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Study of the genetic organisation of a plant viral RNA genome by *in vitro* expression of a full-length DNA copy

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The genetic approach for elucidating functions encoded by RNA plant viruses has been hampered by the lack of methods to select desired mutants following random mutagenesis. An alternative might be to copy RNA genomes into DNA and use methods for site-directed mutagenesis to modify specific regions of the DNA copy. Transcription of the DNA copy will subsequently produce viral RNA with desired mutations. We have constructed a full-length DNA copy of the smaller of the two cowpea mosaic virus (CPMV) RNAs, referred to as M RNA. The DNA copy was positioned downstream from the promoter of bacteriophage SP6 and using SP6 RNA polymerase, this copy and two derivatives of it containing a specific deletion and insertion, respectively, have been transcribed into RNA molecules which are efficiently translated in rabbit reticulocyte lysates. The results obtained show that the subsequent *in vitro* transcription and translation of DNA copies may be a powerful tool to unravel the genetic properties of viral RNA genomes.

Key words: cowpea mosaic virus/cDNA/site-directed mutagenesis/*in vitro* transcription/*in vitro* translation

Introduction

Cowpea mosaic virus (CPMV) is a plant virus with a bipartite, positive-stranded RNA genome that is translated into large polypeptides from which the functional proteins are derived by proteolytic cleavages (Rezelman *et al.*, 1980; Goldbach *et al.*, 1981; Franssen *et al.*, 1982, 1984a). Both *in vivo* and *in vitro* translation studies have resulted in detailed knowledge about the expression of the larger of the two genomic RNAs, the B RNA (5889 nucleotides) (Rezelman *et al.*, 1980; Goldbach *et al.*, 1980; Zabel *et al.*, 1982; Goldbach and Rezelman, 1983; Lomonossoff and Shanks, 1983; Franssen *et al.*, 1984a, 1984b). On the other hand, the expression of the smaller of the two RNAs, the M RNA, (3481 nucleotides) (Van Wezenbeek *et al.*, 1983) is poorly understood. In infected cowpea cells (*Vigna unguiculata*) the only M RNA-specific proteins identified are the two capsid proteins VP37 (37 000 daltons) and VP23 (23 000 daltons). Translation of M RNA *in vitro* yields two overlapping polypeptides of ~105 000 (105 K) and 95 000 (95 K) daltons (Figure 1), which have never been observed *in vivo*, presumably because they are rapidly processed into functional proteins (Pelham, 1979; Franssen *et al.*, 1982, 1984b; Huez *et al.*, 1983). The information available seems to indicate that these polypeptides are produced from two different initiation sites on M RNA (Franssen *et al.*, 1982; Van Wezenbeek *et al.*, 1983). They are recognised and cleaved by a B RNA-encoded protease resulting in cleavage products of 60 000 daltons (60 K), 58 000 daltons (58 K) and 48 000 daltons (48 K) (Figure 1) (Franssen *et al.*, 1982). Whereas the 60-K cleavage product has been shown to represent a common precursor to both capsid proteins (Franssen *et al.*, 1982), the functions of the 58-K and 48-K polypeptides are still unknown. To gain further insight into the expression of M RNA, we decided to construct a full-length cDNA copy of M RNA. This would enable us to study the genetic organisation of M RNA by expression of DNA copies modified by site-directed mutagenesis. Here we report the successfull *in vitro* expression of DNA copies by subsequent *in vitro* transcription and translation. Application of this approach to two mutagenised DNA copies definitely reveals the presence of two translation initiation sites on M RNA and has led to a further mapping of the proteolytic cleavage site used by the B RNA-encoded protease.

