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In vitro expression of a full-length DNA copy of cowpea mosaic virus B RNA: identification of the B RNA encoded 24-kd protein as a viral protease

Jan Verver, Rob Goldbach[1], Juan A.Garcia[2] and Pieter Vos

Department of Molecular Biology, Agricultural University, De Dreijen 11, 6703 BC Wageningen, [1]Department of Virology, Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands, and [2]Centro de Biologia Molecular, Universidad Autonoma de Madrid, 28049 Madrid, Spain

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Double-stranded cDNA was synthesized from B component RNA of cowpea mosaic virus and cloned into appropriate vectors. Using four clones, together representing the entire B RNA sequence, a full-length DNA copy was constructed and subsequently positioned downstream of a phage SP6 or T7 promoter. RNA molecules transcribed from this full-size DNA copy using SP6 or T7 RNA polymerase were efficiently translated in rabbit reticulocyte lysates into a 200-kd polypeptide similar to RNA isolated from viral B components. Moreover this polypeptide was rapidly cleaved into 32-kd and 170-kd polypeptides, exactly like the 200-kd polypeptide encoded by viral B RNA. *In vitro* transcription and translation of a DNA copy in which an 87-bp-long deletion in the coding sequence for the 24-kd polypeptide was introduced revealed that the 24-kd polypeptide bears the proteolytic activity involved in the primary cleavage of the B RNA-encoded polyprotein.

Key words: cowpea mosaic virus/cDNA/*in vitro* transcription/*in vitro* translation/viral protease

Introduction

Cowpea mosaic virus (CPMV) is a plant virus with a genome consisting of two separately encapsidated, positive-sense RNA molecules designated M and B RNA (for a recent review see Goldbach and Van Kammen, 1985). The viral RNAs are characterized by a small protein, denoted VPg, covalently linked to their 5'-end (Stanley *et al.*, 1978; Daubert *et al.*, 1978) and a poly(A)-tail at their 3'-end (El Manna and Bruening, 1973). They are both translated into large polypeptides ('polyproteins') from which the functional proteins are derived by proteolytic cleavages (Rezelman *et al.*, 1980; Goldbach *et al.*, 1981). In all these respects CPMV resembles the animal picornaviruses. The marked similarity in genome organization and the sequence homology among their non-structural proteins, moreover, suggest a genetic relationship (Franssen *et al.*, 1984a).

B RNA is translated both *in vivo* and *in vitro* into a 200-kd polyprotein, which is rapidly cleaved into a 170-kd and a 32-kd polypeptide. The 170-kd polypeptide is then further processed into polypeptides with sizes of 110, 87, 84, 60, 58, 24 and 4 kd (VPg) (Rezelman *et al.*, 1980; Goldbach and Rezelman, 1983; Franssen *et al.*, 1984b and Figure 1). These cleavage products have been mapped on the 200-kd polyprotein by sequencing of their amino-terminal ends (Wellink *et al.*, 1986: Zabel *et al.*, 1984). The results revealed that three different types of cleavage sites are employed during the processing of the B RNA-encoded polyprotein: glutamine–methionine (1x), glutamine–serine (2x) and glutamine–glycine (1x) dipeptide sequences.

Inhibition studies using antisera directed against various viral proteins strongly suggested that the 32-kd protein (see Figure 1) represents a protease involved in the processing of the unique glutamine–methionine cleavage site in the M RNA-encoded polyproteins (Franssen *et al.*, 1984c). Hence involvement of this protease in cleavage at the glutamine–methionine site of the B polyprotein is also very probable. Time course translations have provided indications that the 24-kd polypeptide encoded by a B RNA represents a second viral protease (Franssen *et al.*, 1984b). This suggestion was further supported by the sequence homology of this polypeptide to the picornaviral protease P-3C (Franssen *et al.*, 1984a). We have now constructed and cloned a full-length DNA copy of B RNA in order to introduce mutations on specific sites in this copy and to express mutagenized copies *in vitro* according to the approach described previously for M RNA (Vos *et al.*, 1984). Following this approach we have been able to demonstrate that the 24-kd protein encoded by B RNA indeed represents a protease involved in the primary cleavage of the 200-kd polyprotein.

Results

Construction and molecular cloning of a full-length DNA copy of B RNA

Double-stranded cDNA was synthesized from B RNA (Van Wezenbeek *et al.*, 1983) using an oligonucleotide homologous to the first 16 nucleotides of B RNA to prime second-strand synthesis. The DNA obtained was digested with suitable restriction enzymes at positions which could be deduced from the nucleotide sequence of B RNA (Lomonossoff and Shanks, 1983). In this

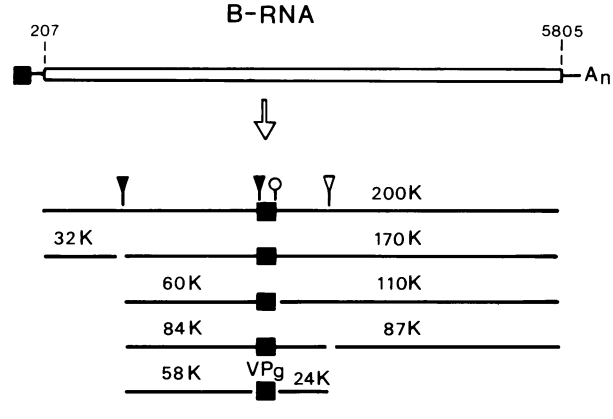

Fig. 1. Model for the translation of the B RNA of CPMV. The double-lined bar in the RNA refers to the long open reading frame running from start codon at position 207 to a stop codon at position 5805 (Lomonossoff and Shanks, 1983). VPg is indicated as a black square, other protein sequences as single lines. Cleavage sites are indicated by open circles (glutamine–methionine), open triangles (glutamine–glycine) or closed triangles (glutamine–serine) (Wellink *et al.*, 1986).

