GENES AND SEQUENCES INVOLVED IN THE REPLICATION OF COWPEA MOSAIC VIRUS RNAs

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STELLINGEN

1 De homologie tussen cowpea mosaic virus (CPMV) en poliovirus geldt voor structurele kenmerken maar niet voor overeenkomende mechanismen van de virusvermenigvuldiging.
Goldbach (1987) Microbiological Sciences 4, 197-202.
Wellink (1987) Proefschrift LU Wageningen.
Dit proefschrift.

2 De door CPMV gecodeerde 87 en 110 kilodalton eiwitten kunnen de synthese van een complementaire RNA keten niet initiëren.
Dit proefschrift.

3 Voor het verkrijgen van stabiele mutaties in specifieke gebieden van het CPMV genoom zal random mutagenese van een DNA cassette een kansrijkere manier zijn dan plaats gerichte mutagenese.

4 De experimenten van Jang et al en Pelletier en Sonenberg tonen onomstotelijk aan dat picornaviraal RNA zich niet conformeert aan het ribosoom scanning model dat is opgesteld voor eukaryotische messenger RNAs.
Kozak et al (1983) Microbiological Reviews 47, 1-45.
Jang et al (1988) J. Virology 62, 2636-2643.
Pelletier en Sonenberg (1988) Nature 334, 320-325.

5 Het artikel van Chu en Westaway waarin zij beweren initiatie van de flavivirus RNA replicatie <u>in vitro</u> waar te nemen puilt uit van `wishfull thinking` en tegenspraken. Bovendien wordt, hoewel zij expliciet het tegendeel beweren, in dit artikel het door hen zelf geponeerde replicatiemodel ondermijnt.
Chu en Westaway (1985) Virology 140, 68-79.
Chu en Westaway (1987) Virology 157, 330-337.

6 Het marginale effect op de smelttemperatuur van eiwitten na het plaatsspecifiek vervangen van aminozuren toont aan dat het inzicht in het mechanisme, waarop de thermostabiliteit van enzymen die werkzaam zijn in extreem thermofiele Archaebacteriën berust, beperkt is.
Nicholson et al (1988) Nature 336, 651-656.
Eggen et al, manuscript in preparation.

7 De interrumperende kracht van de telefoon is dermate groot dat het veelal effectiever is een gesprek met een collega verderop in de gang telefonisch te voeren.

8 Bij het tot expressie komen van een proefschrift blijkt een promotor tegelijkertijd als een terminator te werken.

9 Het vervangen van ervaren universitair docenten door nog onervaren jonge wetenschappers of sterker nog door apparatuur is een bezuinigingsmaatregel die onontkoombaar zal leiden tot een desastreuse daling van het onderzoeks- en onderwijsniveau.

10 Het is een geluk voor vegetariërs dat plantevirussen niet tot het dierenrijk behoren.

Stellingen behorend bij het proefschrift:

Genes and sequences involved in the replication of cowpea mosaic virus RNAs.

Wageningen, 3 mei 1989 Rik Eggen

Ik wil iedereen bedanken die een bijdrage heeft geleverd aan het tot stand komen van dit proefschrift.

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CHAPTER 1

General introduction

GENERAL INTRODUCTION

Plant viruses comprise a diverse group with a broad variation in particle size and morphology as well as in genomic structure and strategy to express their genetic information. The vast majority of these viruses possess a single-stranded, plus-sense RNA as their genome.

In the last few years the knowledge on the molecular genetics and expression mechanism of plant viral genomes has considerably been expanded by the elucidation of complete nucleotide sequences of the RNA genomes and by the development of powerfull techniques to exploit full-length cDNA clones made against the RNA genomes. However, the molecular mechanisms underlying the viral RNA replication are still poorly understood.

For viral RNA replication the production of a virus-encoded replicase is required which must recognize the 3' terminus of RNA templates. Therefore it is plausible to assume that distinct groups of viruses with each a different translation strategy and terminal structures at their genomes will also utilize different mechanisms for viral RNA replication. This matter is discussed in more detail in chapter 2, which specifically reviews the RNA replication of cowpea mosaic virus (CPMV), type member of the comoviruses.

The scope of the investigations described in this thesis, is to unravel the molecular mechanism of CPMV RNA replication.

CPMV has a bipartite genome consisting of two positive stranded RNA molecules that have a small protein, VPg, covalently linked to the 5' end and a poly(A)-tail at the 3' end. The genomic structure and expression strategy have been thoroughly examined and hence this virus lends itself very well for studies on the RNA replication.

Our first approach to analyse the function of viral proteins and the importance of certain nucleotide sequences of the genomic RNAs in the replication was the construction of an *in vitro* replication system that accepted exogenously added RNA templates. Since viral replicase activities isolated from infected cowpea tissue were low, incomplete and were overshadowed by a host-encoded RNA-dependent RNA-polymerase, these *in vitro* RNA replication studies were also done with crude membrane fractions, prepared from another systemic host of CPMV, *Chenopodium amaranticolor* (Chapter 3).

In addition, as an alternative approach to identify viral genes and nucleotide sequences involved in RNA replication, DNA copies of B- and M-RNA have been exploited (Chapters 4, 5 and 6). Firstly, cDNA fragments were used to examine enzymatic activities associated with polymerase proteins, produced in a procaryotic expression system (Chapter 4). Secondly, cDNA clones were used to produce *in vitro* infectious transcripts. By improvement of both the cowpea protoplast inoculation procedure and the specific infectivity of the *in vitro* transcripts (Chapter 5), the systems were made suitable to enable the introduction of site specific mutations in the viral RNAs and the analysis of the effects of such mutations on the RNA replication *in vivo* (Chapter 6).

CHAPTER 2

RNA replication in comoviruses

Rik Eggen and Ab van Kammen.

In: RNA Genetics vol I (Ahlquist, P., Holland, J., and Domingo, E. eds) pp 49-69, CRC Press, 1988.

RNA REPLICATION IN COMOVIRUSES

Rik Eggen and Ab van Kammen

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I. INTRODUCTION

Among the various groups of plant RNA viruses with a positive strand RNA genome are viruses with a mono-, a bi-, and a tripartite genome (see Table 1).[1] Monopartite viruses, like tobamoviruses (e.g., tobacco mosaic virus, TMV), tymoviruses (e.g., turnip yellow mosaic virus, TYMV), and potyviruses (e.g., potato virus Y, PVY), contain the information for virus multiplication within a single RNA molecule, whereas in viruses with a bipartite genome, like comoviruses (e.g., cowpea mosaic virus, CPMV) and nepoviruses (e.g., tomato black ring virus, TBRV), this information is divided among two single-stranded RNA molecules and in viruses with a tripartite genome, like bromoviruses (e.g., brome mosaic virus, BMV) and alfalfa mosaic virus (AMV), among three RNA molecules. With bipartite and tripartite genome viruses the different segments of the genome are separately encapsidated so that infectious preparations of such viruses consist of mixtures of two or three nucleoprotein particles.

The different plant RNA virus groups are further characterized by the structures found at the 5′ and 3′ termini of their genomic RNAs. The predominant structures at the 5′ ends are a cap (= m^7GpppG) similar to the structure found on eukaryotic messenger RNAs and a protein, VPg (viral protein, genome linked), linked to the 5′-terminal phosphate. Twelve different virus groups are known to carry a cap at the 5′ ends of their genomic RNAs; among which are the bromoviruses, the tobamoviruses, the tymoviruses, and alfalfa mosaic virus, whereas for six groups it has been proven that their genomic RNAs are supplied with a VPg. To the latter viruses belong, among others, the comoviruses, the potyviruses, and the nepoviruses.

With respect to the 3′ terminus three different structures are found. For six groups, among which the tobamoviruses, tymoviruses, and bromoviruses, it has been described that they have a tRNA-like structure which can be aminoacylated in vitro, whereas in six other plant virus groups, including the comoviruses, potyviruses, and nepoviruses, the genomic RNAs terminate with a poly(A) tail, and six more groups, including alfalfa mosaic virus and sobemoviruses (e.g., southern bean mosaic virus, SBMV) have neither a poly(A) tail nor a tRNA-like structure. The various RNA segments of a given bi- or tripartite genome virus always have the same structures at their respective 5′ and 3′ ends.

For the expression of their genetic information, three different main strategies are used by various groups.

1. With some groups of viruses the viral RNA is a monocistronic messenger resulting in the synthesis of a polyprotein from which functional smaller proteins are generated by proteolytic cleavages. This is found, for example, in comoviruses and potyviruses.
2. In other groups of viruses the expression of genomic RNA is limited to the 5′ proximal gene and the virus produces subgenomic mRNAs to express other viral genes. This strategy is found, among others, in tobamoviruses, bromoviruses, and alfalfa mosaic virus.
3. Still other groups of plant viruses use a combination of the two previous strategies and express the information in their genomic RNAs partly by polyprotein synthesis and proteolytic processing and partly by synthesis of subgenomic mRNA for the expression of other viral genes. Tymoviruses and sobemoviruses are examples of plant viruses which express their genetic information by this strategy.

There is no clear correlation between the genomic 5′- and 3′-terminal structures and the mode of expression of the genomic RNAs with the exception of viruses which have a 5′ VPg and a 3′ poly(A) tail. This combination has so far been found with the monopartite potyviruses and the bipartite comoviruses and nepoviruses. All three groups happen to use

Table 1
ORGANIZATION AND STRATEGY OF THE POSITIVE-STRAND PLANT RNA VIRUSES

Virus group	Type member[a]	Molecular weight genomic RNAs	Terminal structures of genomic RNAs		Mode of expression
			5′ end	3′ end	
Monopartite Genome Viruses					
Tymoviruses	TYMV	2.0×10^6	cap	tRNA-like	Polyprotein processing subgenomic RNA
Tobamoviruses	TMV	2.0×10^6	cap	tRNA-like	Subgenomic RNA
Potexviruses	PVX	$2.0—2.5 \times 10^6$	cap	polyA	?
Sobemoviruses	SBMV	1.4×10^6	VPg	X_{OH}	Polyprotein processing subgenomic RNA
Luteoviruses	BYDV	2.0×10^6	VPg	X_{OH}	Polyprotein processing subgenomic RNA
Potyviruses	PVY	3.2×10^6	VPg	polyA	Polyprotein processing
Tobacco necrosis virus	TNV	1.4×10^6	ppA	X_{OH}	?
Tombus viruses	TBSV	1.5×10^6	cap	X_{OH}	?
Closteroviruses	SBYV	$2.2—6.5 \times 10^6$	?	?	?
Bipartite Genome Viruses					
Tobraviruses	TRV	2.4×10^6 $0.6—1.4 \times 10^6$	cap	X_{OH}	Readthrough, subgenomic RNA
Furoviruses	BNYVV	2.3×10^6 1.6×10^6	cap	polyA	Readthrough, subgenomic RNA ?
Comoviruses	CPMV	2.02×10^6 1.22×10^6	VPg	polyA	Polyprotein processing
Nepoviruses	TRSV	2.8×10^6 $1.3—2.4 \times 10^6$	VPg	polyA	Polyprotein processing
Pea enation mosaic virus	PEMV	1.8×10^6 1.4×10^6	VPg	?	?
Dianthoviruses	CRSV	1.5×10^6 0.5×10^6	?	?	?
Tripartite Genome Viruses					
Bromoviruses	BMV	1.1×10^6 1.0×10^6 0.7×10^6	cap	tRNA-like	Subgenomic RNA
Cucumoviruses	CMV	1.27×10^6 1.13×10^6 0.75×10^6	cap	tRNA-like	Subgenomic RNA
Hordeiviruses	BSMV	1.5×10^6 1.35×10^6 $1.05—1.35 \times 10^6$	cap	tRNA-like	Subgenomic RNA
Alfalfa mosaic virus	AMV	1.1×10^6 0.8×10^6 0.7×10^6	cap	X_{OH}	Subgenomic RNA

Table 1 (continued)
ORGANIZATION AND STRATEGY OF THE POSITIVE-STRAND PLANT RNA VIRUSES

Virus group	Type member[a]	Molecular weight genomic RNAs	Terminal structures of genomic RNAs		Mode of expression
			5′ end	3′ end	
Tripartite Genome Viruses					
Ilarviruses	TSV	1.1×10^6 0.9×10^6 0.7×10^6	cap	X_{OH}	Subgenomic RNA

[a] AMV: *alfalfa mosaic virus*, BMV: *brome mosaic virus*, BNYVV: *beet necrotic yellow vein virus*, BSMV: barley stripe mosaic virus, BYDV: barley yellow dwarf virus, CMV: cucumber mosaic virus, CPMV: cowpea mosaic virus, CRSV: carnation ringspot virus, PEMV: pea enation mosaic virus, PVX: potato virus X, PVY: potato virus Y, SBYV: sugar beet yellows virus, TBSV: tomato bushy stunt virus, TMV: tobacco mosaic virus, TNV: tobacco necrosis virus, TRSV: tobacco ringspot virus, TRV: tobacco rattle virus, TSV: tobacco streak virus, TSWV: tomato spotted wilt virus, TYMV: turnip yellow mosaic virus.

translation into a polyprotein with subsequent processing of the polyprotein in smaller viral proteins as expression strategy.

Expression of the viral genome is required for virus RNA replication, and it seems plausible that the variation in translation strategies, together with the different number of viral functions encoded by the genome of various groups of viruses, will also result in several mechanisms for viral RNA replication. Naturally there is a common basic pattern of viral RNA replication in the understanding that plant RNA viruses with a positive RNA genome replicate through the formation of a complementary negative strand that subsequently is used as template for the synthesis of progeny viral RNAs. Such a replication mechanism demands an RNA-dependent RNA polymerase (RNA replicase) for the transcription of both the positive and the negative viral RNA strands. In recent years it has become clear that probably most — if not all — plant RNA viruses encode their own specific RNA replicase just as RNA bacteriophages and animal RNA viruses (see Chapter 1). Since the transcription starts at the 3′ end of the template molecule, the viral replicase must be able to specifically recognize the 3′ termini of both positive and negative viral RNA strands, which are not similar. Besides the viral-encoded RNA polymerase, other virus-encoded and/or host cell proteins may be required for the specific selection of viral RNA molecules in the initiation of RNA replication.