Results

Molecular cloning

For the construction of a full-length DNA copy we used a number of cDNA clones described previously (Van Wezenbeek *et al.*, 1983). Since various rearrangements have been shown to occur during cloning of cDNA from M RNA (Van Wezenbeek *et al.*, 1983), this approach was preferred to synthesising new full-length cDNA. Only two new M13 clones, denoted CM131RF and CM414RF were constructed, which contained the 3′ and 5′ terminal sequences of M RNA, respectively (see Figure 2). For the synthesis of double-

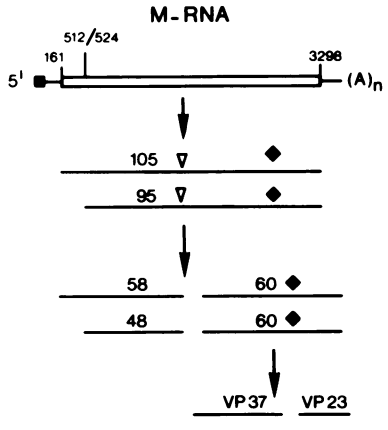

Fig. 1. Model for the translation of the M RNA of CPMV. The open reading frame is indicated with a double-lined bar. The positions of potential start codons (162, 512, 524) and stop codon (3298) are indicated. Open triangles correspond to the putative glutamine-methionine cleavage site recognised by the 32-K B RNA-encoded protease (Franssen *et al.*, 1982; Van Wezenbeek *et al.*, 1983). Black lozenges refer to the glutamine-glycine cleavage site in the 60-K capsid protein precursor, by which VP37 and VP23 are released (Van Wezenbeek *et al.*, 1983). VPg is indicated by a black square.