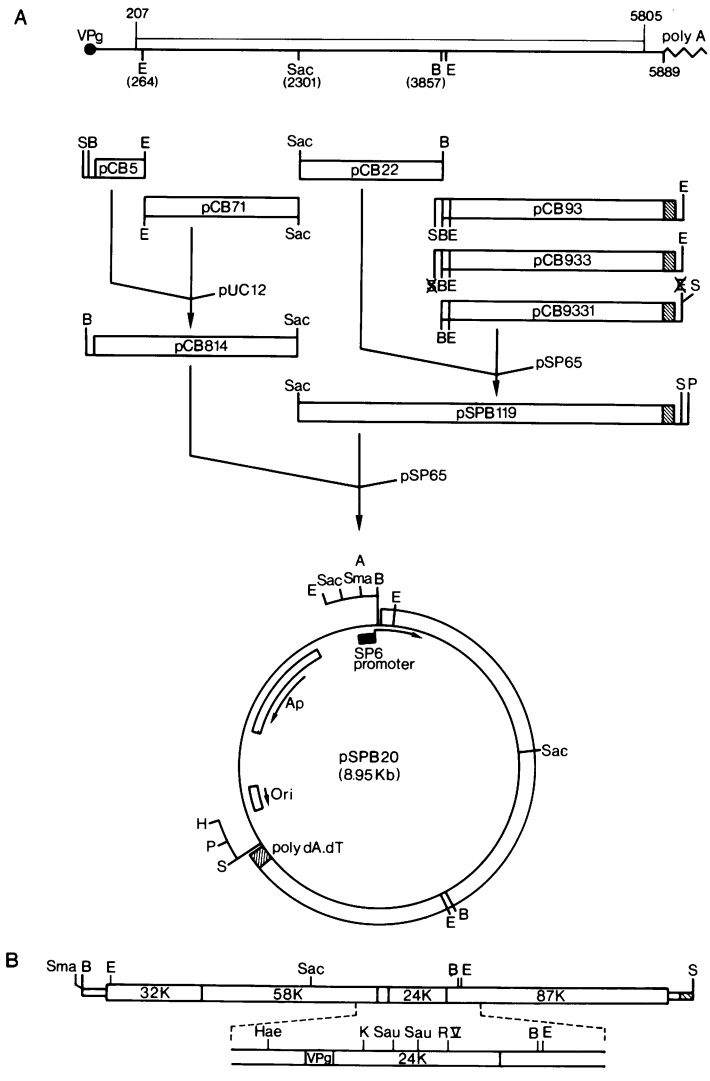

Fig. 2. Construction of a full-length DNA copy of CPMV B RNA. **Panel A:** cloning strategy for construction of a full-size DNA copy from four smaller cDNA clones. The upper line represents the complete B RNA sequence with the double-lined bar indicating the long open reading frame. The positions of relevant recognition sites of *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), *Sal*I (S), *Sac*I (Sac) and *Sma*I (Sma) are shown. In the various cDNA clones obtained vector-derived sequences are indicated by a single line, B RNA-derived sequences by open boxes and the poly d(A-T) track of 55 residues as a dashed box. Double-stranded cDNA was synthesized using oligo(dT) as primer for first strand synthesis and an oligonucleotide homologous to the first 16 nucleotides of B RNA as primer for second strand synthesis. Digestion of the double-stranded cDNA with *Bam*HI and *Sac*I and subsequent ligation into pUC8 linearized with *Sma*I and *Bam*HI and pUC12 digested with *Sac*I and *Bam*HI resulted in cDNA clones pCB93 and pCB22. Clone pCB93 contained a B RNA-specific insert from the *Bam*HI site at position 3857 to the 3′-end including a poly d(A-T) track, cDNA clone pCB22 contained a viral cDNA sequence from the *Sac*I recognition sequence at position 2301 to the *Bam*HI site at position 3857. In addition, double-stranded cDNA was digested with *Sac*I and *Eco*RI and ligated into pUC12 linearized with *Sac*I and *Eco*RI. One of the cDNA clones obtained, pCB71, contained the *Eco*RI−*Sac*I cDNA fragment, corresponding to positions 264−2301 of the B RNA sequence. To obtain a cDNA clone containing the ultimate 5′-end of the B RNA sequence, first strand synthesis was primed with a 388-bp *Kpn*I fragment (positions 2746−3034), derived from cDNA clone pCB22. Double-stranded cDNA synthesized in this way was digested with *Eco*RI and subsequently ligated into *Eco*RI- and *Sma*I-digested pUC8 resulting in clone pCB5, which contained a cDNA insert corresponding to positions 1−264 of the B RNA sequence. cDNA clones pCB5, pCB71, pCB22 and pCB93, together representing the entire B RNA-sequence, were used to construct a full-size DNA copy. For this purpose, first a cDNA clone containing the first 2301 nucleotides of B RNA was constructed (pCB814) by joining the cDNA inserts of clone pCB5, obtained upon digestion with *Sal*I and *Eco*RI, and clone pCB71, obtained upon digestion with *Eco*RI and *Sac*I, via a three-point ligation into pUC12 linearized with *Sal*I and *Sac*I. A unique *Sal*I site was introduced in clone pCB93 downstream of the poly d(A-T) track. Firstly, the *Sal*I site already present in this construct was removed by digesting the DNA with *Sal*I and subsequent religation after filling-in the recessed ends by the Klenow fragment of *E. coli* DNA polymerase I. Secondly, the resulting