of CMV and 3a, respectively (Gould & Symons, 1982; Rezaian et al., 1984, 1985). RNA 3 also encodes the coat protein which is expressed from RNA 4 (1-0 kb), a subgenomic RNA which is 3'-coterminal with RNA 3. All four RNAs are encapsidated.

The PCR (Saiki et al., 1988) was used to obtain full-length clones of the three genomic RNAs of CMV. The 3'-terminal sequences of RNA 1, RNA 2 and RNA 3 are identical. Hence, a single oligonucleotide containing a sequence complementary to the 3'-terminal 16 nucleotides (Fig. 1) was used to prime cDNA synthesis on all the viral RNAs. After electrophoresis of the products of first-strand cDNA synthesis in an alkaline denaturing gel, distinct bands corresponding to full-length cDNA of each RNA were detected. Bands corresponding in size to RNA 1, RNA 2 and RNA 3 were extracted from the gel and each was amplified separately in a PCR. Gel electrophoresis of the PCR products of each RNA is shown in Fig. 2. Single bands of 3-4 kb, 3-0 kb and 2-1 kb were detected for the PCR products of RNA 1, RNA 2 and RNA 3 respectively. Attempts to use PCR on the unpurified first-strand cDNA reaction products were not successful, yielding products of less than 1-5 kb.

Both the first-strand and second-strand primers (Fig. 1) were designed to contain a BamHI site at their extremities to facilitate cloning of the full-length double-stranded cDNA into the BamHI site of pEMBL9(+). The second-strand primers also contained the T7 ϕ10 promoter (Dunn & Studier, 1983) linked to 15 nucleotides corresponding to the 5' sequence of each RNA. Since the 5' sequences of RNA 1, RNA 2 and RNA 3 differ slightly, a separate second-strand primer was needed for each RNA (Fig. 1). Sequence analysis of full-length clones of RNA 1, RNA 2 and RNA 3 in pEMBL9(+(+)) showed that the sequences of the primers were present in all the clones. Five independent clones of each RNA were obtained and are designated as follows: RNA 1 clones, pCMV1-A, pCMV1-B, pCMV1-C, pCMV1-D, pCMV1-E; RNA 2 clones, pCMV2-A, pCMV2-B, pCMV2-C, pCMV2-D, pCMV2-E; RNA 3 clones, pCMV3-A, pCMV3-B, pCMV3-C, pCMV3-D, pCMV3-E.

The T7 class III promoters, which include ϕ10 (Dunn & Studier, 1983), have exactly the same sequence of 23 base pairs located at positions −17 to +6 relative to the start site for the RNA (designated +1). This implies that the transcriptional efficiency may depend on sequences downstream as well as upstream of the start site. The design of the pCMV1, pCMV2 and pCMV3 clones to allow transcription to start precisely at the 5' end of each RNA required a change to the sequence downstream of the transcriptional initiation site of the T7 ϕ10 promoter. For comparison, two further sets of plasmids were constructed, as described in the Methods section, with one or two additional G residues upstream of the 5' end of each RNA, so as to more closely resemble the T7 initiation sequence. These are designated pCMV1G, pCMV2G, pCMV3G and pCMV1GG, pCMV2GG and pCMV3GG, respectively. The sequences around the transcriptional initiation sites of the three plasmids are compared with that of the T7 ϕ10 promoter in Fig. 3.

In vitro transcription

Transcription of BamHI-linearized pCMV1-(A to E), pCMV2-(A to E) and pCMV3-(A to E) by T7 RNA polymerase generated RNAs that comigrated with the CMV virion RNAs 1, 2 and 3, respectively, in denaturing gels (shown for pCMV1-A, pCMV2-A and pCMV3-A in Fig. 4). The amount of transcript obtained corresponded to about five transcripts per plasmid. Similar results were obtained whether or not the cap analogue m'GpppG was present in the reaction mixture.

Transcription of linearized pCMV1G, pCMV2G, pCMV3G, pCMV1GG, pCMV2GG and pCMV3GG with T7 RNA polymerase gave similar results (not shown), except that the yields of transcripts were higher. In the case of the latter three plasmids, yields of up to 20 transcripts per plasmid were obtained.
Infectivity and symptom induction

The results of inoculation of *N. tabacum* cv. Samsun, a systemic host for CMV, with transcripts produced from different plasmids are shown in Table 1. Infectivity was demonstrated for capped transcripts of the combinations: pCMV1-A, pCMV2-A, pCMV3-A; pCMV1-B, pCMV2-B, pCMV3-B; pCMV1-C, pCMV2-C, pCMV3-C; pCMV1-D, pCMV2-D, pCMV3-D; pCMV1-E, pCMV2-E, pCMV3-E. Infectivity was reduced either by the absence of a cap structure or by the addition of one or two G residues to the 5' end of the transcripts.

With one exception the symptoms induced in the *N. tabacum* plants by the transcripts were similar to those induced by virions or RNA of CMV-Q and consisted of a mild mosaic on the systemically infected leaves. However, the symptoms induced by transcripts of pCMV1-E, pCMV2-E and pCMV3-E were much more severe. A bright yellow mosaic appeared on the systemic leaves which eventually became completely chlorotic (Fig. 5).