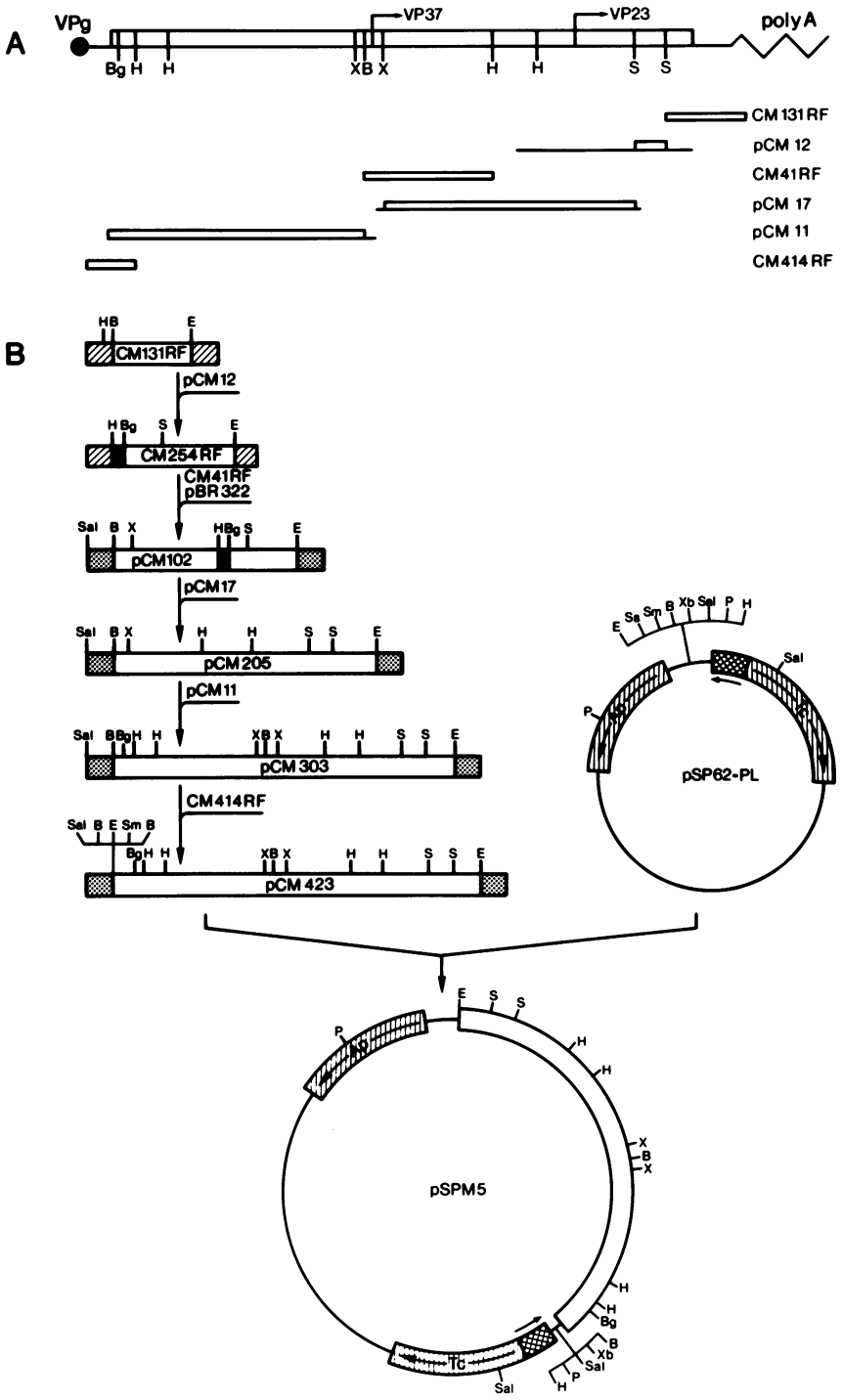

Fig. 2. Construction of a full-length DNA copy of CPMV M RNA. **Panel A:** schematic representation of the various cDNA clones used for the construction of the full-size DNA copy. The upper line represents the complete M RNA sequence with the double-lined bar indicating the position of the long open reading frame. The positions of the N-terminal ends of VP37 and VP23 are indicated. Prefix p refers to pBR322-derived clones, RF refers to M13mp9-derived clones. The positions of relevant recognition sites of *Bam*HI (B), *Bgl*I (Bg), *Eco*RI (E), *Hind*III (H), *Pst*I (P), *Sal*I (Sal), *Sau*3A (S), *Sma* (Sm), *Sst*I (Ss), *Xba*I (Xb) and *Xho*I (X) are shown. The regions of the M RNA-derived inserts in various clones are shown, with the segments used for the construction indicated by double-lined bars. M RNA-derived cDNA clones pCM11, pCM12, pCM17 and CM41RF have been described previously (Van Wezenbeek *et al.*, 1983). **Panel B:** construction of a full-size DNA copy from various cDNA clones. Sources of DNA fragments are indicated as follows: open boxes, M RNA-specific sequences; dotted boxes, adjacent pBR322 sequences; dashed boxes, adjacent M13 vector sequences; black box, *Bgl*II-*Hind*III adaptor fragment (see below); chequered box, SP6 promoter fragment. The figures are not drawn to scale. For explanation of symbols see **panel A**. For further details see text.

stranded cDNA containing the ultimate 5′ end, an oligonucleotide primer for second strand DNA synthesis homologous to the first 16 nucleotides of M RNA was used. Double-stranded cDNA obtained in this way was digested with *Hind*III and ligated into M13mp9 digested with *Hind*III and *Hind*II, producing clone CM327RF. The polylinker sequence of M13mp7 was subsequently inserted into the *Eco*RI site of CM327RF, resulting in clone CM414RF (Figure 2). In this way a number of restriction sites were positioned upstream of the 5′ end of the CPMV M cDNA facilitating manipulations with clone CM414RF. The DNA clone containing the 3′ end (CM131RF) was constructed by insertion

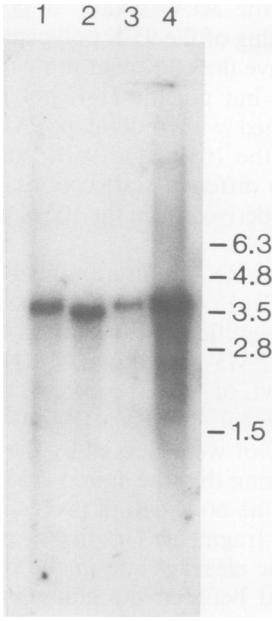

Fig. 3. Northern blot analysis of *in vitro* transcripts, obtained with phage SP6 RNA polymerase. RNA samples were glyoxylated and electrophoresed in a 1% agarose gel according to McMaster and Carmichael (1977) and transferred to nitrocellulose (Thomas, 1980). The blot was hybridised to nick-translated pCM423, washed as described (Thomas, 1980) and exposed to X-ray film (Sakura) for 16 h. **Lanes 1, 2** and **3** contain *in vitro* transcripts obtained from *Eco*RI-digested 0.05 μg pSPM5, pSPM5△*Xho*I and pSPM5△*Bgl*II respectively; **Lane 4**, 0.2 μg M RNA extracted from CPMV M components (Klootwijk *et al.*, 1977; Davies *et al.*, 1978). The numbers at the right side of the figure indicate the sizes (in kilobases) of glyoxylated marker DNA fragments derived from plasmid pCM423.