clone pCB933 was partially digested with *Eco*RI, made blunt-end with S1 nuclease and the linearized DNA, obtained by digestion of one of the two *Eco*RI sites eluted from the gel (Tautz and Renz, 1984). Finally *Sal*I linkers were coupled to the DNA, cohesive ends were generated by digestion with *Sal*I and the DNA was religated. One of the clones obtained in this way, pCB9331, contained a unique *Sal*I site downstream of the poly d(A-T) track as intended. The *Bam*HI−*Sal*I fragment from this clone and the *Sac*I−*Bam*HI fragment from clone pCB22 were joined via a three-point ligation with *Sac*I and *Sal*I digested pSP65 to produce clone pSPB119, containing a cDNA copy corresponding to the 3′-end of B RNA from position 2301 downstream. Finally a full-size cDNA clone was constructed, denoted pSPB20, by joining of the *Bam*HI−*Sac*I fragment of pCB814 with the *Sac*I−*Pst*I fragment of pSPB119 in a three-point ligation with *Bam*HI- and *Pst*I-digested pSP65. pSPB20 has a total length of 8.95 kb and the B RNA-specific cDNA can be excised from this plasmid with *Sma*I and *Sal*I (see also panel B). This 5.95-kb *Sma*I−*Sal*I fragment, containing the full-length DNA copy, was inserted into plasmid pT7-1 digested with *Sma*I and *Sal*I, resulting in clone pTB3 (not shown). Using this clone B RNA-like transcripts could be synthesized initiated at the T7 promoter present in the construct. **Panel B:** schematic representation of the full-length DNA copy. The long open reading frame with the positions of various B RNA-specific proteins is indicated by a large open bar, other B RNA-specific sequences are indicated by double lines. The region in the DNA copy used in the construction of a mutant with a deletion in the coding region for the 24-kd polypeptide is presented enlarged (see Materials and methods). Relevant recognition sites for restriction enzymes are indicated: Hae (*Hae*III), K (*Kpn*I), Sau (*Sau*3A), RV (*Eco*RV), for other enzymes see panel A.

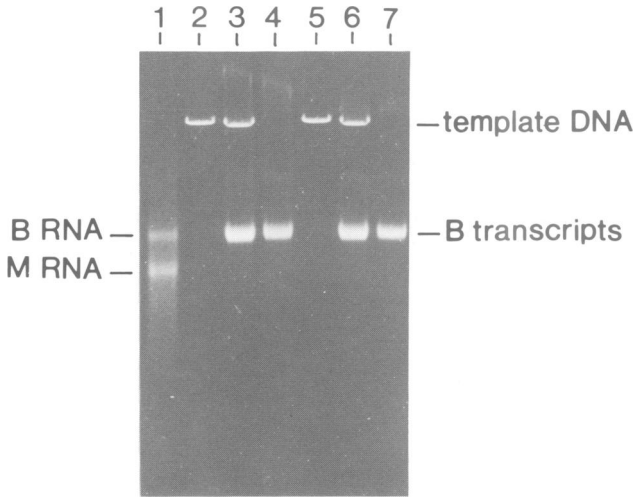

Fig. 3. Native 1.2% agrarose gel with reaction products of several *in vitro* translation assays using T7 RNA polymerase. On the right-hand side the positions of template DNA and RNA transcripts are shown. On the left-hand side the positions of M and B RNA isolated from virus particles are indicated. **Lane 1** shows M and B RNA isolated from virus particles. **Lanes 2** and **5** show linearized template DNA of pTB3 and pTB3Δ1 respectively. Reaction products of *in vitro* transcriptions with pTB3 and pTB3Δ1 are shown without DNase treatment (**lanes 3** and **6** respectively) or DNase treated (**lanes 4** and **7** respectively). Reaction conditions were as described in Materials and methods using 20 units T7 RNA polymerase per μg of template DNA. Template DNA was removed by DNase treatment as described previously (Vos *et al.*, 1984).