Having explained to a certain extent the diversity of plant RNA viruses with their variation in genome structure, expression, and replication we shall exclusively engage further in this chapter with one virus group and discuss the present state of knowledge on the replication of comoviruses.

II. COWPEA MOSAIC VIRUS IS TYPE MEMBER OF THE COMOVIRUS GROUP

The comoviruses are a group of 14 different plant viruses that have the same structural organization of genomic RNAs and virus particles, and use the same mechanism for expression and replication of the viral RNAs (see Reference 2 for review). Cowpea mosaic virus (CPMV) is type member of the comoviruses and the only comovirus that has been thoroughly examined with respect to genome structure, translation, and replication strategy.[2,3] In the following, we shall therefore mainly discuss the results obtained from studies on CPMV. CPMV has acquired this exceptional position because in many respects it is an easy virus

to work with. CPMV multiplies rapidly in its natural host *Vigna unguiculata* (L.) and as a result, purified CPMV can be obtained in gram amounts.[2-4] Therefore, the virus has lent itself very well for studies on the molecular properties of the virus particles and the genomic RNAs. Moreover, cowpea mesophyll protoplasts are in vitro efficiently and approximately synchronously infected with CPMV and this has greatly contributed to the understanding of the expression and replication mechanism of the virus.[5]

CPMV has a bipartite genome consisting of two positive strand RNA molecules that each are encapsidated in icosahedral particles with a diameter of 28 nm. The two nucleoprotein particles are denoted B and M components and have similar capsids composed of 60 copies of each of two different coat proteins, a large one with a molecular weight of 37 kdaltons, VP37, and a smaller one with a molecular weight of 23 kdaltons, VP23.[6-8] The B and M components differ in nucleic acid content, B containing a single RNA molecule (B-RNA) with a molecular weight of 2.04×10^6, and M, a RNA molecule (M-RNA) with a molecular weight of 1.22×10^6.[9,10] Both B- and M-RNA have a small protein, VPg, covalently linked to the 5′ end and a poly(A) tail at the 3′ end.[11-15] The RNAs are translated in vitro and in vivo into large polyproteins that are subsequently cleaved through a number of steps into several functional proteins.[2,3]

Both B and M components, or their RNAs, are required for virus multiplication in plants, but B-RNA is self supporting with respect to RNA replication in cowpea protoplasts.[16-19] B-RNA that is expressed and replicated in cowpea protoplasts in the absence of M-RNA is not assembled to virions.[18,19] Conversely, M-RNA is completely dependent on B-RNA expression for its replication. These findings demonstrate that B-RNA encodes functions required for replication whereas M-RNA carries information for the virus capsid proteins.

III. EXPRESSION OF COMOVIRUS RNAs

So far as different comoviruses have been examined, the genomic RNAs of comoviruses are in vitro translated as monocistronic messengers into large polyproteins corresponding to about 80% of their estimated coding capacity (see Reference 3 for review). The primary translation products are then processed into smaller viral proteins by specific proteolytic cleavages.[2,3]

Only for CPMV there is a rather complete picture of the expression mechanism of the viral RNAs, substantiated by knowledge of the nucleotide sequence of M- and B-RNA, a detailed analysis of the polyprotein processing and characterization of the viral proteins produced.[2,3,9,10] For CPMV the viral protein synthesis has been studied both in vitro and in vivo, resulting in the current model for the expression of the two CPMV RNAs as drawn in Figure 1 and briefly discussed in the following.

A. Expression of CPMV-B-RNA

The sequence of B-RNA, 5889 nucleotides excluding the poly(A) tail contains a single open reading frame of 5598 nucleotides, spanning from the AUG-codon at position 207 to the UAG-stopcodon at position 5805 (Figure 1).[10] In full agreement with the single long open reading frame, B-RNA is translated in vitro as well as in vivo into a 200-kdalton protein. The 200-kdalton protein is rapidly cleaved into 32- and 170-kdalton proteins, and this processing can even start before the chain of the 200-kdalton primary translation product is completed.[20-24] The 170-kdalton protein is then further processed via two alternative routes: either the 170-kdalton is cleaved into 60- and 110-kdalton proteins or, by another cleavage, into 84- and 87-kdalton proteins.[24,25] The 110- and 84-kdalton proteins can undergo an additional cleavage to give rise to the 87- and 60-kilodalton proteins, respectively, together with a 24-kdalton protein.[23-26] The 60-kdalton protein is the direct precursor of VPg.[27,28] Whereas the cleavage steps mentioned so far occur in vivo and in vitro, processing of the

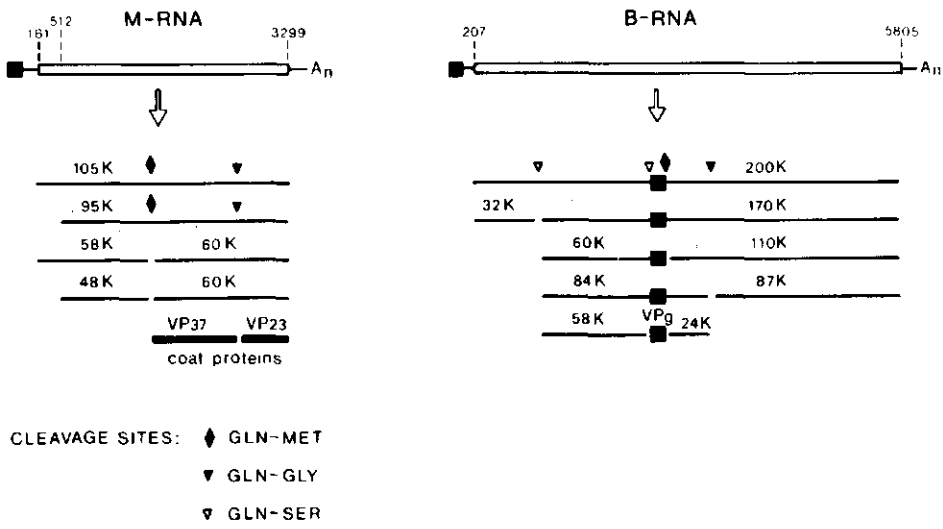

FIGURE 1. Expression of M- and B-RNA of CPMV. M- and B-RNA each contain a single, open reading frame represented by the open bars. The position of the translational start and stop codons is indicated. Translation of M-RNA in vitro starts at the AUG codon at position 161, but more efficiently at the AUG codon at position 512. B-RNA is translated into a 200-K (Kilodalton) polyprotein and M-RNA into 105- and 95-K polyproteins which are subsequently processed by specific proteolytic cleavages at the indicated sites into smaller functional proteins. (K = kilodalton.)

60-kdalton protein into 58-kdalton with the release of VPg has never been observed in vitro. All five final cleavage products of the 200-kdalton polyprotein encoded by B-RNA (see Figure 1) are detectable in CPMV-infected protoplasts, if in varying amounts. Free VPg has not been detected in vivo, but it occurs either in precursor form or linked to the 5′ phosphate of the terminal uridyl-residue of B- and M-RNA. Beside the final cleavage products, the processing intermediates 170-, 110-, 84-, and 60-kdalton are also found in considerable amounts in infected cells which suggests that they may also represent functional molecules.[2,18]

The order of the cleavage products NH_2-32 kdaltons-58 kdaltons-4 kdaltons (= VPg)-24 kdaltons-87 kdaltons-COOH, in the 200-kdalton B-RNA-encoded protein, initially established by comparison of the different proteins by peptide mapping and by immunological techniques, has been confirmed by determining the amino terminal sequences of the various B-RNA-encoded proteins and locating the coding regions for these proteins on the B-RNA sequence.[21,25,27-29] Such sequence analysis, moreover, revealed the cleavage sites used in the proteolytic processing of the 200-kdalton polyprotein. It was found that three types of proteolytic cleavage sites were used: a glutamine-serine pair (2x), a glutamine-methionine pair (1x), and a glutamine-glycine pair (1x) (see Figure 2).[29] The proteolytic activity involved in cleaving at all three pairs is located in the 24-k/dalton protein encoded by B-RNA.[31,31a,31b] In contrast to what was thought previously, the 32-kdalton protein does not bear proteolytic activity.[30,32]

B. Expression of CPMV-M-RNA

The sequence of CPMV-M-RNA is 3481 nucleotides long, not including the poly(A) tail, and also contains a single, large, open reading frame running from the AUG codon at position 161 to the UAG stop codon at position 3299.[9] Nevertheless, M-RNA produces upon in vitro translation two polyproteins with molecular weights of 105 and 95-kdaltons. These two proteins have overlapping carboxy-terminal ends and arise because initiation of translation does not only start at the AUG codon at position 161, but also, and even to a greater

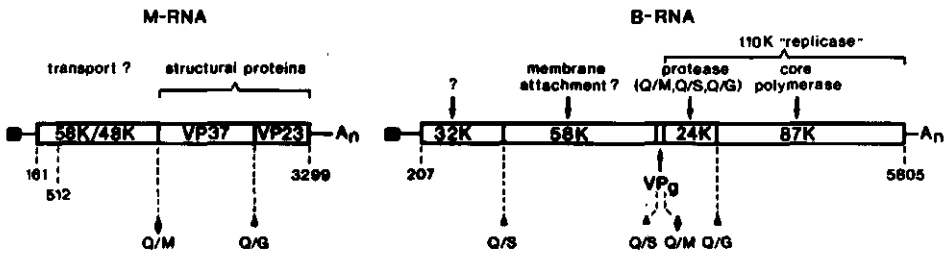

FIGURE 2. Genetic map of CPMV. The single, open reading frames in M- and B-RNA are represented by the open bars. The indicated positions of the coding regions of the different functional protein domains in the reading frames are drawn to scale. (K = kilodalton.)

extent, at the AUG codon at position 512 in phase with the open reading frame.[9,20,21,30,33] Both M-RNA-encoded polyproteins are proteolytically cleaved and produce overlapping 58- and 48-kdalton proteins and a 60-kdalton protein which is the direct precursor of the two capsid proteins VP37 and VP23 (see Figure 1). A second cleavage generates the two coat proteins from the 60-kdalton protein.[20,30,32]

By locating the coding region of VP37 and VP23 on the nucleotide sequence of M-RNA, the proteolytic cleavage sites used to release the capsid proteins from the 105- and 95-kdalton primary translation products have been determined to be a glutamine-methionine pair and a glutamine-glycine pair (see Figure 2).[34] The cleavages at both sides are achieved by the B-RNA-encoded, 24-kdalton protease. Cleavage at the glutamine-methionine pair requires, moreover, the 32-kdalton encoded by B-RNA as a cofactor.[31a,31b]

The model for CPMV M-RNA expression depicted in Figure 1 has been derived from in vitro translation studies. In vivo, the capsid proteins are the only M-RNA-encoded products which are easily detectable. To verify whether this model also holds in vivo, a search in CPMV-infected cells was undertaken for other proteins occurring in the processing scheme. Using specific antibodies, it appeared possible to detect in CPMV-infected protoplasts small amounts of the 60-kdalton capsid precursor, and also of the 48-kdalton protein, but the 58-kdalton protein has not been found.[35] This demonstrates that in any case, the 95-kdalton polyprotein is synthesized in vivo, which is then rapidly cleaved into 48- and 60-kdalton products, followed by a second rapid cleavage of the 60-kdalton precursor to release the two capsid proteins. The 105-kdalton polyprotein is either not produced in vivo or only in amounts which are below the level of detection. It is therefore unclear if expression of M-RNA into a 105-kdalton protein has a role in vivo. In this connection it is striking that M-RNA of four other comoviruses, cowpea severe mosaic virus, bean pod mottle virus, red clover mottle virus, and squash mosaic virus, also direct in vitro translation of two polypeptides of approximately the same size as CPMV M-RNA.[36-39] The occurrence of two AUG codons which give rise to translation in two large-sized proteins is apparently a common feature of comovirus M-RNA, which suggests that it may have biological significance.

IV. DISTINCTIVE FEATURES OF THE STRUCTURE OF CPMV RNA AND ITS REPLICATIVE FORM

The two genomic RNAs of CPMV are templates for both translation and replication. Once virus particles have invaded a host cell, the viral RNAs are released and first translated to produce proteins necessary for specific replication of virus RNA. From then onward the viral RNA also functions as template for the production of progeny RNA strands. Since in

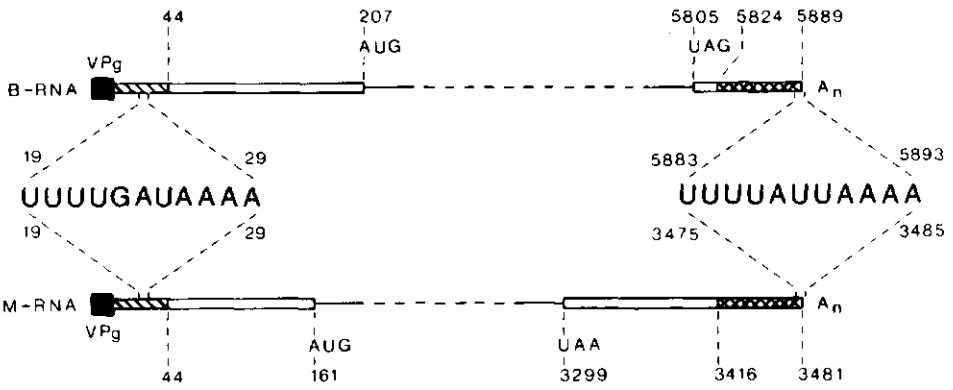

FIGURE 3. Structural organization of the nontranslated regions of the CPMV genome. The 5′ and 3′ nontranslated regions in B- and M-RNA are represented as open bars. Within these bars two stretches with more than 80% nucleotide sequence homology between the RNAs are indicated (shaded areas); for further details see text.