Virus preparations were made from plants infected by transcripts from pCMV1-A, pCMV2-A and pCMV3-A (A transcripts) and from pCMV1-E, pCMV2-E and pCMV3-E (E transcripts) and were designated CMV-TA and CMV-TE respectively. Particles of CMV-TA and CMV-TE were indistinguishable from each other and from CMV-Q by electron microscopy and by gel diffusion serology. Plants inoculated with CMV-TA or CMV-TE became infected and the symptoms induced were the same as those induced by A transcripts or E transcripts respectively.

Table 1. Infectivity of transcripts synthesized in vitro

<table>
<thead>
<tr>
<th>Templates for T7 RNA polymerase transcription*</th>
<th>Infected Samsun/total†</th>
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<tbody>
<tr>
<td>pCMV1A + pCMV2A + pCMV3A</td>
<td>18/20</td>
</tr>
<tr>
<td>pCMV1B + pCMV2B + pCMV3B</td>
<td>8/10</td>
</tr>
<tr>
<td>pCMV1C + pCMV2C + pCMV3C</td>
<td>7/10</td>
</tr>
<tr>
<td>pCMV1D + pCMV2D + pCMV3D</td>
<td>8/10</td>
</tr>
<tr>
<td>pCMV1E + pCMV2E + pCMV3E</td>
<td>17/20</td>
</tr>
<tr>
<td>pCMV1G + pCMV2G + pCMV3G</td>
<td>13/20</td>
</tr>
<tr>
<td>pCMV1GG + pCMV2GG + pCMV3GG</td>
<td>1/20</td>
</tr>
<tr>
<td>pCMV1A + pCMV2A + pCMV3A (No cap)</td>
<td>3/10</td>
</tr>
<tr>
<td>CMV-Q RNA</td>
<td>19/20</td>
</tr>
</tbody>
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* Tobacco plants were inoculated as described by Osman & Buck (1989) using either a mixture of *in vitro* transcripts (100 ng) synthesized from the templates shown or 100 ng virion RNA.
† Number of plants showing symptoms after 10 days.

RNA was extracted from preparations of CMV-TA or CMV-TE and examined by electrophoresis in a denaturing agarose gel. Bands with mobilities identical to RNAs 1, 2, 3 and 4 of CMV-Q were detected in each case (not shown). Analysis of protein from virus preparations of CMV-TA or CMV-TE by SDS-PAGE in each case gave a major band with the same mobility as that of protein from preparations of CMV-Q.
Fig. 5. Symptoms induced in *N. tabacum* cv. Samsun plants by CMV-Q RNA and transcripts synthesized *in vitro*. (a) Leaf of healthy plant. (b to d) Systemically infected leaves of plants 10 days after inoculation with (b) RNA from purified virus particles of CMV-Q, (c) transcripts synthesized *in vitro* from pCMV1-A, pCMV2-A and pCMV3-A or (d) transcripts synthesized *in vitro* from pCMV1-E, pCMV2-E and pCMV3-E.

**Discussion**

We have found that cloning of full-length cDNAs of CMV RNAs is facilitated by amplification of cDNAs using the PCR and that infectious transcripts can be synthesized *in vitro* from such clones. The error rate (mutations per base duplication) of *Taq* polymerase used in the PCR reactions has been estimated to be $2 \times 10^{-4}$ (Keohavong & Thilly, 1989). Mutations may therefore be expected to accumulate in a population of DNA molecules as a function of the number of cycles of amplification. It is clear that any mutations introduced during the PCR in the present study were not lethal to the virus because all the clones tested gave rise to infectious RNA transcripts. Furthermore, most of the clones gave symptoms indistinguishable from CMV-Q, which was used as the source of RNA for cDNA synthesis. However, it is noteworthy that transcripts from clones pCMV1-E, pCMV2-E and pCMV3-E induced more severe symptoms than CMV-Q and a mutation must therefore be present in at least one of these clones. We cannot be certain whether this mutation arose during the PCR, during reverse transcription or as a spontaneous mutation in the population of CMV-Q RNA molecules during propagation of the virus. Reverse transcriptases and RNA-dependent RNA polymerases are also known to have relatively high error rates (Gopinathan *et al.*, 1979; Steinhauer & Holland, 1987). Nevertheless amplification of cDNA by the PCR may be worthy of investigation as a method of generating virus mutants.

The finding that infectivity was reduced by addition of one G residue, and to a greater extent by two G residues, to the 5' end of the transcripts (Table 1) confirms the importance of the 5'-terminal structure of transcripts for infectivity (Dawson *et al.*, 1986; Van der Werf *et al.*, 1987; Janda *et al.*, 1987; Rice *et al.*, 1987; Shaklee *et al.*, 1988; Eggen *et al.*, 1989a; Heaton *et al.*, 1989) but, in contrast, infectivity appears to be relatively insensitive to 3'-terminal extensions (Janda *et al.*, 1987; Eggen *et al.*, 1989b). *BamHI*-cleaved pCMV1-(A to E), pCMV2-(A to E) and pCMV3-(A to E) contained four additional nucleotides beyond the 3' terminus of the RNAs; however, there was little difference between the infectivity of the transcripts and that of CMV-Q RNA. As with *in vitro* transcripts of other viruses, the presence of a 5'-terminal cap structure was necessary for maximum infectivity.

The availability of cDNA clones from which infectious RNA can be transcribed will greatly facilitate molecular genetic studies with CMV. Furthermore, analysis of the mutant clones which give more severe symptoms on *N. tabacum* should be informative with regard to the molecular basis of the pathogenicity of this virus.

We thank BP Nutrition for financial support and P. Atkey for electron microscopy.

**References**