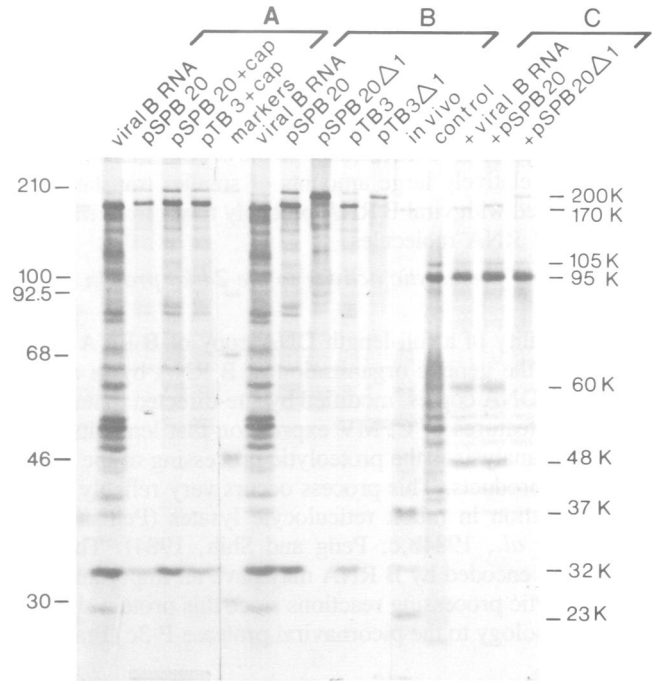

Fig. 4. *In vitro* translation products of transcripts obtained from various CPMV-specific cDNA clones using SP6 or T7 polymerase. Numbers at the left-hand side indicate the sizes (in kd) of marker proteins used: myosin, 210; phosphorylase b, 100 and 92.5; bovine serum albumin, 68; ovalbumin 46; carbonic anhydrase, 30. Numbers at the right-hand side correspond to the positions of relevant viral proteins encoded by natural M or B RNA. **Panel A:** *in vitro* translation products of viral B RNA and of transcripts synthesized by SP6 RNA polymerase from pSPB20 or by T7 RNA polymerase from pTB3, with (+ cap) or without a cap structure at the 5′ end. **Panel B:** *in vitro* translation products of viral B RNA and of transcripts synthesized by SP6 or T7 RNA polymerase using 'wild type' DNA copies as template DNA (pSPB20, pTB3) or DNA copies in which a deletion in the coding region for the 24-kd polypeptide was introduced (pSPB20Δ1 and pTB3Δ1). **Panel C:** *in vitro* translation products of viral M RNA, untreated (control) or incubated with unlabelled translation products of viral B RNA, or of transcripts from pSPB20 or pSB20Δ1. M RNA was translated in the presence of [^{35}S]methionine as described previously (Goldbach *et al.*, 1981) and after 1 h a 3 times excess of unlabelled translation products of viral B RNA or B RNA transcript was added and incubation continued at 30°C for another 5 h. In the case of the control sample, incubation was also continued for 5 h. The lane between panels B and C shows the [^{35}S]methionine-labelled proteins extracted from CPMV-infected protoplasts.

way a set of restriction fragments was generated from the cDNA synthesized which, after cloning in suitable vectors, represented the entire B RNA sequence. After verifying their integrity by restriction enzyme and/or sequence analysis the cDNA inserts of four different clones were selected and ligated to generate a full-length cDNA clone of B RNA. The strategy of this multi-step cloning procedure is outlined in Figure 2A.

The resulting clone pSPB20 had a total length of 8.95 kb with a unique *Sal*I-site downstream of a poly d(A-T) track of 55 bp that could be utilized to synthesize run-off transcripts initiated at the SP6-promoter by phage SP6 RNA polymerase (Green *et al.*, 1983; Vos *et al.*, 1984). Alternatively the viral insert of clone pSPB20 was positioned downstream of a phage T7 promoter, resulting in clone pTB3, to allow *in vitro* transcription by RNA polymerase from phage T7 instead of SP6 (Figure 2).

In vitro expression

To generate run-off transcripts plasmids pSPB20 and pTB3 were linearized downstream of their poly d (A-T) track by digestion with *Sal*I (Figure 2) and trancribed using SP6 or T7 RNA polymerase, repectively (Vos *et al.*, 1984; Davanloo *et al.*, 1984) (see Figure 3). Discrete transcripts of 6.0 kb were produced containing, in addition to B RNA-specific sequences, ~35 extra nucleotides to the 5′ end, derived from the SP6 or T7 promoter and multiple cloning site, and nine nucleotides at the 3′ end of the 55 residue-long poly(A) tail. A further difference with B RNA from virions was the absence at the 5′-end of the genome-linked protein VPg. Depending on the amount of SP6 or T7 polymerase added the efficiency of the *in vitro* transcription reactions could be increased, yielding up to 20 μg of RNA from 1 μg of template DNA. Occasionally transcripts were capped by addition of the cap precursor m^7GpppG to the transcription reaction (Konarska *et al.*, 1984). The efficiency of capping depended on the ratio of m^7GpppG to GTP in the reaction mixture. In the case of SP6 polymerase capping was very efficient giving 50% capped transcripts with equal amounts of GTP and m^7GpppG. For the T7 polymerase a four times molar excess of m^7GpppG to GTP had to be used to achieve 50% capping (results not shown).