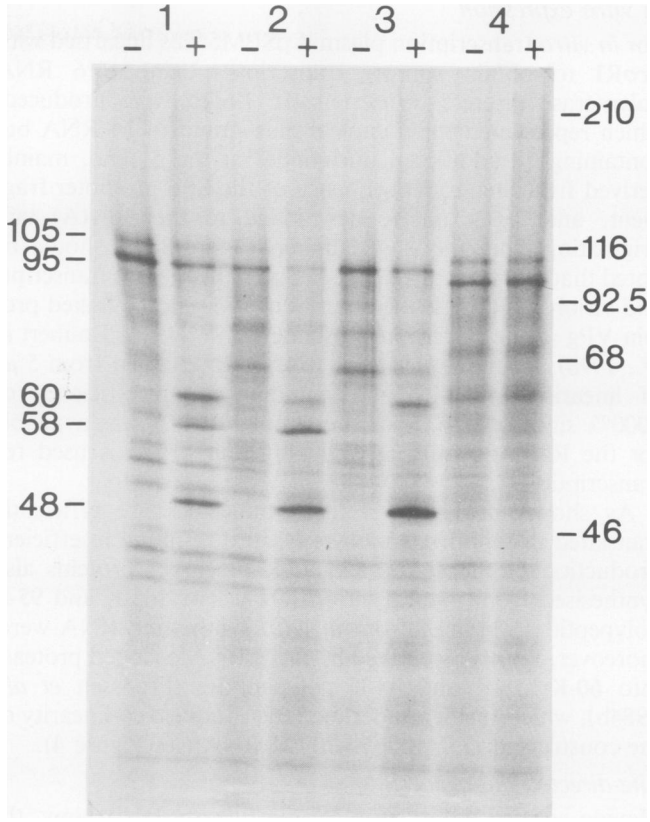

Fig. 4. *In vitro* translation and processing of transcripts obtained with SP6 polymerase. In each assay 0.5 μg of RNA was translated *in vitro* in 10 μl rabbit reticulocyte lysate using [^{35}S]methionine as radioactively labeled amino acid (Goldbach *et al.*, 1981). The *in vitro* translation products were incubated with an equal volume of 30 000 *g* supernatant fraction from B components inoculated protoplasts (+), or left untreated (−) and analysed on a 12.5% SDS-polyacrylamide gel. **Lane 1**, translation products from natural M-RNA; **lanes 2, 3** and **4** translation products from *in vitro* transcripts obtained with SP6 RNA polymerase from pSPM5, pSPM5△*Bgl*II and pSPM5△*Xho*I, respectively. The numbers at the right side of the gel indicate the sizes (in kd) of the marker proteins used: myosin, 210; *E. coli* β-galactosidase, 116; phosphorylase b, 92.5; bovine serum albumin, 68; ovalbumin,46. The numbers at the left side indicate the positions of the 105 and 95 primary translation products of M RNA and their proteolytic processing products (60, 58 and 48). The actual mol. masses of 105 and 95 are 116 and 102 kd, respectively (Van Wezenbeek *et al.*, 1983).

of a 360-bp DNA fragment, obtained upon digestion of double-stranded cDNA with *Sau*3A, into *Bam*HI and *Sma*I digested M13mp9. CM131RF contained 27 residues of the poly(A) tail and since a C residue was present adjacent to the *Sau*3A recognition sequence, the *Bam*HI site of M13mp9 was restored in this clone. The cDNA clones CM131RF, CM414RF and four previously described clones were used for the construction of a full-size cDNA clone of CPMV M RNA.