CPMV-infection B- and M-RNA are multiplied by the same B-RNA-encoded replication machinery, it may be expected that both RNAs have features in common for their function in RNA replication. Indeed the 5′- and 3′-terminal noncoding regions of B- and M-RNA show conspicious sequence homology suggesting that these sequences contain recognition signals for various interactions with different viral and/or host protein involved in RNA replication (Figure 3).[9,10,40,41] The first 44 nucleotides in the two 5′ leader sequences show 89% homology, and the last 65 nucleotides preceding the poly(A) tails show 82% homology. Particularly striking is a stretch of 11 nucleotides, UUUUGAUAAAA, in the homologous parts of the 5′ leader sequences of both RNAs, which is complementary to the first four A's of the poly(A) tail and the last seven nucleotides before the beginning of the poly(A), allowing one G-U base pairing. Hence, it follows that the complementary negative strands of each genome segment have sequences at their 3′ termini similar to those of the 3′ ends of the positive viral RNAs. Such sequences may therefore constitute a recognition sequence for the viral RNA replicase.

The poly(A) tails of B- and M-RNA are heterogeneous in size and vary between 10 to 170 residues for B-RNA and between 20 to 400 residues for M-RNA.[42] It is not known whether the poly(A) tail is required for virus infectivity. The poly(A) tails are transcribed in the replication process, as is suggested by the absence of a polyadenylation recognition sequence AAUAAA in the region preceding the poly(A) tail, but has become apparent by the finding that poly(U) stretches are present at the 5′ termini of negative strands of CPMV RNA replicative-form molecules.[9,10,40,43]

The functional significance of the protein VPg covalently linked to the 5′ ends of B- and M-RNA is also not clear. Removal of VPg from the 5′ termini by incubation with proteinase K does not lead to loss of infectivity of the viral RNA.[12] The protease treatment of isolated CPMV RNAs has equally been shown not to influence the messenger activity of the viral RNAs in cell-free systems.[12] Moreover, in rabbit reticulocyte lysates more than 90% of the VPg linked to CPMV RNA is removed and degraded within the first 10 min of incubation without any noticeable effect on the translational activity of the RNA.[44] A role of VPg in the translation of RNA is therefore not likely, and it appears that VPg has no function in establishing virus infection and replication.

Another possibility is that VPg, when linked to the virion RNAs, has no actual function, but represents a vestige of the process of RNA replication in which the RNA has been produced. VPg is encoded by B-RNA of CPMV as described in the previous section. It consists of a chain of 28 amino acid residues which is released from its precursors by

cleavages at a glutamine-methionine pair and a glutamine-serine pair, respectively (Figure 2). VPg is linked to the 5′-terminal uridylic acid residue of the CPMV RNAs by a phosphodiester linkage with the –OH-side chain of its amino terminal serine.[45] Since B-RNA encodes proteins involved in viral RNA replication, it seems plausible that VPg is linked to the 5′ ends during viral RNA synthesis. A role of VPg, or the generation of VPg, in viral RNA synthesis is substantiated by the finding that complementary negative strands in viral RNA replicative form are also provided with VPg at their 5′ ends.[43] A possible role of VPg in viral RNA replication will be discussed in Section IX of this chapter.

V. REPLICATION OF CPMV RNA

Replication of CPMV RNA is associated with vesicular membranes of characteristic cytopathic structures in the cytoplasm of infected cells.[46] These cytopathic structures, consisting of arrays of vesicles surrounded by electron-dense material, the chemical nature of which is unknown, appear in cells early in infection with CPMV.[46-48] By fractionation of virus-infected cells it has been demonstrated that these structures contain CPMV-specific double-stranded RNAs and viral RNA replicase capable of synthesizing in vitro double-stranded RNA, and possibly some single-stranded viral RNA on endogeneous template RNA.[4,46,47] In cowpea protoplasts inoculated in vitro with B components of CPMV alone, B-RNA is expressed and replicated and the development of similar cytopathic membrane structures is observed.[49] This suggests that the induction of the membrane proliferation for the vesicular structures is achieved by a B-RNA-encoded function and may fulfill an essential role in viral RNA synthesis.

During the early stages of infection, CPMV replication is inhibited by actinomycin D, but no longer once virus replication is established at about 8 hr after infection.[8,50] Similar inhibition of virus replication by actinomycin D has been reported for bean pod mottle virus, another member of the comovirus group.[51,52] This inhibition of CPMV replication at an early stage of infection indicates that host DNA-dependent RNA synthesis is required to allow virus RNA replication and suggests that some host-specified component, the synthesis of which is induced by viral infection, is essential at that stage.

Involvement of a host factor in CPMV RNA replication is also demonstrated by a CPMV mutant that is no longer able to grow in cowpea, but still able to replicate in bean plants, *Phaseolus vulgaris* var. Pinto.[53] The mutation responsible for this behavior is located in B-RNA which codes for functions involved in viral RNA replication. The effect of the mutation may be interpreted as a defect in a specific interaction between a B-RNA encoded protein and a host factor necessary for the formation of a functional viral RNA replication complex. By the mutation, this interaction has become defective in cowpea, but is apparently still effective in beans.

Since addition of actinomycin D during active RNA replication does not affect virus synthesis and neither is there any effect on the activity of viral RNA replication complexes in vitro, actinomycin D does not seem to interfere with the process of viral RNA replication per se, but rather with the establishment of viral RNA synthesis in the infected cell. It is possible that host-dependent RNA synthesis is required for the development of the vesicular membrane structures as sites for virus RNA replication. Another possibility is that *de novo* synthesis of a host protein is necessary to supply an essential function for virus RNA replication.

In accordance with the location of viral RNA replication in the membranes of the cytopathic structures in CPMV-infected cells, the crude membrane fraction of CPMV infected leaves was found to contain RNA-dependent RNA polymerase activity capable in vitro of fully elongating nascent viral RNA chains initiated in vivo.[54] The completed chains are all positive strands and exclusively found in double stranded replicative forms. Beside the RNA replicase

associated with negative strand viral RNA as template, the crude membrane fraction appeared to hold another RNA-dependent RNA polymerase. This second RNA-dependent RNA polymerase activity is also detectable in the membrane fraction of uninfected leaves in very small amounts, but is greatly increased in CPMV-infected leaves.[55] This host-encoded RNA polymerase transcribes endogenous plant RNA, and in infected plants also viral RNAs, into small (4 to 5 S) negative strand RNA molecules.[54] In CPMV-infected cowpea leaves the activity of the host-dependent RNA polymerase activity is enhanced at least 20-fold and almost overshadows the viral RNA replicase activity which represents less than 5% of the total RNA-dependent RNA polymerase activity in the crude membrane fraction. The viral replicase and the host RNA polymerase are, however, distinguished by virtue of the different products of their respective activities, which show that they are functionally different.[54]

The host RNA-dependent RNA polymerase activity can be separated from the CPMV RNA replication complex, as the binding of the host RNA polymerase to the membranes is much weaker than the binding of the replication complex. As a result, the host enzyme can be readily released from the membranes by washing with Mg^{++}-deficient buffer whereas the CPMV RNA replication complex remains firmly bound under these conditions. The host-encoded RNA polymerase has been purified by successive steps as outlined in Figure 4, and the purified enzyme proved to be a monomeric protein with a molecular weight of 130,000 daltons (130 kdaltons).[55] Using antibodies raised against purified enzyme preparations in an antibody-linked polymerase assay on nitrocellulose blots, the 130,000-dalton protein could definitely be identified as a RNA-dependent RNA polymerase.[56] It was also demonstrated, using the antibodies against the host RNA polymerase, that the increase of its activity in CPMV-infected leaves is indeed due to an increase in the amount of the 130-kdalton protein and not to activation of enzyme already present.[57] The increase has been further shown to be restricted to CPMV-infected cells within the leaf tissue and does not occur in leaf cells in which no virus multiplication takes place.[57]

Strikingly, no increase of 130-kdalton host-encoded, RNA-dependent RNA polymerase was found to accompany CPMV-RNA replication in cowpea mesophyll protoplasts upon inoculation with CPMV in vitro, indicating that the increased production of host RNA-dependent RNA polymerase is not a prerequisite for CPMV-RNA replication.[57]

VI. CHARACTERIZATION OF PURIFIED CPMV RNA REPLICATION COMPLEX

By definition, the native CPMV RNA replication complex consists of RNA replicase molecules bound to template viral RNA and is detectable by its capacity in vitro to elongate nascent in vivo-initiated viral RNA chains to full-length molecules. In CPMV-infected cowpea leaves this viral RNA replicase activity is first detectable in the crude membrane fraction one day after inoculation and then increases to reach a maximum 2 to 3 days later. At the time that the replicase activity has attained its maximum, the 130-kdalton host-encoded, RNA-dependent RNA polymerase has also strongly increased, and that makes the separation of the host-encoded RNA polymerase activity from the CPMV RNA replication complex the major problem to surmount in the purification of the viral RNA replicase.[54] The different steps in a procedure which has resulted in a highly purified CPMV RNA replication complex are summarized in Figure 4.[54,58] The main feature of this procedure is that in the purification the replication complex is maintained functionally intact, which allows distinguishing the viral RNA replicase activity from the host-encoded, RNA-dependent RNA polymerase. Washing of the crude membrane fraction of CPMV infected leaves with Mg^{++}-deficient buffer removes the vast majority of the 130-kdalton host RNA polymerase and leaves the viral replication complex intact in the membranes. The CPMV RNA replication complex can then be solubilized by treating the washed membranes with Triton®X-100,

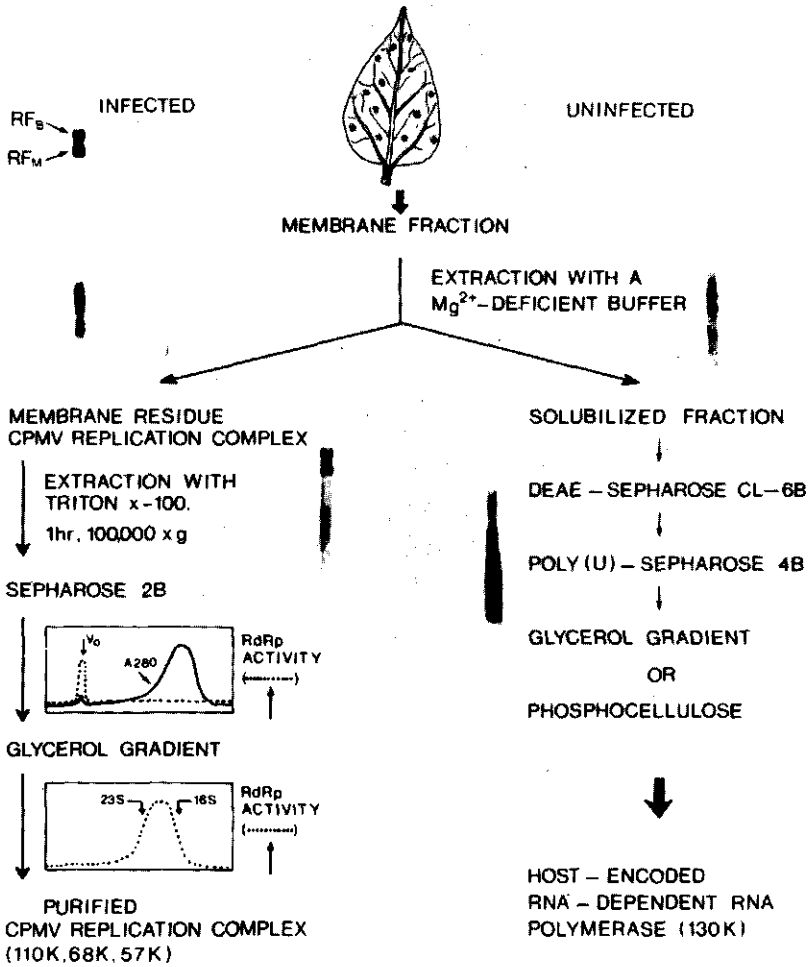

FIGURE 4. Separation and purification of CPMV RNA replication complex and host-encoded, RNA-dependent RNA polymerase from CPMV infected cowpea leaves. The host-encoded activity produces short (4- to 55-base), negative strand RNAs from plant and viral RNA templates. The virus-encoded replicase elongates in vitro nascent chains initiated in vivo to full-length, plus-type viral RNA mainly found in double-strand form (RF_B and RF_M). For details see text and references therein. (K = kilodalton.)

whereupon most of the contaminating proteins are removed by Sepharose®2B chromatography. After this step, the CPMV replication complex was free of the 130-kdalton host enzyme which was no longer detectable using Western blot analysis.[57] Further purification of the CPMV RNA replication complex was obtained by glycerol gradient centrifugation while the capacity of elongating nascent RNA chains to full-length viral RNAs was still preserved.[58] The active preparation after glycerol gradient centrifugation contained three major polypeptides of 110, 68, and 57 kdaltons. Using antisera against various viral proteins, the 110-kdalton, protein was proven to be a viral protein encoded by CPMV B-RNA. (Figure 2). The 68- and 57-kdalton polypeptides did not react with antibodies against viral-encoded proteins and presumably are host proteins which may either have a function in the viral RNA replication complex or be contaminating proteins. Since the amount of B-RNA encoded protein associated with the replication complex is correlated with the polymerase activity in the purified complex, the 110-kdalton protein has been assigned to represent the viral-encoded core polymerase in the CPMV RNA replication complex.