The *in vitro* transcripts obtained were translated in rabbit reticulocyte lysates resulting in efficient production of a 200-kd protein, co-migrating with the 200-kd polyprotein produced from natural B RNA (Figure 4A). Moreover this 200-kd polypeptide was cleaved into two polypeptides with sizes of 170 and 32 kd, similar to the primary cleavage of B RNA-encoded 200-kd polyprotein (Figure 4A). These results show that the DNA copy contained a single long open reading frame similar to viral B RNA and that the translation product contains the proteolytic activity for cleavage of the polyprotein at the right position. No differences were observed between the *in vitro* translation products of *in vitro* RNAs synthesized by T7 or SP6 RNA polymerase. The presence of the cap structure at the 5′ end of

the *in vitro* transcripts did not significantly increase the translational activity of the transcripts, which is in contrast to results obtained with several *in vitro* synthesized eukaryotic mRNAs (Krieg and Melton, 1984). The minor differences in the amounts of translation products obtained (Figure 4A) are probably due to variations in RNA concentration in the *in vitro* translation reactions. The relatively large amounts of smaller translation products obtained with viral B RNA probably result from translation of degraded RNA molecules.

Assignment of proteolytic activity to the 24-kd protein encoded by B RNA

The availability of a full-length DNA copy of B RNA enabled us to study the genetic organization of B RNA by *in vitro* expression of DNA copies, modified by site-directed mutagenesis. One of the features of CPMV expression that lends itself well for *in vitro* analysis is the proteolytic processing of the primary translation products. This process occurs very reliably upon *in vitro* translation in rabbit reticulocyte lysates (Pelham, 1979; Franssen *et al.*, 1984b,c; Peng and Shih, 1984). The 24-kd polypeptide encoded by B RNA may have an important role in the proteolytic processing reactions since this protein shows sequence homology to the picornaviral protease P-3c (Franssen *et al.*, 1984a).

To test whether the 24-kd polypeptide indeed possesses proteolytic activity we introduced a small deletion in the coding region for this protein within the DNA copy of B RNA and determined possible defects in proteolytic cleavage of the translation product of this deletion mutant. For this purpose an 87-bp *Sau*3A fragment, corresponding to positions 3240–3327 of the B RNA sequence, was excised from the full-size DNA copy (Figure 2B and Materials and methods). This deletion did not change the reading frame, but resulted in the loss of the coding sequence for 29 amino acids of the 24-kd polypeptide. The resulting deletion mutant, denoted pSPB20Δ1 or pTB3Δ1 depending on the choice of the promoter, was subjected to subsequent *in vitro* transcription and translation after linearization with *Sal*I. Compared to the primary translation products of both viral B RNA and transcripts of clones pSPB20 or pTB3, the translation product of the deletion mutant was, as expected, slightly smaller (see Figure 4B). Furthermore this product did not undergo the faithful proteolytic processing as observed with the translation products of pSPB20 and pTB3 (figure 4B). This finding indicates that the 24-kd polypeptide indeed represents a proteolytic enzyme, involved in the primary cleavage of the 200-kd viral polyprotein, which occurs at a glutamine–serine pair (Wellink *et al.*, 1986).

The 32-kd protein released by the primary cleavage has been reported to represent also a viral protease, involved in the cleavage of the M RNA encoded polyproteins at a glutamine–methionine site (Franssen *et al.*, 1982, 1984c). We were interested in the effect of the deletion in the 24-kd protease, i.e. blocking of the release of the 32-kd protease, on the activity of this second protease. For this purpose *in vitro* translation products of viral B RNA or of transcripts from the cDNA clones pSPB20 and pSPB20Δ1, respectively, were mixed with the M RNA-encoded polyproteins and incubated for 5 h at 30°C (Figure 4C). Significant processing of the M RNA-encoded 95-kd polyprotein to polypeptides of 60 and 48 kd (Franssen *et al.*, 1982) was observed when *in vitro* translation products of viral B RNA or of transcript from clone pSPB20 were added, but upon addition of translation product from the mutant clone pSPB20Δ1 processing could not be detected (Figure 4C). A possible explanation for this effect of the B RNA-encoded 24-kd polypeptide on the processing of the primary translation products of M RNA at a glutamine–methionine site is discussed below.

Discussion

Various animal and plant RNA viruses use proteolytic processing to synthesize their proteins. Most of the current knowledge on viral protein cleavage reactions is derived from studies with picornaviruses (for recent review see Toyoda *et al.*, 1986; Nicklin *et al.*, 1986; Jackson, 1986) and CPMV (Goldbach and Van Kammen, 1985). Until recently it was generally believed that in the proteolytic processing of the picornaviral polyproteins, in addition to the viral protease 3C, a cellular enzyme was involved (Korant *et al.*, 1980; Burroughs *et al.*, 1984). By analogy to the picornaviruses a similar involvement of a host-encoded protease in the cleavage of the CPMV polyproteins may be surmized. In this paper we report the construction of a full-length cDNA clone of CPMV B RNA and its subsequent exploitation for studying the proteolytic processing of the CPMV translation products. The *in vitro* transcription and translation experiments described here demonstrate that the B RNA-encoded 24-kd polypeptide represents a viral protease, being involved in the primary cleavage of the 200-kd polyprotein, thereby excluding the possible involvement of a cellular enzyme in this process.