Construction of a full-length cDNA copy

The construction of a full-size DNA copy of M RNA is outlined in Figure 2. The first step in the construction was a three-point ligation of the large *Hind*III-*Bam*HI fragment of CM131RF, a 169-bp *Sau*3A fragment of pCM12 and an 83-bp *Hind*III-*Bgl*II adaptor fragment, derived from clone pCM11, resulting in clone CM254RF. As the *Sau*3A recognition sequence of the 169-bp fragment was flanked by an A residue the adaptor fragment obtained from pCM11 introduced a *Bgl*II site allowing unique cleavage next to the inserted *Sau*3A fragment in a later stage of the construction. Clone pCM102 was constructed by insertion of the 606-bp *Eco*RI-*Hind*III fragment of CM254RF and the 707-bp *Hind*III-*Bam*HI fragment of CM414RF into pBR322 digested with *Eco*RI and *Bam*HI. Insertion of the 1366-bp *Xho*I-*Sau*3A fragment of pCM17 into *Xho*I and *Bgl*II digested pCM102 resulted in clone pCM205, which now contained an M RNA-derived sequence from the *Bam*HI site at position 1504 downstream to the 3′ end. Next, the 1557-bp *Bam*HI fragment of pCM11 was ligated to pCM205, linearised with *Bam*HI, resulting in clone pCM303, which contained almost the entire cDNA except for the ultimate 5′ end. To construct a full-size clone, the *Sal*I-*Bgl*II fragment of CM414RF, containing the 5′ end, was ligated to *Sal*I and *Bgl*II digested pCM303, resulting in clone pCM423. Both restriction enzyme analysis and nucleotide sequence analysis revealed that clone pCM423 contained the complete sequence of M RNA including 27 residues of the 3′-terminal poly(A) tail.

Since bacteriophage SP6 RNA polymerase has been shown to be very useful for *in vitro* transcription (Kassavetis *et al.*, 1982; Green *et al.*, 1983), the full-length copy was positioned downstream of the SP6 promoter sequence in plasmid pSP62-PL. For this purpose the cDNA insert of pCM423, as obtained upon total digestion with *Eco*RI and partial digestion with *Bam*HI, was ligated to *Bam*HI, and *Eco*RI-digested pSP62-PL (a gift of J. Robertson). The resulting clone pSPM5 (Figure 2) and its derivatives were used for *in vitro* transcription experiments.

In vitro expression

For *in vitro* transcription plasmid pSPM5 was linearised with *Eco*RI to obtain run-off transcripts. Using SP6 RNA polymerase discrete transcripts of 3.6 kb were produced, which represented the complete plus-strand of M RNA but containing 78 additional nucleotides at the 5′ end, mainly derived from the leader sequence of the SP6 promoter fragment, and seven nucleotides added to the poly(A) tail, originating from the *Eco*RI linker (Figure 3). It should be noted that a further difference between the *in vitro* transcripts and virion M RNA is the absence of the genome-linked protein VPg at the 5′ terminus (Stanley *et al.*, 1978; Daubert *et al.*, 1978). Routinely 10 μg of RNA was obtained from 5 μg of linearised pSPM5, corresponding to an efficiency of 1000% since only 40% of the template DNA was available for the RNA polymerase and only one strand is used for transcription.

As shown in Figure 4 these molecules are efficiently translated in rabbit reticulocyte lysates, resulting in efficient production of the 105-K and 95-K precursor proteins also synthesised from natural virion RNA. The 105-K and 95-K polypeptides obtained from *in vitro* synthesised RNA were, moreover, faithfully cleaved by the B RNA-encoded protease into 60-K, 58-K and 48-K polypeptides (Franssen *et al.*, 1984b), which further underlined the complete co-linearity of the constructed DNA copy with M RNA (see Figure 4).