In the next section we shall first discuss the similarities between plant comoviruses and animal picornaviruses and, more specifically, the analogy in genome structure, expression, and replication mechanism between CPMV and poliovirus. These similarities have considerably influenced the ideas about the possible involvement of viral proteins in the replication of CPMV RNA.

VII. SIMILARITIES BETWEEN CPMV AND PICORNAVIRUSES

The detailed studies on the expression and replication of CPMV have revealed a striking similarity between the genome strategy of comoviruses and that of animal picornaviruses (see also the chapter by Semler, Kuhn, and Wimmer in this volume).[2] Apart from their host range and other biological properties, the major difference between comoviruses and picornaviruses is that in comoviruses the genome is divided in two RNA molecules whereas that in picornaviruses is a single RNA molecule. On the other hand, the genomic RNAs of comoviruses and picornaviruses have both a VPg at their 5′ terminus and a 3′ terminal poly(A) tail. The genomic RNAs of the two virus groups are expressed by translation into large-sized polyproteins which are processed to functional proteins by virus-encoded protease activities (this chapter).[59] Both CPMV and polioviruses induce in inoculated cells the formation of vesicular membrane structures which represent the sites of viral RNA replication.[46-48,60] The capsids of CPMV are built of two proteins present in 60 copies, each which are processed from a common precursor protein.[6-8] Similarly, the capsid of poliovirus is made up of 60 copies of each of four proteins derived from a common precursor.[60] More recent crystallographic studies on CPMV and poliovirus have shown that the polypeptide chain of the two capsid proteins of CPMV are folded in such a way as to produce three distinct β-barrel domains similarly arranged as in the poliovirus capsid.[61,62]

The analogy between CPMV and poliovirus is not confined to the structural organization of the capsids, features of the genomic RNAs, and their mode of expression. In addition, there is significant sequence homology between nonstructural (58-, VPg, 24-, and 87-kdalton) proteins contained in the CPMV B-RNA-encoded polyprotein and four nonstructural proteins of poliovirus, 2C, VPg, 3C, and 3D, which are found in similar relative positions in the genetic map and probably provide analogous functions in RNA replication (Figure 5).[63] Protein 3D has been identified as the core polymerase of the polioviral RNA replicase. The protein exhibits 20.9% amino acid sequence homology to the sequence of the 87-kdalton protein of CPMV. This homologous region of the 87-kdalton protein moreover contains a block of 14 amino acid residues consisting of a GDD (Gly–Asp–Asp) sequence flanked by hydrophobic residues at both sides, and a second block with the conserved sequence $\frac{S}{T}GxxxTxxxN\frac{S}{T}$ (in which x may be any amino acid residue) 37 residues upstream from the first conserved region, found in all viral RNA-dependent RNA polymerases characterized so far.[64]

This provides strong evidence that the 87-kdalton B-RNA encoded protein represents the core polymerase of CPMV replicase. This is in agreement with the occurrence of the B-RNA-encoded 110-kdalton protein in purified CPMV RNA replication complexes; for the 110-kdalton protein contains the sequences of the 87- and the 24-kdalton proteins.

The homology between the 24-kdalton CPMV protein, which has been shown to carry specific protease activity, and the poliovirus protease 3C adjoining the polymerase sequence is located in their C terminal sequences. In that part of the polypeptide chain both proteases have the features of the active site of a thiol protease, consisting of a Cys residue and a His residue approximately 14 to 18 residues apart.

The function of poliovirus protein 2C (formerly P2-x) has not yet been biochemically defined, but there is evidence that this protein, or derivatives thereof, is associated with the

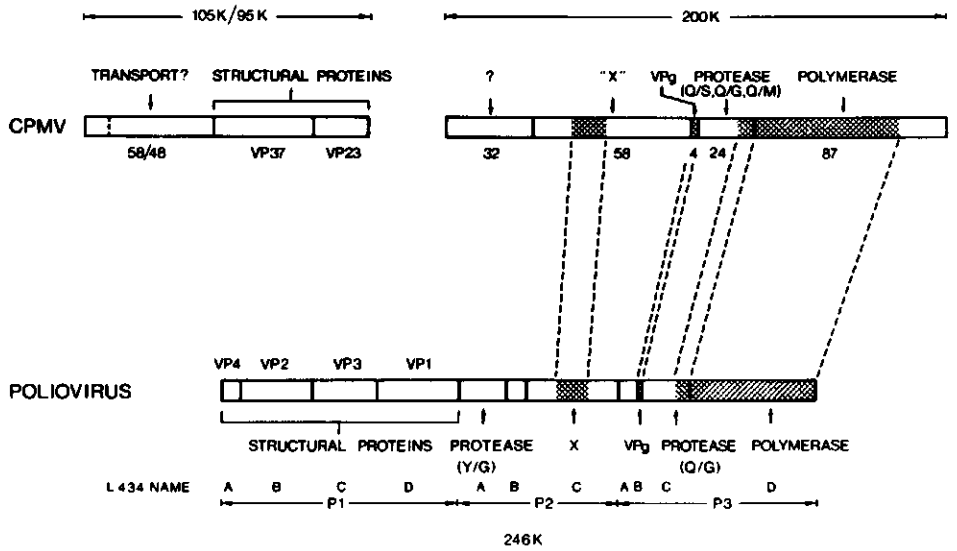

FIGURE 5. Comparison of the functional organization in CPMV and polioviral polyproteins. Similar functions are found in similar relative positions in the genomes of CPMV and poliovirus. Moreover, in the B-RNA-encoded polyprotein, three regions show more than 20% amino acid sequence homology to regions in the polioviral polyprotein (hatched boxes). These homologous regions reside in proteins which probably have similar functions in viral RNA replication. (K = kilodalton). (From Franssen, H., Leunissen, J., Goldbach, R., Lomonossoff, G., and Zimmern, D., *EMBO J.*, 3, 855, 1984. With permission.)

vesicular membranes where polioviral RNA replication is located in infected cells.[65] Moreover, recent analyses of guanidine-resistant and guanidine-dependent isolates of poliovirus revealed that these contain a mutation in the central domain of about 140 amino acid residues of the protein 2C, which is strongly conserved among picornaviruses and shows 30% sequence homology to the central region of the 58-kdalton protein of CPMV.[63,66-68] Since the major effect of guanidine appears to be blocking of viral RNA synthesis, these observations provide additional evidence that the protein 2C of poliovirus plays a role in this process. By analogy, a similar role may be attributed to the 58-kdalton protein encoded by CPMV B-RNA.

The strong analogy between CPMV and poliovirus in structural organization and expression strategy, together with similar genetic organization and amino acid homology of viral proteins involved in RNA replication, prove that CPMV and poliovirus are somehow evolutionary related. Therefore, in view of the similarities between CPMV and poliovirus, it is natural to anticipate that the mechanism of CPMV RNA replication will be very similar to that of poliovirus RNA replication. In the following sections we shall take that line in further discussion of the replication of CPMV RNA.

VIII. CPMV RNA REPLICATION DIFFERS FROM THE RNA REPLICATION OF TMV, BMV, AlMV, AND CMV

If sequence conservation in nonstructural viral proteins which are involved in viral RNA replication defines a group of positive RNA viruses with a similar mechanism of viral RNA replication, then CPMV belongs to a different group of plant viruses than TMV, BMV, AlMV, and CMV. Although TMV is a monopartite genome virus and BMV, CMV, and AlMV are tripartite genome viruses, TMV is similar to BMV, CMV, and AlMV in containing cistrons for four viral proteins. Two of these proteins — the coat protein and a protein

thought to be involved in transfer of virus from cell to cell in infected plants — are not required for RNA replication, whereas both other proteins appear to be involved in viral RNA synthesis. The latter two proteins of TMV, BMV, and AlMV contain distinct domains with sequence homology, but do not show homology with the virus-encoded proteins involved in replication of CPMV RNA beyond the GDD-associated sequences noted above.[63,69,70] Obviously then, the plant RNA viruses of which the genome structure and expression mechanism has been elucidated can so far be classified into two groups: one containing viruses like TMV, BMV, CMV, and AlMV, and another represented by CPMV and viruses such as the nepoviruses, which appear similar to CPMV in genome structure and translation strategy. Each group has a mechanism of viral RNA replication with characteristics based on similar functions of the proteins or the domains of proteins with conserved function. This would imply that functionally equivalent replication complexes are formed for the various viruses of each group. A major difference between the two groups could be that the viral RNA replicase of CPMV, just as the replicase of poliovirus, is a RNA-dependent RNA polymerase that requires for its activity not only a template, but also a primer.[60] In contrast, the replicase of the other group of viruses, as has been demonstrated for BMV replicase and AlMV replicase, do not require a primer, but can transcribe a template starting at a specific recognition sequence.[71-73] Such difference will imply a different mechanism of initiation of viral RNA replication for each group.

IX. INITIATION OF CPMV RNA REPLICATION

Although the CPMV RNA replication complex has been purified, and the virus-encoded RNA polymerase identified, virtually nothing is known about the mechanism of initiation of viral RNA synthesis. It is tempting to ascribe to the small protein, VPg, a role in the initiation of viral RNA replication, if only to explain its occurrence at the 5′ end of the viral RNA. There is, however, as yet no direct evidence for such a role of VPg. Neither for poliovirus is the mechanism of initiation of viral RNA replication and the possible role of VPg in this process completely understood. On the one hand it has been shown that a crude membrane fraction from poliovirus-infected HeLa cells is capable of synthesizing in vitro the uridylated proteins VPgpU and VPgpUpU, which under conditions of RNA synthesis are further elongated into much longer stretches of 5′ terminal poliovirus RNA.[74] Such data suggest that VPgpU might function as a primer for viral RNA synthesis. On the other hand, Flanegan and co-workers have reported that purified poliovirus replicase synthesizes in vitro dimer products with virus RNA as a template in the presence of a host factor.[75,76] According to these authors, the host factor represents a terminal uridylyl transferase that elongates the poly(A) tail at the 3′ end of the template RNA with a number of U residues which can form a hairpin with the poly(A) tail.[77,78] The virus-encoded replicase then starts elongation at the hairpin primer and further transcribes the viral RNA template producing RNA products up to twice the length of the genome. Addition of VPg subsequently resulted in cleavage of the dimer molecule at the hairpin and linkage of VPg to the 5′ end of the newly synthesized RNA strand. Such data conflict with a primer role for VPg, but, on the contrary, propose a role of VPg in the nucleolytic cleavage of the hairpin.

For CPMV there is no experimental evidence for either mechanism since no in vitro initiating system for CPMV RNA elongation has been developed. Free VPgpU or uridylation of VPg or uridylation of its direct 60-kdalton precursor has never been observed, nor has the occurrence of dimer length RNA molecules as possible intermediates in viral RNA synthesis so far been demonstrated. The only observation to suggest a role of VPg in an early stage of CPMV RNA replication is that VPg is found both at the 5′ end of negative and positive RNA strands in the replicative forms isolated from virus-infected leaves.[43]

X. ROLE OF PROTEIN PROCESSING IN STARTING VIRAL RNA REPLICATION

The occurrence of VPg at the 5′ end of each progeny viral RNA strand raises more questions concerning the viral RNA replication mechanisms. VPg of CPMV is encoded by B-RNA which is translated into a 200-kdalton primary translation product. This polyprotein has to complete several successive processing steps (Figure 1) to make VPg available for RNA synthesis. Only a single VPg is released each time a 200-kdalton translation product is processed, and this must happen for each molecule of B and M-RNA to be produced. In this way protein processing appears to have a dominant role in the replication of CPMV RNA, and both processes seem to be closely connected. Indeed, the translation-expression mechanism used by CPMV implies the production of equimolar amounts of polymerase molecules and other B-RNA-encoded proteins involved in replication, i.e., since VPg, the core polymerases (110 or 87 kdaltons) and the 58-kdalton membrane protein, together with the 32-kdalton protein are produced from a common B-RNA-encoded polyprotein, the latter proteins are simultaneously produced with VPg. For that reason a viral polymerase molecule needs on the average to synthesize only a single RNA molecule to keep step with the production of VPg. On that line of reasoning it is conceivable that the viral RNA polymerase molecules lose their activity upon releasing their first template. For example, it may be imagined that the 110-kdalton protein is involved in binding to template RNA and initiation of transcription. If subsequently the 110-kdalton protein is processed into 24- and 87-kdalton proteins by an intramolecular cleavage carried out by the proteolytic activity in the 24-kdalton domain of the 110-kdalton protein, reinitiation will be prevented. Such restriction of the polymerase molecules would fit with the observation that isolated CPMV replication complexes contain polymerase molecules which are only able to complete nascent chains from their endogenous template, but are not capable of accepting added template molecules.