Bacteriophage SP6 RNA polymerase has been demonstrated to be very suitable for *in vitro* transcription of DNA templates (e.g. Green *et al.*, 1983; Melton *et al.*, 1984; Vos *et al.*, 1984; Tabler and Sänger, 1985). In addition to SP6 RNA polymerase we now also have used T7 RNA polymerase to synthesize RNA from templates provided with a T7 promoter fragment. RNA synthesis by T7 RNA polymerase also appeared to be very efficient, giving yields comparable with those of SP6 polymerase-dependent reactions and, furthermore, efficient capping of the RNA transcripts could be achieved similar to the SP6 polymerase (Konarska *et al.*, 1984). The advantage of the use of T7 instead of SP6 RNA polymerase in *in vitro* transcription assays may be the thorough understanding of the genetics of phage T7. Transcription of T7 genes under the control of T7 promoters has been extensively studied (see for instance Dunn and Studier, 1983). Moreover, plasmids are available containing the T7 RNA polymerase gene under the control of the λP_L promoter, permitting simple, large-scale purification of the enzyme from induced *Escherichia coli* cells (Tabor and Richardson, 1985; this paper).

Recently the proteolytic cleavage sites in the 200-kd polyprotein from B RNA have been identified (Wellink *et al.*, 1986). Three types of cleavage sites are used for processing, i.e. glutamine–serine, glutamine–glycine and glutamine–methionine dipeptide sequences. For processing of the M RNA-encoded polyproteins only two of these three different cleavage sites are used: a glutamine–methionine dipeptide sequence to release the 60-kd capsid protein precursor, and a glutamine–glycine dipeptide sequence to release the two capsid proteins from this precursor (Van Wezenbeek *et al.*, 1983; Vos *et al.*, 1984). Inhibition studies using antisera raised against various viral proteins and *in vitro* processing studies using partially purified proteins from B component-inoculated protoplasts strongly suggested that the 32-kd protein encoded by B RNA (see Figure 1) is involved in the cleavage of the M RNA-encoded polyproteins at the glutamine–methionine site (Franssen *et al.*, 1984b,c). If this protein indeed represents a protease recognizing this site then it is most probably also involved in cleavage of the single glutamine–

methionine site in the B polyprotein, which is situated at the left border of the 24-kd polypeptide sequence (Figure 1).

Since we have now demonstrated that the 24-kd polypeptide also possesses proteolytic activity, responsible for the primary cleavage of the 200-kd polyprotein at a glutamine−serine dipeptide sequence, the question arises which of the two proteases recognizes the glutamine−glycine cleavage sites. Comparison of the three types of cleavage sites used in the processing of the CPMV polyproteins shows that they fall into two categories. Although all cleavage sites share a glutamine residue at the first position of the dipeptide sequence the nature of the residue at the second position is quite variable, being either small and polar (glycine and serine) or large and non-polar (methionine). It is therefore tempting to assume that the 32-kd polypeptide recognizes only glutamine−methionine cleavage sites, while the 24-kd polypeptide may be involved in cleavage of both the glutamine−serine and glutamine−glycine cleavage sites. Indeed, preliminary experiments seem to indicate that the 24-kd polypeptide is also involved in the cleavage at a glutamine−glycine site in the M RNA-encoded polyproteins (data not shown).

Surprizingly the small deletion in the coding region of the 24-kd polypeptide also affected the processing of the M RNA-encoded polyproteins at a glutamine−methionine site, which is supposed to be recognized by the 32-kd protease (Franssen *et al.*, 1984c). While such processing was observed upon addition of translation products from clone pSPB20 this was clearly not the case with translation products from clone pSPB20Δ1. The most plausible explanation for this inhibitory effect is that release of the 32-kd polypeptide from the 200-kd polyprotein (by the 24-kd protease) is a prerequisite for the 32-kd polypeptide to become also proteolytically active. Clearly further experiments are necessary to confirm this latter hypothesis and to understand fully how the two different B RNA-encoded proteases co-operate in the release of all mature proteins from the viral polyproteins.

Materials and methods

Nucleic acids and enzymes

CPMV was propagated in *Vigna unguiculata* L., 'California Blackeye' and B components were purified as described previously (Klootwijk *et al.*, 1977). CPMV B RNA was extracted from purified B components as described by Zimmern (1975) or Davies *et al.* (1978). Plasmid DNA isolations on large or small scale were performed by the alkaline lysis method (Birnboim and Doly, 1979). All enzymes used were purchased from Boehringer Mannheim B.V. or Anglian Biotechnology Corp. and were used according to the manufacturer's indications. T7 RNA polymerase was isolated as described below. Plasmid pT7-1 was purchased from United States Biochemical Corp. The 16-mer d(TATTAAAATCAATACA) was synthesized by the chemical phosphotriester method (Van der Marel *et al.*, 1982).