Site-directed mutagenesis

Having verified the integrity of the full-size DNA copy, the approach of successive *in vitro* transcription and translation was subsequently applied to DNA copies modified by site-directed mutagenesis. Two different mutant copies were constructed. One to test whether indeed two translation initiation sites are present on the M RNA, another to confirm the previous localisation of the recognition sequence for the B RNA encoded protease in the primary translation products of M RNA. The unique *Bgl*II site present in pSPM5 (at position 189 of M RNA, see Figure 2) was exploited to construct the first mutant. After digestion of pSPM5, the protruding ends of the linearised plasmid were filled in and re-ligated resulting in plasmid pSPM5△*Bgl*II. The result of the addition of four nucleotides in this mutant is a shift in the reading frame of M RNA by which a translational start at the first AUG codon at position 161 of the M RNA sequence is expected to be terminated 22 codons downstream at an UAA stop codon. The second mutant was made by deleting the 174-bp *Xho*I fragment, corresponding to the M RNA sequence between positions 1446 and 1620 (see Figure 2). This deletion does not alter the reading frame, but results in the loss of the coding sequence for 58 amino acids surrounding the glutamine-methione dipeptide sequence, proposed to be the cleavage site used for the release of the 60-K coat protein precursor (Van Wezenbeek *et al.*, 1983). Both modified DNA copies, linearised with *Eco*RI, were transcribed using SP6 polymerase and the transcripts obtained were translated in rabbit reticulocyte lysates. Compared with pSPM5 transcripts, RNA transcribed from pSPM5△*Bgl*II had the same length whereas, as expected, transcripts obtained from pSPM5△*Xho*I were slightly smaller (Figure 3). Upon translation of RNA obtained from plasmid pSPM5△*Bgl*II only the 95-K polypeptide was produced. The absence of synthesis of the 105-K polypeptide indicates that the AUG codon of position 161 represents the initiation codon for this polypeptide. Since the synthesis of the 95-K polypeptide remained unaffected translation must have initiated at the AUG codon at position 512 or 524 (Figure 1). Processing of the 95-K polypeptide produced from pSPM5△*Bgl*II, gave the 60-K coat protein precursor and the 48-K polypeptide, but not the 58-K polypeptide (Figure 4). The results obtained with mutant pSPM5△*Bgl*II definitely demonstrate that the 105-K and 95-K polypeptides are synthesised from two different start codons, and that the 95-K polypeptide is not derived from the 105-K polypeptide by proteolytic cleavage.

Translation of the RNA transcript from the second mutant clone, pSPM5△*Xho*I, resulted in the synthesis of two polypeptides with significantly lower mol. wt. compared with the products of pSPM5 and natural M RNA (Figure 4). This reduction in mol. wt. of ~7 kd conforms to the length of the deleted *Xho*I fragment. Neither of the two translation products of pSPM5*Xho*I were cleaved by the protease specified by B RNA, indicating that the amino acid sequence required for the release of the 60-K capsid protein precursor was encoded by the *Xho*I fragment. This fits the previous suggestion that the proteolytic cleavage site in the primary translation products is located between the glutamine and methionine residues corresponding to positions 1536–1541 of the M RNA.

Discussion

Here we report the successful *in vitro* expression of a full-size DNA copy of CPMV M RNA. Application of this method to DNA copies modified by site-directed mutagenesis has contributed to a further understanding of the genetic organisation of the M RNA. First, it has now been definitely demonstrated that the 105-K and 95-K polypeptides obtained upon *in vitro* translation of M RNA are synthesized from two different AUG codons. Secondly, the position of the cleavage site used by the B RNA-encoded protease to release the 60-K coat protein precursor from the 105-K and 95-K polypeptides has been determined.

As proposed by the scanning model of Kozak (1978, 1981) ribosomal subunits attach to the 5′ end of mRNAs and move downstream the leader sequence. AUG codons are preferentially recognised as initiator codons when a purine, A rather than G, is present at position −3 and a guanine is present at position +4. The AUG codon at position 161 of the M RNA, representing the initiator codon for the 105-K polypeptide, has an adenine residue at position −3, but a uracil residue at position +4 and only partially matches the consensus sequence for initiator codons. This may account for the fact that many ribosomes do not recognise this AUG triplet as initiator codon and start translation at a second 'in phase' initiator codon, presumably the AUG codon at position 512. This codon matches the consensus sequence very well, since guanine residues are present at positions −3 and +4. It may also explain why, upon *in vitro* translation of M RNA, synthesis of 95-K polypeptide is favoured over the synthesis of the 105-K polypeptide (see Figure 4 and Goldbach *et al.*, 1981; Franssen *et al.*, 1982). The occurrence of two different translation initiation sites on M RNA might be a general feature of comoviruses, since the M RNAs of all comoviruses tested so far are translated *in vitro* into two large polypeptides (Goldbach and Krijt, 1982). Furthermore, it is interesting to note that in the case of CPMV B RNA (Lomonossoff and Shanks, 1983), which specifies only one unique precursor polypeptide, the sequence surrounding the initiator AUG conforms perfectly to the 'Kozak rule'.