XI. A MODEL FOR CPMV RNA REPLICATION

In Figure 6 a model is depicted in which the possible linkage between processing and CPMV RNA replication is illustrated. In this model a complex is formed between the 60- and 110-kdalton B-RNA-encoded proteins, with the 60-kdalton protein tightly associated with the membranes of the cytopathic structure in infected cells. Template RNA and possibly a host factor are bound to the complex in such a way to allow initiation of transcription. The 60-kdalton protein has to supply VPg, while the 110-kdalton protein contains the 24-kdalton domain which is able to release VPg by proteolytic cleavage. VPg may become uridylated at its N-terminal serine prior to initiation. Since the 60- and 110-kdalton proteins are derived from the common 170-kdalton precursor, formation of the initiation complex may also start with the binding of 170-kdalton protein to the membrane, whereupon cleavage into 60- and 110-kdalton protein occurs. Following the release of VPg, the 87-kdalton polymerase domain in the 110-kdalton protein starts transcription of the template, possibly using uridylated VPg as a primer. It has been noticed that the 58-kdalton protein contains an amino acid sequence, $GxxxxGK\frac{T}{S}$, which is similar to the consensus sequence found in proteins with ATPase/GTPase activity and in virus-encoded proteins involved in viral RNA replication for several viruses.[79] This sequence may represent a binding site for nucleoside triphosphates. While remaining attached to the 58-kdalton protein, the 87-kdalton polymerase domain within the 110-kdalton proteins continues the elongation of the RNA strand which is transcribed from the template. When transcription is completed, template RNA is released from the protein complex. In the meantime, the 3′ terminal end of the template RNA may be used in the formation of another initiation complex. This has to take place at another 60-

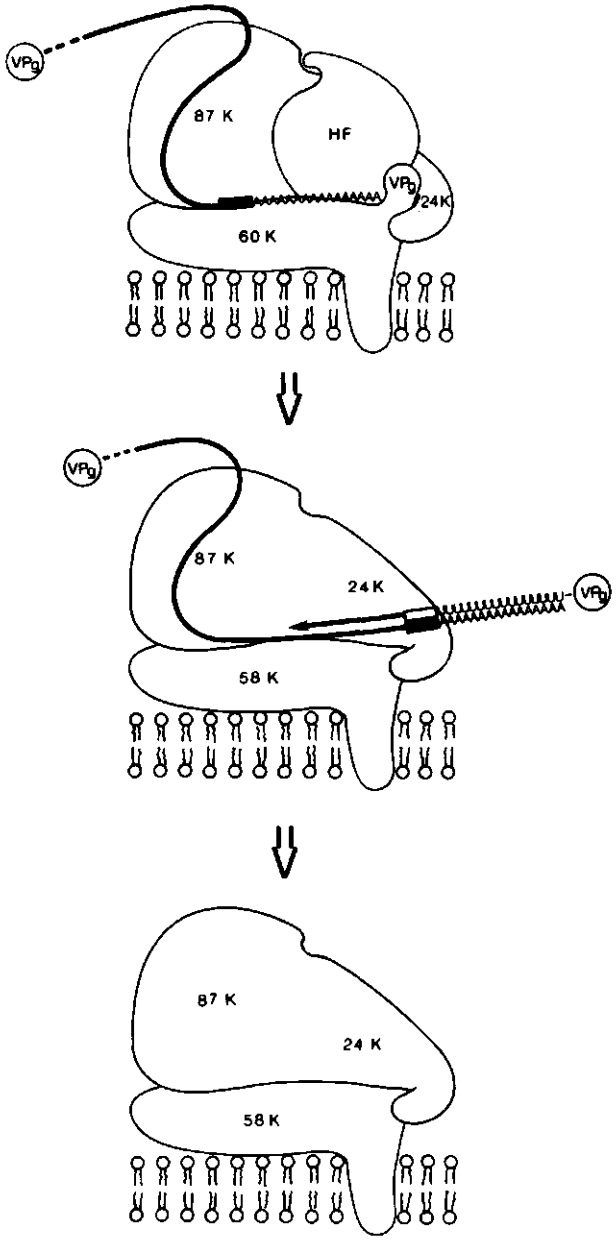

FIGURE 6. Model for membrane-bound CPMV RNA replication complex. Three stages in CPMV RNA replication are outlined: the replication complex just before RNA synthesis is initiated, an intermediate stage in which VPg has been linked to the 5′ end of the nascent RNA chain, and the protein complex remaining when the newly synthesized viral RNA and the template have been released. For discussion of the model, see the text. (K = kilodalton.)

to 110-kdalton complex, as the site used (see Figure 6) is now devoid of VPg and, therefore, not capable of initiating the synthesis of a viral RNA strand.

The model may account both for the synthesis of negative and positive strands. It proposes an essential role for VPg in the initiation of viral RNA synthesis, which is speculative and needs to be tested. In the model are, however, incorporated the different observations which must be accounted for in CPMV RNA synthesis. The essential part of the model is the close linkage between protein processing and viral RNA replication. The host factor in the model may have a role in the uridylation of VPg for the priming of RNA synthesis. Alternatively, if the initiation of viral RNA synthesis proceeds by hairpin priming, as has been proposed for poliovirus, the host factor may represent the terminal uridylyl transferase activity for elongating the 3′ end of the template. The occurrence of terminal uridylyl transferase has been demonstrated in cowpea.[80]

The model does not explain the presence in infected cells of B-RNA-encoded 84- and 87-kdalton proteins, which are produced by alternative cleavage of the 170-kdalton polypeptides. Possibly it does not make much difference whether the 170-kdalton protein is first cleaved into 60- and 110-kdalton polypeptides or into 84- and 87-kdalton polypeptides if viral RNA replication as caricatured in Figure 6 can be established from either pair of polypeptides. This would imply that the 84-kdalton polypeptide also can supply VPg and the 87-kdalton polypeptide is active as polymerase. Alternatively, it must be seriously considered that a second pathway for cleaving the 170-kdalton polypeptide may be significant in regulating viral RNA replication in a way not yet understood.

XII. CONCLUSION

It will be clear from the discussion above that if we think we know the alphabet of CPMV RNA replication, we do not yet understand its grammar. Further biochemical identification of the activities of the various virus-encoded polypeptides and host factors involved is required to elucidate their role in CPMV RNA replication. It will be necessary to develop a system in which the initiation of CPMV RNA replication is reconstituted to resolve the speculations about the mechanism of viral RNA replication and to answer the questions raised by the model. For elucidating the functioning of the virus-encoded proteins involved in RNA replication, it may be equally helpful to have access to mutants of CPMV which each bear a single mutation at a well-defined site in the genomic RNA or in one of the virus-encoded proteins. Recently, full-size DNA copies of B- and M-RNA have been cloned in our laboratory and it was shown that such DNA copies can be transcribed in vitro into RNA molecules which were found to be infectious upon inoculation of cowpea mesophyll protoplasts (Vos et al., to be published).[31,33] With this, it seems that for CPMV a system has become available for producing site specific mutations in B-RNA, of which the effect on viral RNA replication can subsequently be tested. This genetic approach together with biochemical studies may result in further unravelling of the molecular mechanism of CPMV RNA replication.

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REFERENCES

1. **Dougherty, W. G. and Hiebert, E.,** Genome structure and gene expression of plant RNA viruses, in *Molecular Plant Virology*, Vol. 2, Davies, J. W., Ed., CRC Press, Boca Raton, Fl., 1985, chap. 2.
2. **Goldbach, R. and Van Kammen, A.,** Structure, Replication and Expression of the bipartite genome of cowpea mosaic virus, in *Molecular Plant Virology*, Vol. 2, Davies, J. W., Ed., CRC Press, Boca Raton, Fl., 1985, chap. 3.
3. **Goldbach, R.,** Comoviruses: molecular biology and replication, in *Plant Viruses: Viruses with Bipartite Genomes and Isometric Particles*, Harrison, B. D. and Murant, A. F., Eds., Plenum Press, New York, in press.
4. **Zabel, P., Weenen-Swaans, H., and Van Kammen, A.,** In vitro replication of cowpea mosaic virus RNA I. Isolation and properties of the membrane-bound replicase, *J. Virol.*, 14, 1049, 1974.
5. **Rottier, P. J. M., Rezelman, G., and Van Kammen, A.,** Protein synthesis in cowpea mosaic virus-infected cowpea protoplasts: detection of virus-related proteins, *J. Gen. Virol.*, 51, 359, 1980.
6. **Wu, G. J. and Bruening, G.,** Two proteins from cowpea mosaic virus, *Virology*, 46, 596, 1971.
7. **Geelen, J. L. M. C.,** Structuur en Eigenschappen van Cowpea-Mozaiekvirus, Ph.D. thesis, Agricultural University, Wageningen, 1974.
8. **Rottier, P. J. M., Rezelman, G., and Van Kammen, A.,** The inhibition of cowpea mosaic virus replication by actinomycin D, *Virology*, 92, 299, 1979.
9. **Van Weezenbeek, P., Verver, J., Harmsen, J., Vos, P., and Van Kammen, A.,** Primary structure and gene organization of the middle component RNA of cowpea mosaic virus, *EMBO J.*, 2, 941, 1983.
10. **Lomonossoff, G. P. and Shanks, M.,** The nucleotide sequence of cowpea mosaic virus B RNA, *EMBO J.*, 2, 2153, 1983.
11. **Daubert, S. D., Bruening, G., and Najarian, R. C.,** Protein bound to the genome RNAs of cowpea mosaic virus, *Eur. J. Biochem.*, 92, 45, 1978.
12. **Stanley, J., Rottier, P., Davies, J. W., Zabel, P., and Van Kammen, A.,** A protein linked to the 5′ termini of both RNA components of the cowpea mosaic virus genome, *Nucleic Acids Res.*, 5, 4505, 1978.
13. **Daubert, S. D. and Bruening, G.,** Genome-associated protein of comoviruses, *Virology*, 98, 246, 1979.
14. **El Manna, M. M. and Bruening, G.,** Polyadenylate sequences in the ribonucleic acids of cowpea mosaic virus, *Virology*, 56, 198, 1973.
15. **Steele, K. P. and Frist, R. H.,** Characterization of the 3′ termini of the RNAs of cowpea mosaic virus, *J. Virol.*, 26, 243, 1978.
16. **Van Kammen, A.** The relationship between the component of cowpea mosaic virus. I. Two ribonucleoprotein particles necessary for the infectivity of CPMV, *Virology*, 34, 312, 1968.
17. **De Jager, C. P.,** Genetic analysis of cowpea mosaic virus mutants by supplementation and reassortment tests, *Virology*, 70, 151, 1976.
18. **Goldbach, R., Rezelman, G., and Van Kammen, A.,** Independent replication and expression of B-component RNA of cowpea mosaic virus, *Nature (London)*, 286, 297, 1980.
19. **De Varennes, A. and Maule, A. J.,** Independent replication of cowpea mosaic virus of bottom component RNA: in vivo instability of the viral RNAs, *Virology*, 144, 495, 1985.
20. **Pelham, H. R. B.,** Synthesis and proteolytic processing of cowpea mosaic virus proteins in reticulocyte lysates, *Virology*, 96, 463, 1979.
21. **Goldbach, R. W., Schilthuis, J. G., and Rezelman, G.,** Comparison of in vivo and in vitro translation of cowpea mosaic virus RNAs, *Biochem. Biophys. Res. Comm.*, 99, 89, 1981.
22. **Goldbach, R. and Rezelman, G.,** Orientation of the cleavage map of the 200-kilodalton polypeptide encoded by the bottom-component RNA of cowpea mosaic virus, *J. Virol.*, 46, 614, 1983.
23. **Franssen, H., Goldbach, R., and Van Kammen, A.,** Translation of bottom component RNA of cowpea mosaic virus in reticulocyte lysates: faithful proteolytic processing of the primary translation product, *Virus Res.*, 1, 39, 1984.
24. **Peng, X. X. and Shih, D. S.,** Proteolytic processing of the proteins translated from the bottom component RNA of cowpea mosaic virus. The primary and secondary cleavage reactions, *J. Biol. Chem.*, 259, 3197, 1984.
25. **Rezelman, G., Goldbach, R., and Van Kammen, A.,** Expression of bottom component RNA of cowpea mosaic virus in cowpea protoplasts., *J. Virol.*, 36, 366, 1980.
26. **Wellink, J., Jaegle, M., and Goldbach, R.,** Detection of a 24K protein encoded by B-RNA of cowpea mosaic virus using antibodies raised against a synthetic peptide, *J. Virol.*, submitted.
27. **Zabel, P., Moerman, M., Van Straaten, F., Goldbach, R., and Van Kammen, A.,** Antibodies against the genome linked protein VPg of cowpea mosaic virus recognize a 60,000-Dalton precursor polypeptide, *J. Virol.*, 41, 1083, 1982.
28. **Goldbach, R., Rezelman, G., Zabel, P., and Van Kammen, A.,** Expression of the bottom-component RNA of cowpea mosaic virus. Evidence that the 60-kilodalton VPg precursor is cleaved into single VPg and a 58-kilodalton polypeptide, *J. Virol.*, 42, 630, 1982.