Molecular cloning

Double-stranded cDNA was synthesized from CPMV RNA as described previously (Van Wezenbeek *et al.*, 1983). DNA fragments were joined by T4 DNA ligase and transformed into competent *E. coli* HB101 cells as described by Van Wezenbeek *et al.* (1983). DNA fragments used for ligation were generally purified either by extraction from agarose gels (Tautz and Renz, 1984; Weislander, 1979) or by elution from polyacrylamide gel slices (Maxam and Gilbert, 1977). Recombinant DNA clones were analysed by restriction enzyme mapping and/or nucleotide sequencing (Sanger *et al.*, 1977, 1980).

Introduction of a deletion in the full-length DNA copy of B RNA

A *Hae*III−*Eco*RI fragment (corresponding to positions 2815−3865 of the B RNA sequence) (see Figure 2B) was isolated from a full-length cDNA clone of B RNA (pSPB20) and inserted in plasmid pUC9 previously digested with *Sma*I and *Eco*RI. The resulting clone, pLB281, was cut with *Kpn*I and *Eco*RV, both having unique recognition sites in this plasmid, yielding a small DNA fragment of 301 bp corresponding to positions 3134 (*Kpn*I) to 3435 (*Eco*RV) of the B RNA sequence (see Figure 2B), together with a large fragment. The 301-bp fragment was subsequently digested with *Sau*3A resulting in three fragments of 106 bp (*Kpn*I, 3134, to *Sau*3A, 3240). 87 bp (*Sau*3A, 3240, to *Sau*3A, 3326) and 108 bp (*Sau*3A, 3327, to *Eco*RV, 3435). In a three-point ligation the 106-bp *Kpn*I−*Sau*3A fragment and 108-bp *Sau*3A−*Eco*RV fragment were joined together to the large *Kpn*I−*Eco*RV fragment of pLB281. In this way a clone was constructed, pLB281ΔSau87, that contained a *Hae*III−*Eco*RI fragment (corresponding to positions 2815−3865 of the B RNA sequence), missing the 87-bp *Sau*3A fragment (positions 3240−3327). To construct a full-length cDNA clone of B RNA containing this deletion, the *Kpn*I−*Eco*RI fragment (positions 3134−3865) of clone pLB281ΔSau87 was exchanged for the corresponding fragment of pSPB20, yielding clone pSPB20Δ1. The *Sma*I−*Sal*I fragment of pSPB20Δ1, containing the full-size DNA copy of B RNA with the desired deletion was also positioned downstream of the T7 promoter in plasmid pT7-1 in a similar way as decribed for clone pTB3 (see Results and Figure 2) yielding clone pTB3Δ1.

Purification of T7 RNA polymerase

As source for the purification of T7 RNA polymerase plasmid pT7P2 was used in *E. coli* strain NF1 (Bernard *et al.*, 1979). Isolation was according to Tabor and Richardson (1985) with some modifications. Plasmid pT7P2 contained a *BamHI*−*Eco*RI fragment from the low copy number plasmid pGP1 (a kind gift of Stanley Tabor; described in Tabor and Richardson, 1985) cloned into the high copy number plasmid pUC8. This fragment contained the T7 RNA polymerase gene under control of the λP_L promoter. Due to a temperature-sensitive cI repressor gene in bacterial strain NF1 (Bernard *et al.*, 1979) synthesis of T7 polymerase can be triggered by raising the temperature from 28 to 42°C.

Cells were grown at 28°C until an OD_{600} of 0.4 was reached and then incubated for 90 min at a temperature of 42°C. Cells were collected by centrifugation and lysed according to the procedure of Butler and Chamberlin (1982). The lysate was centrifuged for 30 min at 20 000 *g* and $(NH_4)_2SO_4$ was added to the supernatant to a saturation level of 30%. The $(NH_4)_2SO_4$ suspension was centrifuged for 30 min at 20 000 *g* and the pellet fraction containing most of the T7 RNA polymerase was further purified as described by Tabor and Richardson (1985).

In vitro transcription and translation

In vitro transcription using SP6 polymerase was performed as described previously (Vos *et al.*, 1984). *In vitro* transcription with T7 RNA polymerase was carried out in 100 μl containing 40 mM Tris−HCl pH 8.0, 20 mM $MgCl_2$, 10 mM DTT, 100 μl/ml BSA, 1 mM of each four NTPs, 5 μg linearized template DNA, 5−50 U of T7 polymerase and 50 U of RNasin. To synthesize capped transcripts the GTP concentration was lowered to 0.2 mM and the cap precursor m^7GpppG was added to a final concentration of 2 mM.

Aliquots of the transcription mixture were translated directly, without any purification, in rabbit reticulocyte lysates using [^{35}S]methionine as radioactive amino acid as described previously (Goldbach *et al.*, 1981). *In vitro* translation products were analysed on a 12.5% SDS−polyacrylamide gel (Franssen *et al.*, 1982).