The large capsid protein VP37 has been shown to possess a N-acetylated methionine residue at its N terminus (Bruening, 1981). From previous work (Van Wezenbeek *et al.*, 1983) it has been proposed that the coding region of VP37 starts with the AUG codon of position 1538. This would lead however to a protein with a mol. mass of 41.2 kd, instead of 37 kd. Therefore the AUG triplet of position 1643 remained an attractive alternative, since it would give a protein with a mol. mass of ~37.2 kd. Our results with cDNA clone pSPM5△ *Xho*I, in which the AUG codon of position 1538 has been removed and not that of position 1643, demonstrate however that the N-terminal amino acid of the 37-K protein is encoded by the AUG codon at position 1538. Recently analysis of the C-terminal amino acids of the 48-K polypeptide has confirmed the position of the cleavage site.

The results presented in this paper show that certain aspects of the genetic organisation and expression of viral RNA genomes can be studied by site-directed mutagenesis of full-length DNA copies and their subsequent transcription and translation *in vitro*. Finally, the genetic studies on the functions of the CPMV genome may even be further extended *in vivo* by transfection of cowpea cells with mutagenised RNA molecules obtained by the procedure described here.

Materials and methods

Nucleic acids and enzymes

CPMV was grown in *Vigna unguiculata L.*, 'California Blackeye' and M components were purified as described previously (Klootwijk *et al.*, 1977). CPMV RNA molecules were extracted from separated components as described by Davies *et al.* (1978). Plasmid DNA was isolated as described by Godson and Vapnek (1973) or by the method of Klein *et al.* (1980). Sources of enzymes were as described previously (Van Wezenbeek *et al.*, 1983). The 16-mer d(TATTAAAATCTTAATA) was synthesized by the chemical phosphotriester method (Van der Marel *et al.*, 1982).

Molecular cloning

Double-stranded cDNA was synthesised as described previously (Van Wezenbeek *et al.*, 1983). Conditions used for ligation of DNA fragments and subsequent transformation of competent *Escherichia coli* cells have been described (Van Wezenbeek *et al.*, 1983). DNA fragments used for ligation were purified either by extraction from low-melting agarose (Weislander, 1979) or by elution from polyacrylamide gel slices (Maxam and Gilbert, 1977). Recombinant DNA clones were characterised by restriction enzyme analysis and/or nucleotide sequencing (Sanger *et al.*, 1977, 1980).

In vitro transcription

In vitro transcriptions were carried out in a volume of 100 μl containing 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 10 mM dithiothreitol, 4 mM spermidine, 0.5 mM of each of four NTPs, 5 μg template DNA, 15 units of SP6 polymerase (New England Nuclear Corp.) and 50 units of RNasin (Biotec Inc.). After a 30 min incubation at 37°C, 5 μg of DNase I (Worthington), freed of contaminating RNase activity by absorption to macaloid (Maniatis *et al.*, 1982), was added and incubation was continued for a further 15 min at 37°C. The reaction mixture was extracted once with phenol/chloroform, passed through Sephadex G-50 and precipitated with ethanol.

In vitro translation

M RNA extracted from CPMV M components and *in vitro* transcripts were translated in rabbit reticulocyte lysate using [^{35}S]methionine as radioactive amino acid as described previously (Goldbach *et al.*, 1981). Translation mixtures were incubated with an equal volume of 30 000 *g* supernatant fraction from cowpea protoplasts inoculated with B components as described by Franssen *et al.* (1982). *In vitro* translation products were analysed on a 12.5% SDS-polyacrylamide gel (Franssen *et al.*, 1982).

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