29. **Wellink, J., Rezelman, G., Goldbach, R., and Beyreuther, K.,** Determination of the sites of proteolytic processing in the polyprotein encoded by the B-RNA of cowpea mosaic virus, *J. Virol.*, 59, 50, 1986.
30. **Franssen, H., Moerman, M., Rezelman, G., and Goldbach, R.,** Evidence that the 32,000-Dalton protein encoded by bottom-component RNA of cowpea mosaic virus is a proteolytic processing enzyme, *J. Virol.*, 50, 183, 1984.
31. **Verver, J., Goldbach, R., Garcia, J. A., and Vos, P.,** In vitro expression of a full-length DNA copy of cowpea mosaic virus B-RNA: identification of the B RNA-encoded 24-kilodalton protein as a viral protease, *EMBO J.*, 6, 549, 1987.
31a. **Vos, P.** Molecular Cloning and Expression of Full-Length DNA Copies of the Genomic RNAs of Cowpea Mosaic Virus, Ph. D. thesis, Agricultural University, Wageningen, 1987.
31b. **Vos, P., Verver, J., Jaegle, M., Wellink, J., van Kammen, A., and Goldbach, R.,** Role of the cowpea mosiac virus B RNA-encoded 24K and 32K polypeptides in the proteolytic processing of the viral proteins, submitted.
32. **Franssen, H., Goldbach, R., Broekhuijsen, M., Moerman, M., and Van Kammen, A.,** Expression of middle-component RNA of cowpea mosaic virus: in vitro generation of a precursor to both capsid proteins by a bottom-component RNA-encoded protease from infected cells, *J. Virol.*, 41, 8, 1982.
33. **Vos, P., Verver, J., Van Weezenbeek, P., Van Kammen, A., and Goldbach, R.,** Study of the genetic organisation of a plant viral RNA genome by in vitro expression of a full length DNA copy, *EMBO J.*, 3, 3049, 1984.
34. **Franssen, H., Roelofsen, W., and Goldbach, R.,** Mapping of coding regions for the capsid proteins of cowpea mosaic virus on the nucleotide sequence of middle component RNA, *Chin. J. Virol.*, 2, 46, 1984.
35. **Wellink, J., Jaegle, M., Prinz, H., Van Kammen, A., and Goldbach, R.,** Expression of the middle component RNA of cowpea mosaic in vivo, *J. Gen. Virol.*, 68, 2577, 1987.
36. **Hiebert, E. and Purcifull, D. E.,** Mapping of the two coat protein genes on the middle RNA component of squash mosaic virus (comovirus group), *Virology*, 113, 630, 1981.
37. **Beier, H., Issinger, D. G., Deuschle, M., and Mundry, K. W.,** Translation of the RNA of cowpea severe mosaic virus in vitro and in cowpea protoplasts, *J. Gen. Virol.*, 54, 379, 1981.
38. **Gabriel, C. J., Derrick, K. S., and Shih, D. S.,** The synthesis and processing of the proteins of bean pod mottle virus in rabbit reticulocyte lysates, *Virology*, 122, 476, 1982.
39. **Goldbach, R. and Krijt, J.,** Cowpea mosaic virus-encoded protease does not recognize primary translation products of M-RNA from other comoviruses, *J. Virol.*, 43, 1151, 1982.
40. **Davies, J. W., Stanley, J., and Van Kammen, A.,** Sequence homology adjacent to the 3′-terminal poly(A) of cowpea mosaic virus RNAs. *Nucleic Acids Res.*, 7, 493, 1979.
41. **Najarian, R. C. and Bruening, G.,** Similar sequences from the 5′ end of cowpea mosaic virus RNAs, *Virology*, 106, 301, 1980.
42. **Ahlquist, P. and Kaesberg, P.,** Determination of the length distribution of poly(A) at the 3′-terminus of the virion RNAs of EMC virus, poliovirus, rhinovirus, RAV-61 and CPMV and of mouse globin mRNA, *Nucleic Acids Res.*, 7, 1195, 1979.
43. **Lomonossoff, G., Shanks, M., and Evans, D.,** The structure of cowpea mosaic virus replicative form RNA, *Virology*, 144, 351, 1985.
44. **Goldbach, R.,** Unpublished results.
45. **Jaegle, M., Wellink, J., and Goldbach, R.,** The genome-linked protein of cowpea mosaic virus is bound into the 5′-terminus of virus RNA by a phosphodiester linkage to serine, *J. Gen. Virol.*, 68, 627, 1987.
46. **De Zoeten, G. A., Assink, A. M., and Van Kammen, A.,** Association of cowpea mosaic virus-induced double stranded RNA with a cytopathological structure in infected cells, *Virology*, 59, 341, 1974.
47. **Assink, A. M., Swaans, H., and Van Kammen, A.,** The localisation of virus specific double-stranded RNA of cowpea mosaic virus in subcellular fractions of infected Vigna leaves, *Virology*, 53, 384, 1973.
48. **Hibi, T., Rezelman, G., and Van Kammen, A.,** Infection of cowpea mesophyll protoplasts with cowpea mosaic virus, *Virology*, 64, 308, 1975.
49. **Rezelman, G., Franssen, H. J., Goldbach, R. W., Ie, T-S., and Van Kammen, A.,** Limits to the independence of bottom component RNA of cowpea mosaic virus, *J. Gen. Virol.*, 60, 335, 1982.
50. **De Varennes, A., Davies, J. W., Shaw, J. G., and Maule, A. J.,** A reappraisal of the effect of actinomycin D and cordycepin on the multiplication of cowpea mosaic virus in cowpea protoplasts, *J. Gen. Virol.*, 66, 817, 1985.
51. **Lockhart, B. E. L. and Semancik, J. S.,** Inhibition of a plant virus by actinomycin D, *Virology*, 36, 504, 1968.
52. **Lockhart, B. E. L. and Semancik, J. S.,** Differential effect of actinomycin D on plant virus multiplication, *Virology*, 39, 62, 1969.
53. **Evans, D.,** Isolation of a mutant of cowpea mosaic virus which is unable to grow in cowpeas, *J. Gen. Virol.*, 66, 339, 1985.

54. **Dorssers, L., Van der Meer, J., Van Kammen, A., and Zabel, P.,** The cowpea mosaic virus RNA replicase complex and the host-encoded RNA-dependent RNA polymerase-template complex are functionally different, *Virology*, 125, 155, 1983.
55. **Dorssers, L., Zabel, P., Van der Meer, J., and Van Kammen, A.,** Purification of a host-encoded RNA-polymerase from cowpea mosaic virus-infected cowpea leaves, *Virology*, 116, 236, 1982.
56. **Van der Meer, J., Dorssers, L., and Zabel, P.,** Antibody-linked polymerase assay on protein blots: a novel method for identifying polymerase following SDS-polyacrylamide gel electrophoresis, *EMBO J.*, 2, 233, 1983.
57. **Van der Meer, J., Dorssers, L., Van Kammen, A., and Zabel, P.,** The RNA-dependent RNA polymerase of cowpea is not involved in cowpea mosaic virus RNA replication: immunologic evidence, *Virology*, 132, 413, 1984.
58. **Dorssers, L., Van der Krol, S., Van der Meer, J., Van Kammen, A., and Zabel, P.,** Purification of cowpea mosaic virus RNA replication complex: identification of a virus-encoded 110,000 dalton polypeptide responsible for RNA chain elongation, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 1951, 1984.
59. **Toyada, H., Nicklin, M. J. H., Murray, M. G., Anderson, C. W., Dunn, J. J., Studier, F. W., and Wimmer, E.,** A second virus encoded proteinase involved in proteolytic processing of poliovirus polyprotein, *Cell*, 45, 761, 1986.
60. **Strauss, E. G. and Strauss, J. H.,** Replication strategies of the single-stranded RNA viruses of eukaryotes, in *Current Topics in Microbiology and Immunology 105*, Cooper, M., Hofschneider, M., Koprowski, H., Melchers, F., Rott, R., Schweiger, H. G., Vogt, P. K., and Zinkernagel, R., Eds., Springer Verlag, Berlin, 1983, 1.
61. **Hogle, J., Chow, M., and Filman, D. J.,** The high resolution structure of the Mahoney strain of type 1 poliovirus, *Science*, 229, 1359, 1985.
62. **Stauffacher, C. V., Usha, R., Harrington, M., Schmidt, T., Hosur, M. V., and Johnson, J. E.,** The structure of cowpea mosaic virus at 3.5. Å resolution, in *Crystallography in Molecular Biology*, Mora, D., Ed., Plenum Press, New York, in press.
63. **Franssen, H., Leunissen, J., Goldbach, R., Lomonossoff, G., and Zimmern, D.,** Homologous sequences in non-structural proteins from cowpea mosaic virus and picornaviruses, *EMBO J.*, 3, 855, 1984.
64. **Kamer, G. and Argos, P.,** Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses, *Nucleic Acids Res.*, 12, 7269, 1984.
65. **Tershak, D. R.,** Association of poliovirus proteins with the endoplasmic reticulum, *J. Virol.*, 52, 777, 1984.
66. **Emini, E. A., Schleif, W. A., Colonno, R. J., and Wimmer, E.,** Antigenic conservation and divergence between the viral-specific proteins of poliovirus type 1 and various picornaviruses, *Virology*, 140, 13, 1985.
67. **Pincus, S. E., Diamond, D. C., Emini, E. A., and Wimmer, E.,** Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C, *J. Virol.*, 57, 638, 1986.
68. **Argos, P., Kamer, G., Nicklin, M. J. H., and Wimmer, E.,** Similarity in gene organization and homology between proteins of animal picornaviruses and a plant comovirus suggest common ancestry of these virus families, *Nucleic Acids Res.*, 12, 7251, 1984.
69. **Haseloff, J., Goelet, P., Zimmern, D., Ahlquist, P., Dasgupta, R., and Kaesberg, P.,** Striking similarities in amino acid sequence among nonstructural proteins encoded by RNA viruses that have dissimilar genomic organization, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 4358, 1984.
70. **Goldbach, R. W.,** Molecular evolution of plant RNA viruses, *Annu. Rev. Phytopathol.*, 24, 289, 1986.
71. **Miller, W. A., Bujarski, J. J., Dreher, T. W., and Hall, T. C.,** Minus-strand initiation by Brome mosaic virus replicase within the 3′ tRNA-like structure of native and modified RNA templates, *J. Mol. Biol.*, 187, 537, 1986.
72. **Houwing, C. J. and Jaspars, E. M. J.,** Coat protein blocks the in vitro transcription of the virion RNAs of alfalfa mosaic virus, *FEBS Lett.*, 209, 284, 1986.
73. **Berna, A., Doré, J.-M. Collendavelloo, J., Mugnier, M.-A., and Pinck, L.,** EMBO Workshop, Molecular Plant Virology, (Abstr.), 52, 1986.
74. **Takeda, N., Kuhn, R. J., Yang, C.-F., Takegami, T., and Wimmer, E.,** Initiation of poliovirus plus-strand RNA synthesis in a membrane complex of infected HeLa cells, *J. Virol.*, 60, 43, 1986.
75. **Young, D. C., Tuschall, D. M., and Flanegan, J. B.,** Poliovirus RNA-dependent RNA polymerase and host cell protein synthesize product RNA twice the size of poliovirion RNA in vitro, *J. Virol.*, 54, 256, 1985.
76. **Young, D. C., Dunn, B. M., Tobin, G. J., and Flanegan, J. B.,** Anti-VPg antibody precipitation of product RNA synthesized in vitro by the poliovirus polymerase and host factor is mediated by VPg on the poliovirion RNA template, *J. Virol.*, 58, 715, 1986.
77. **Andrews, N. C., Levin, D. H., and Baltimore, D.,** Poliovirus replicase stimulation by terminal uridylyl transferase, *J. Biol. Chem.*, 260, 7628, 1985.

78. **Andrews, N. C. and Baltimore, D.,** Purification of a terminal uridylyltransferase that acts as host factor in the in vitro poliovirus replicase reaction, *Proc. Natl. Acad. Sci. U.S.A.*, 83, 221, 1986.
79. **Gorbalenya, A. E., Blinov, V. M., and Koomin, E. V.,** Prediction of nucleotide-binding properties of virus-specific proteins from their primary structure, *Mol. Genetika*, N11, 30, 1985.
80. **Zabel, P., Dorssers, L., Wernars, K., and Van Kammen, A.,** Terminal uridylyl transferase of Vigna unguiculata: purification and characterization of an enzyme catalyzing the addition of a single UMP residue to the 3'-end of an RNA primer, *Nucleic Acids Res.*, 9, 2433, 1981.

CHAPTER 3

CPMV RNA replication in crude membrane fractions from infected cowpea and *Chenopodium amaranticolor*

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Cowpea Mosaic Virus RNA Replication in Crude Membrane Fractions from Infected Cowpea and *Chenopodium amaranticolor*

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SUMMARY

The replication of cowpea mosaic virus (CPMV) RNA was studied in crude membrane fractions prepared from leaves of CPMV-infected cowpea and *Chenopodium amaranticolor*. *In vitro* replicase assays showed that in the cowpea extract only the replicative intermediate (RI) and replicative form (RF) were synthesized. In the *C. amaranticolor* extract however, single-stranded progeny RNA was produced in addition to RI and RF. Production of the ssRNA in the *C. amaranticolor* extract was a result of the greater stability of the CPMV replication complex in this host. Comparison of the viral replicase activity and the amount of virus-encoded proteins in cowpea and *C. amaranticolor* crude membrane fractions indicated that only a small fraction of the non-structural proteins detected in cowpea is active in RNA replication. This suggests that viral replication proteins are used only once, perhaps because of a stringent coupling of polyprotein processing and replication.

INTRODUCTION

The comovirus cowpea mosaic virus (CPMV) resembles animal picornaviruses in having a plus-stranded RNA genome with a small protein (VPg) at the 5′ end and a poly(A) tail at the 3′ end, and which contains a single long open reading frame, coding for a polyprotein from which the functional polypeptides are derived by specific proteolytic cleavages. The structure and expression of the two genomic RNAs of CPMV, denoted M- and B-RNA, have been studied in detail (for recent reviews, see Goldbach & van Kammen, 1985; van Kammen *et al.*, 1987) and are shown in Fig. 1.

Non-structural proteins of CPMV and poliovirus share significant amino acid sequence homology, display probably similar functions in RNA replication and are similarly arranged in the genome (Franssen *et al.*, 1984; Argos *et al.*, 1984; Goldbach, 1986, 1987). Therefore, it is tempting to assume that the mechanisms of replication of CPMV RNA and poliovirus RNA will be very similar.