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References

Bernard,H.U., Remaut,E., Vickers Hersfield,M.V., Das,H.K., Helinski,D.R., Yanofsky,C. and Franklin,N. (1979) *Gene*, **5**, 59−76.
Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513−1523.
Burroughs,J.N., Sanger,D.V., Clarke,B.E., Rowlands,D.J., Billian,A. and Collen,D. (1984) *J. Virol.*, **50**, 878−883.
Butler,E.T. and Chamberlin,M.J. (1982) *J. Biol. Chem.*, **257**, 5772−5778.
Daubert,S.D., Bruening,G. and Najarian,R.C. (1978) *Eur. J. Biochem.*, **92**, 45−51.
Davanloo,P., Rosenberg,A.H., Dunn,J.J. and Studier,F.W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2035−2039.
Davies,J.W., Verver,J.W.G., Goldbach,R.W. and Van Kammen,A. (1978) *Nucleic Acids Res.*, **5**, 4643−4661.
Dunn,J.J. and Studier,F.W. (1983) *J. Mol. Biol.*, **166**, 477−535.
El Manna,M.M. and Bruening,G. (1973) *Virology*, **56**, 198−206.
Franssen,H., Goldbach,R., Broekhuijsen,M., Moerman,M. and Van Kammen,A. (1982) *J. Virol.*, **41**, 8−17.
Franssen,H., Leunissen,J., Goldbach,R., Lomonossoff,G. and Zimmern,D. (1984a) *EMBO J.*, **3**, 855−861.
Franssen,H., Goldbach,R. and Van Kammen,A. (1984b) *Virus Res.*, **1**, 39−49.
Franssen,H., Moerman,M., Rezelman,G. and Goldbach,R. (1984c) *J. Virol.*, **50**, 183−190.

Goldbach,R. and Rezelman,G. (1983) *J. Virol.*, **46**, 614–619.
Goldbach,R.W. and Van Kammen,A. (1985) In Davies,J.W. (ed.) *Molecular Plant Virology.* CRC Press, Boca Raton, FL, Vol. II, pp. 83–120.
Goldbach,R.W., Schilthuis,J.G. and Rezelman,G. (1981) *Biochem. Biophys. Res. Commun.*, **99**, 89–94.
Green,M.R., Maniatis,T. and Melton,D.A. (1983) *Cell*, **432**, 681–694.
Jackson,R.J. (1986) *Virology*, **149**, 114–127.
Klootwijk,J., Klein,I., Zabel,P. and Van Kammen,A. (1977) *Cell*, **11**, 73–82.
Konarska,M.M., Padgett,R.A. and Sharp,P.A. (1984) *Cell,*, **38**, 731–736.
Korant,B.D., Chow,N.L., Lively,M.O. and Powers,J.C. (1980) *Ann. NY Acad. Sci.*, **343**, 304–318.
Krieg,P.A. and Melton,D.A. (1984) *Nucleic Acids Res.*, **11**, 7057–7070.
Lomonossoff,G.P. and Shanks,M. (1983) *EMBO J.*, **2**, 2253–2258.
Van der Marel,C.A., Wille,G., Marugg,J.E., De Vroom,E., Tromp,M., Van Boekel,C.A.A. and Van Boom,J.H. (1982) *Rec. Trav. Chim. Pays-Bas*, **10**, 239–241.
Maxam,A.M. and Gilbert,W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 560–564.
Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
Nicklin,M.J.H., Toyoda,H., Murray,M.G. and Wimmer,E. (1986) *Biotechnology*, **4**, 33–42.
Pelham,H.R.B. (1979) *Virology*, **96**, 463–477.
Peng,X.X. and Shih,D.S. (1984) *J. Biol. Chem.*, **259**, 3197–3201.
Rezelman,G., Goldbach,R. and Van Kammen,A. (1980) *J. Virol.*, **36**, 366–373.
Sanger,F., Nickler,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Sanger,F., Coulson,A.R., Barrel,B.G., Smith,A.J.H. and Roe,B.A. (1980) *J. Mol. Biol.*, **143**, 161–178.
Stanley,J., Rottier,P., Davies,J.W., Zabel,P. and Van Kammen,A. (1978) *Nucleic Acids Res.*, **5**, 4505–4522.
Tabler,M. and Sänger,H.L. (1985) *EMBO J.*, **4**, 2191–2199.
Tabor,S. and Richardson,C.C. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 1074–1078.
Tautz,D. and Renz,M. (1983) *Anal. Biochem.*, **132**, 14–19.
Toyoda,H., Nicklin,M.J.H., Murray,M.G. and Wimmer,E. (1986) In Inouye,M. and Sarma,M. (eds), *Protein Engineering: Application in Science, Medicine and Industry.* Academic Press, New York, in press.
Vos,P., Verver,J., Van Wezenbeek,P., Van Kammen,A. and Goldbach,R. (1984) *EMBO J.*, **3**, 3049–3053.
Weislander,L. (1979) *Anal. Biochem.*, **98**, 305–309.
Wellink,J., Rezelman,G., Goldbach,R. and Beyreuther,K. (1986) *J. Virol.*, **59**, 50–58.
Van Wezenbeek,P., Verver,J., Harmsen,J., Vos,P. and Van Kammen,A. (1983) *EMBO J.*, **2**, 941–946.
Zabel,P., Moerman,M., Lomonossoff,G., Shanks,M. and Beyreuther,K. (1984) *EMBO J.*, **3**, 1629–1634.
Zimmern,D. (1975) *Nucleic Acids Res.*, **2**, 1189–1201.

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