The replication of CPMV RNA has been studied extensively with crude membrane fractions prepared from CPMV-infected cowpea plants (for reviews, see van Kammen & Eggen, 1986; Eggen & van Kammen, 1988). These studies showed the existence of two functionally distinct and physically separable RNA-dependent RNA polymerase (RdRp) activities. The first has been identified as a host-encoded RdRp, which can be readily released from the membranes by washing with a Mg^{2+}-deficient buffer. Although the biological function of this host-encoded enzyme (M_r 130K) still remains to be determined, it has been shown to transcribe plant RNAs, and in infected cowpea plants also viral RNAs, into short RNA molecules of negative polarity (Dorssers *et al.*, 1982, 1983; Van der Meer *et al.*, 1983, 1984). The second RdRp, representing only 5% of the total RdRp activity, is the virus-specific, tightly membrane-bound RNA

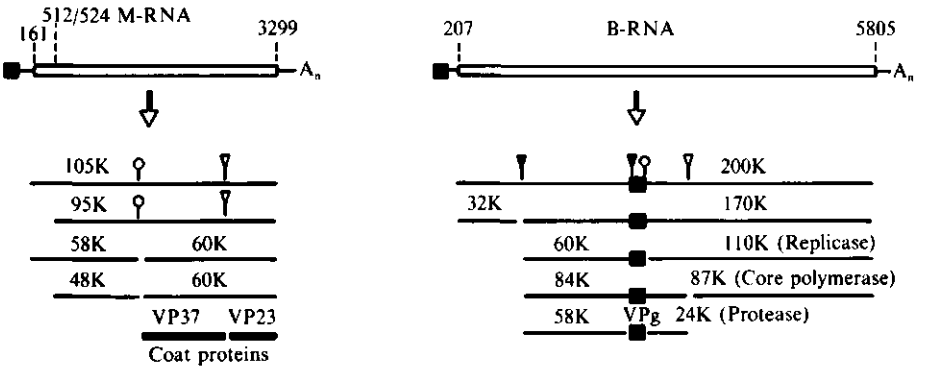

Fig. 1. Expression and genetic organization of the RNAs of CPMV. The long open reading frames in the RNAs are represented by open bars on which the positions of the start and stop codons are indicated. B- and M-RNA are translated into polyproteins which are subsequently processed by specific proteolytic cleavages at the indicated sites (○, Gln-Met; ▽, Gln-Gly; ▼, Gln-Ser) into smaller functional proteins.

replication complex. Protein analysis has demonstrated the presence in the complex of the B-RNA-encoded 110K polypeptide, constituting the viral replicase and two host proteins with M_r values of 68K and 57K (Dorssers *et al.*, 1984). Whether these host proteins are contaminants in the replication complex preparation or are functional subunits has not yet been investigated. *In vitro* the viral RNA replicase is capable of elongating only viral plus-sense RNA chains that have already been initiated *in vivo*. The completed chains are only detectable in double-stranded RNA (replicative form; RF). Moreover, the viral polymerase activity does not correlate with the amount of B-RNA-encoded 110K protein in CPMV-infected cowpea plants (Dorssers *et al.*, 1984).

Since the viral polymerase isolated from cowpea plants has only low activity, almost overshadowed by the host RdRp, extracts from another systemic host of CPMV, *Chenopodium amaranticolor*, were prepared to examine whether in this host additional viral RNA species and lower amounts of host RdRp products are synthesized. In this paper we report that the viral RNA-synthesizing activity extracted from this plant can produce single-stranded progeny RNA. These ssRNA molecules were produced in addition to RF and the replicative intermediate (RI), which is in contrast to extracts from CPMV-infected cowpea leaves that synthesized only RF and RI. To understand this difference, the structure and stability of the replication complexes in cowpea and *C. amaranticolor* have been studied. The host-encoded RdRp activity was lower in the *C. amaranticolor* extract than in cowpea extracts. The viral RNA polymerase activity and the amount of viral-encoded proteins involved in viral RNA replication were compared in extracts of the two hosts. This analysis indicated that the bulk of viral replication proteins in cowpea are inactive in viral RNA replication.

METHODS

Plants and virus. The primary leaves of 8-day-old cowpea (*Vigna unguiculata*, 'California Blackeye') or all the leaves of 40-day-old *C. amaranticolor* plants were inoculated with sap from CPMV (Sb isolate)-infected cowpea leaves, extracted in 0·1 M-sodium phosphate pH 7·0. Healthy samples were taken from plants inoculated with buffer alone. Propagation of the virus was as described by Zabel *et al.* (1974).

Preparation of the crude membrane fractions. The method was modified from Zabel *et al.* (1974). All operations were performed at 4 °C. Portions of freshly harvested leaves (12 g), from which the biggest ribs had been removed, were rinsed with distilled water, blotted with paper and homogenized in 35 ml buffer (50 mM-Tris–acetate pH 7·4, 10 mM-potassium acetate, 1 mM-EDTA, 5 mM-dithiothreitol and 0·5 mM-PMSF).

The homogenate was filtered through two layers of 'Miracloth' and subsequently centrifuged for 15 min at 1000 *g*. The supernatant fraction was adjusted to 20% (v/v) glycerol and was centrifuged for 30 min at 31000 *g*. The resulting pellet was resuspended thoroughly using a Dounce homogenizer in 0·5 ml buffer (50 mM-Tris–

acetate pH 8·0, 25% glycerol, 50 mM-potassium acetate, 1 mM-EDTA, 5 mM-dithiothreitol and 0·5 mM-PMSF) per g of fresh leaf. Small samples were stored at −80 °C.

Micrococcal nuclease treatment. Calcium acetate was added to 30 µl crude membrane fractions of cowpea or *C. amaranticolor* to a final concentration of 1 mM. After the addition of 90 units micrococcal nuclease, the mixture was incubated for 30 min at 30 °C. The micrococcal nuclease was inactivated by the addition of EGTA (final concentration 5 mM).

RdRp assay and product purification. The standard assay (60 µl) contained the following: 30 µl of the crude membrane fraction, 50 mM-Tris–acetate pH 8·2, 8 mM-magnesium acetate, 1 mM-EDTA, 25 mM-ammonium sulphate, 1·25 µg actinomycin D, 2 µCi [α-^{32}P]UTP (sp. act. 3000 Ci/mmol) and 1 mM each of ATP, CTP and GTP. The reaction mixtures were incubated at 30 °C for 60 min.

After incubation, the RNAs synthesized *in vitro* were extracted with an equal volume of a phenol/chloroform (1:1) mixture in the presence of 2 µg yeast RNA, 0·5% SDS and 10 mM-EDTA, and re-extracted without adding further SDS and EDTA. RNA was recovered by ammonium acetate/isopropanol precipitation (Maniatis *et al.*, 1982) and washed twice with 70% ethanol.

RNA gel electrophoresis. RNA samples were analysed either native in a 1% agarose gel containing 40 mM-Tris–acetate, 20 mM-sodium acetate and 2 mM-EDTA, pH 7·4, or fully denatured, using glyoxal and DMSO, in a 1% agarose gel in 10 mM-sodium phosphate pH 7·0, according to McMaster & Carmichael (1977).

SDS–polyacrylamide gels and immunoblot analysis. Protein samples were mixed with one-third volume of a fourfold concentrated sample buffer (4 × SB; 40 mM-Tris–HCl pH 8·0, 4 mM-EDTA, 40% v/v glycerol, 8% w/v SDS, 20% v/v 2-mercaptoethanol and 0·004% w/v bromophenol blue). After heating for 3 min at 100 °C, protein samples were separated by electrophoresis in a 12·5% SDS–polyacrylamide gel and blotted onto nitrocellulose. The resulting blot was subsequently incubated with antisera and ^{125}I-labelled Protein A for the detection of immune complexes as described by Zabel *et al.* (1982). The antisera used were raised against purified viral protein (anti-VP23, Franssen *et al.*, 1982), or synthetic peptides (anti-24K, Wellink *et al.*, 1987; anti-VPg, M. Jaegle & M. Van den Broek, unpublished data).

RESULTS

Time course of development of enzyme activity in infected plants

To compare the viral replicase activities in *C. amaranticolor* and cowpea, crude membrane fractions containing viral replication complexes active *in vitro* were prepared from leaves at daily intervals after inoculation with CPMV. As with cowpeas, the replicase activity isolated from infected *C. amaranticolor* leaves could only be detected in the membrane fraction (Dorssers *et al.*, 1983). The level of viral replicase activity *in vitro* in *C. amaranticolor* extracts was 25% or more of that in cowpea (data not shown). Differences in RNA polymerase activity and products made in the two hosts are shown in Fig. 2. In cowpea extracts there was a slower decrease of the viral replicase activity from days 2 to 6 after infection, and a stimulation of the host-encoded RdRp resulting in synthesis of products of low M_r. In *C. amaranticolor* extracts additional RNA species were synthesized; in addition to RI and RF, also produced in cowpea extracts (Dorssers *et al.*, 1983), labelled RNAs comigrating with CPMV B- and M-RNA were detected, suggesting the *in vitro* production of ss progeny RNA.

Characterization of the ssRNA products synthesized in C. amaranticolor *extracts*

To identify the nature of the putative ssRNAs produced *in vitro* by the viral replication complex from *C. amaranticolor* leaves, harvested on day 3 after inoculation, the products comigrating with viral B- and M-RNA were isolated from an agarose gel (Fig. 2) by the freeze–squeeze method (Tautz & Renz, 1983) and subjected to electrophoresis under denaturing conditions. Under these conditions the putative ssRNA products comigrated with the corresponding ss viral RNAs (Fig. 3). This result indicates that they were genome-length and excludes the possibility that the ssRNA bands were formed as a result of aggregation of labelled, negative-sense RNA fragments (e.g. produced by the host-encoded RdRp) and unlabelled viral RNA, as seen by Dorssers *et al.* (1983) and Jaspars *et al.* (1985) with CPMV and alfalfa mosaic virus respectively.

Isolated labelled replication products hybridized with ss M13 DNA containing negative-sense sequences of CPMV M-RNA but not with DNA containing positive-sense sequences (data not shown). These experiments thus show that the label was incorporated into plus-stranded RNA.

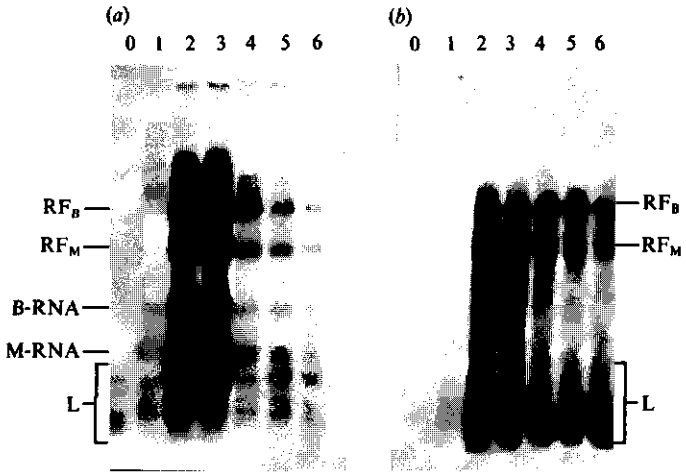

Fig. 2. Autoradiogram showing the time course of development of RdRp activity in crude membrane fractions prepared from infected *C. amaranticolor* (*a*) and cowpea (*b*) leaves on successive days (indicated by lane number) after inoculation. Total RNA samples were analysed on a 1% agarose gel under non-denaturing conditions. RF_B and RF_M indicate the positions of the dsRNA; B-RNA and M-RNA indicate the ssB- and ssM-RNA respectively. L indicates the low M_r RNA products produced by the host-encoded RdRp.

No labelled RI, RF or ssRNA could be detected when the *in vitro* RdRp assays were carried out in the presence of [^{32}P]UTP or [^{32}P]UTP plus ATP as the only ribonucleotides (data not shown). This result eliminated terminal addition of labelled UTP as a possible mechanism for the production of labelled ssRNA (Zabel *et al.*, 1981), and indicates that the labelled ssRNAs arose by *in vitro* elongation and correct termination of shorter endogenous RNA chains already synthesized *in vivo*.

Analysis of RNA precursor–product relationships

The elongation, termination and possible initiation of viral RNA synthesis in *C. amaranticolor* extracts (prepared 3 days after inoculation) were examined by investigating the kinetics of appearance of the different virus RNA species, including RI, RF and ssRNA. For this purpose RNA produced in crude membrane fractions after different times of incubation was analysed by agarose gel electrophoresis under native (Fig. 4*a*) and denaturing conditions (Fig. 4*b*). Analysis under native conditions showed that RI, forming a diffuse band migrating more slowly than RFs (Dorssers *et al.*, 1983), had formed by 1 min of incubation and that the RI disappeared after prolonged incubation, apparently being chased into RF_B, RF_M and the ssB- and ssM-RNA (Fig. 4*a*). Analysis under denaturing conditions showed that during incubation the RNA products increased in size with time until full length genomic RNAs appeared (Fig. 4*b*, lane 5).

These results, together with those described in the previous paragraph, provide evidence that, following elongation, correct termination of viral RNA replication had taken place in membrane fractions from CPMV-infected *C. amaranticolor* leaves.

After an incubation of 30 min virtually all labelled RI was converted into ds- and ssRNA (Fig. 4*a*, lane 6), but no further increase of the amount of ssRNA was observed in the following 30 min (Fig. 4*a*, lane 7). This result indicates that if any *de novo* initiation of viral RNA replication *in vitro* had occurred, followed by elongation, it had been at a low undetectable frequency.

Structure and stability of the replication complex

For infected cowpea, it has been suggested that upon fractionation of the plant or during *in vitro* RNA synthesis, the nascent RNA chains in the RI molecule are released or degraded (Dorssers *et al.*, 1983) resulting in most of the RNA synthesized in cowpea extracts being ds RF. To examine whether the CPMV replication complex in the membrane fraction of *C.*

Fig. 3

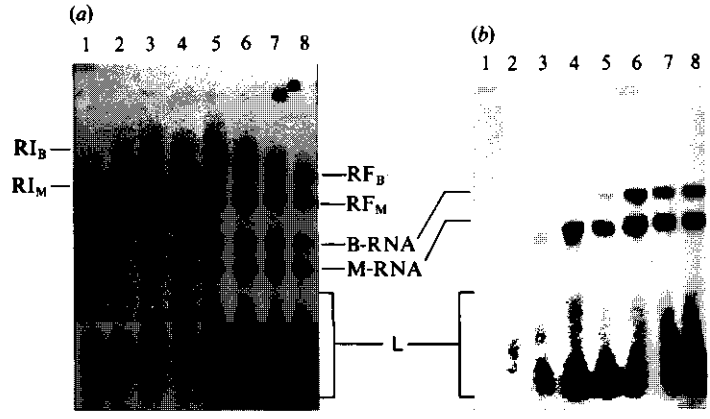

Fig. 4

Fig. 3. Autoradiogram showing the characterization of ssRNA products synthesized in the *C. amaranticolor* extract, by electrophoresis in a 1% agarose gel under denaturing conditions. Lanes 1 and 2 contain isolated RNA products which comigrated with B- and M-RNA respectively (see Fig. 2). The positions of CPMV B- and M-RNA are indicated at the side.

Fig. 4. Autoradiogram showing the time course of RNA-dependent RNA synthesis by crude membrane fractions of infected *C. amaranticolor* leaves. Reaction mixtures were incubated for different times (lanes 1, 1 min; lanes 2, 2 min; lanes 3, 5 min; lanes 4, 10 min; lanes 5, 20 min; lanes 6, 30 min; lanes 7, 60 min). After 30 min of incubation, one mixture was adjusted to 1 mM-UTP and incubated for a further 30 min (lanes 8). After extraction of the RNA products, the RNA samples were subjected to electrophoresis in a 1% agarose gel under native (*a*) or denaturing (*b*) conditions. Labels are as in Fig. 2, RI_B and RI_M indicate the positions of the replicative intermediates.

amaranticolor is more resistant to degradation, the products synthesized *in vitro* in a mixture of both plant extracts were analysed (Fig. 5). The production of ssRNA in *C. amaranticolor* extract (lane 2) was not affected by the addition of cowpea extract to the assay mixture (lane 3). Thus the replication complex in *C. amaranticolor* is more stable and less susceptible to nuclease than that in cowpeas.

This interpretation was further supported by attempts to remove endogenous RNA in cowpea and *C. amaranticolor* replication complexes. Micrococcal nuclease treatment abolished the synthesis of any labelled RNA species in cowpea extracts, whereas in *C. amaranticolor*, although ssRNA synthesis was affected, some RF was produced (Fig. 6, lanes 1 to 6). Even after micrococcal nuclease treatment in the presence of the non-ionic detergents Triton X-100 or dodecyl β-D-maltoside, which is more efficient in combination with dodecyl β-D-maltoside than with Triton X-100, some viral RNA species were produced in the *C. amaranticolor* extract (lanes 8 and 10). Addition of the detergents alone reduced the RF and ssRNA production at the same rate (lanes 7 and 9).

Detection of virus-encoded proteins in crude membrane fractions

In an attempt to understand the difference between the RNA-synthesizing activities in replication complexes isolated from cowpea and *C. amaranticolor*, the viral protein constituents of the replication-active membrane fractions of the respective hosts were analysed by immunoblotting (Fig. 7). Incubation with anti-VP23 serum revealed that in cowpea extracts two to three times more structural proteins could be detected than in *C. amaranticolor* extracts (Fig.

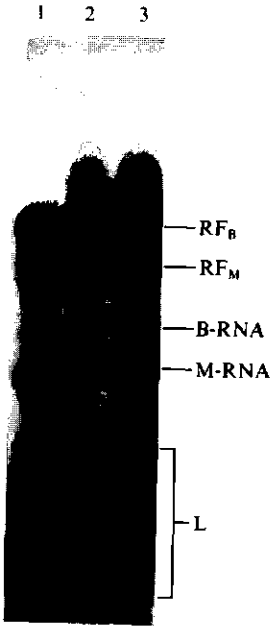

Fig. 5. Autoradiogram showing the analysis of RNA species produced *in vitro* using 15 µl crude membrane fractions prepared from CPMV-infected leaves of cowpea (lane 1), *C. amaranticolor* (lane 2), and a mixture of 15 µl as in lane 1 and 15 µl as in lane 2 (lane 3). Labels are as in Fig. 2.

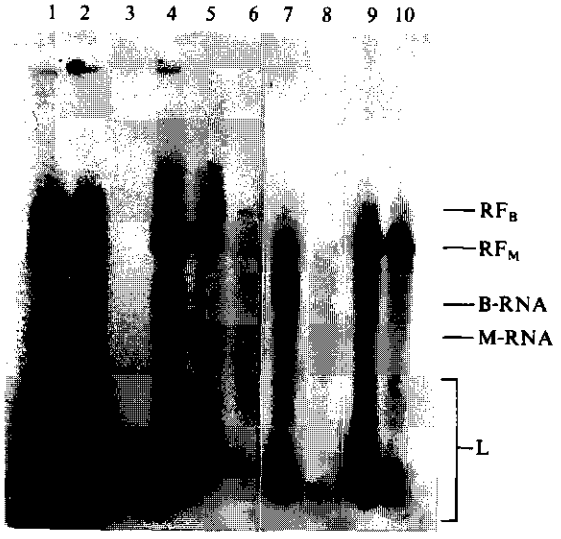

Fig. 6. Autoradiogram showing the effect of micrococcal nuclease and/or detergent on the RNA synthesis in cowpea (lanes 1 to 3) or *C. amaranticolor* (lanes 4 to 10) extracts *in vitro*. Crude membrane fractions were incubated with (lanes 3, 6, 8 and 10) or without (lanes 1, 2, 4, 5, 7 and 9) micrococcal nuclease. To some of these incubations with *C. amaranticolor* extract, 0·1% (w/v) dodecyl β-D-maltoside (lanes 7 and 8) or 0·1% (v/v) Triton X-100 (lanes 9 and 10) was added. The RNA products were analysed on a 1% agarose gel under non-denaturing conditions. Labels are as in Fig. 2.

7*a*, lanes 2 and 4). The 23K and 22K bands represent VP23 and its shortened product VP22 respectively (Franssen *et al.*, 1982). We do not know the identity of the more slowly migrating bands.

The quantities of the non-structural viral proteins, however, were strikingly different; that in *C. amaranticolor* was at most 5% of that found in cowpea. Anti-VPg serum reacted weakly with

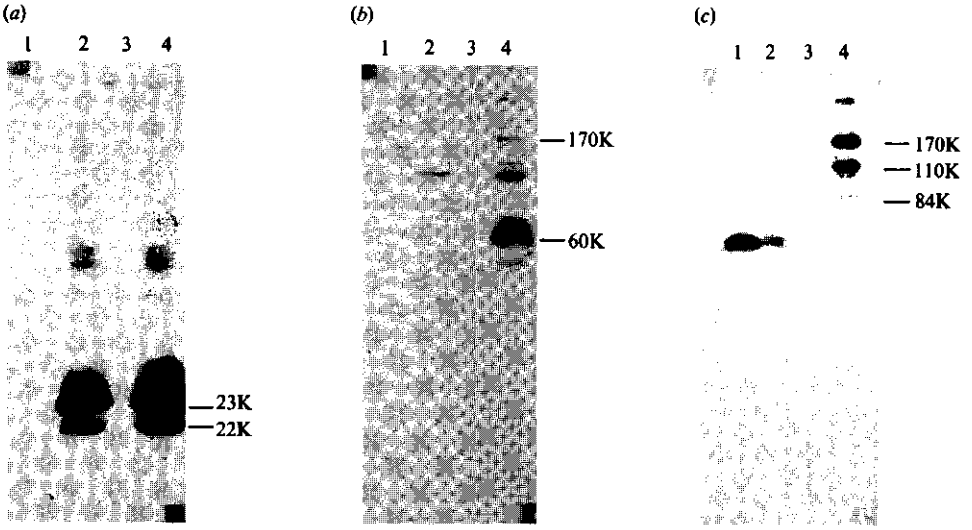

Fig. 7. Autoradiogram showing the immunological detection of CPMV-encoded proteins. Protein samples were from 30 µl of crude membrane fractions, prepared from healthy (odd-numbered lanes) or CPMV-infected (even-numbered lanes) leaves of *C. amaranticolor* (lanes 1 and 2) or cowpea (lanes 3 and 4). They were analysed on a 12.5% SDS–polyacrylamide gel and immunoblotted with (*a*) anti-VP23, (*b*) anti-VPg or (*c*) anti-24K antisera. The M_r values of polypeptides, calculated from mobilities of marker proteins are indicated to the right of each panel.

170K and 84K proteins and very strongly with the 60K VPg precursor proteins. These proteins were only detected in the crude membrane fractions prepared from inoculated cowpea leaves (Fig. 7*b*). Also, the non-structural polypeptides visualized with the anti-24K serum (e.g. 170K, 110K and 84K) were detectable only in extracts from inoculated cowpea leaves (Fig. 7*c*). The nature of the immunoreactive proteins also present in crude membrane fractions prepared from both healthy and inoculated *C. amaranticolor* leaves is not known (Fig. 7*c*, lanes 1 and 2).

The polypeptide with an M_r of about 100K that reacted with both the anti-VPg and anti-24K sera might represent a B-RNA-encoded protein arising by an alternative cleavage of the 170K polypeptide (Fig. 7*b* and *c*).

Radiolabelled virus-encoded non-structural proteins produced in cowpea protoplasts were degraded more rapidly when added to extracts of *C. amaranticolor* leaves than when added to extracts of cowpea leaves (data not shown). This indicated that the different amounts of non-structural proteins in the two hosts could be explained by rapid proteolytic breakdown occurring in *C. amaranticolor*. When added to extracts of both plants, labelled viral structural proteins were unaffected by the higher proteolytic activity in *C. amaranticolor*, which is not surprising because such stability would reflect their genome-protecting function.

DISCUSSION

Viruses with ss plus-sense RNA genomes that infect eukaryotic cells replicate via the synthesis of a minus-sense strand, which in turn is the template for the production of progeny virus RNA strands. Replication intermediates include RI and RF molecules. In extracts of CPMV-infected cowpea leaves RI and RF have been detected in *in vitro* replicase assays but viral ssRNA has not (Dorssers *et al.*, 1983). In this paper we show that crude membrane fractions prepared from CPMV-infected *C. amaranticolor* leaves contained CPMV replication complexes able to produce full length progeny ssRNA as well as the RI and RF synthesized in cowpea extracts.

Analysis of the stability of the replication complex, using micrococcal nuclease and non-ionic

detergents, showed that the structure and membranous environment may be responsible for the differences detected. Besides the greater stability of the replication complex in *C. amaranticolor* extract, the lower level of host-encoded RdRp activity is an improvement in comparison with the cowpea extract.

Examination of the precursor–product relationships of the different virus RNA species produced in *C. amaranticolor* extracts suggests that RI is the functional intermediate in viral RNA replication *in vivo*, as indicated by its immediate appearance and efficient chase into RF and ssRNA *in vitro*.

After prolonged incubation *in vitro*, RF and ssRNA were produced in equal amounts. From this one can speculate that at least two (as labelled RF and ssRNA are produced), but probably less than five polymerase molecules will be present per RI structure. This number has been estimated assuming that the viral polymerases work processively and taking into account the possible lower intensity of ssRNA bands due to degradation. This estimate is in agreement with the analysed RI structures of poliovirus, for which four to eight polymerase molecules per RI have been proposed (Richards *et al.*, 1984).

RF labelled *in vitro* is detectable only after increasing incubation times, which suggests that CPMV RF is probably a 'dead-end' molecule. This conclusion is supported by the observation that CPMV RF cannot infect cowpea plants unless it is first denatured (Shanks *et al.*, 1985). Earlier suggestions for functions of RF are that it represents a functional replication intermediate (Koch & Koch, 1985), an isolation artefact (Hall *et al.*, 1982; Richards *et al.*, 1984) or a 'dead-end' molecule arising either in non-optimally replicating *in vitro* systems (Chu & Westaway, 1987; Hall *et al.*, 1982; Jaspars *et al.*, 1985; Kuhn & Wimmer, 1987; Morrow *et al.*, 1985; Mouchès *et al.*, 1974; Watanabe & Okada, 1986; Young & Zaitlin, 1986) or accumulating *in vivo* at the end of the infection cycle (Koch & Koch, 1985).

Chenopodium amaranticolor extracts did not support initiation of RNA synthesis, because no new RI molecules were produced during the replication assay. The initiation of viral RNA replication of VPg-containing viruses is poorly understood. Even in the intensively studied poliovirus RNA replication system this aspect is still a matter of debate (for a recent review, see Kuhn & Wimmer, 1987).

Although there was a striking difference between the amount of non-structural proteins present, the viral replicase activities in both plant extracts were of the same order of magnitude. This supports the proposal presented previously (Dorssers *et al.*, 1984) that only a small proportion of the non-structural proteins detected in cowpea plants are active. The bulk of such molecules found in infected cells is either irrelevant or, as we think, had been functional earlier during the infection and accumulated as inactive molecules in the cytoplasm (Wellink *et al.*, 1988). This idea has led to a replication model in which viral replication proteins are used only once by a stringent coupling of polyprotein processing and replication (van Kammen & Eggen, 1986; Eggen & van Kammen, 1988). The hypothesis of viral proteins with limited activity is supported by the lack of success in our laboratory of attempts to prepare a template-dependent *in vitro* RNA replicating system, for which re-usable polymerase molecules would be necessary (R. Eggen, unpublished results).

Cowpeas of cv. Arlington do not support detectable production of CPMV (Eastwell *et al.*, 1983). For this lack of CPMV multiplication three possibilities have been suggested: inhibition of the CPMV-encoded protease, inhibition of the CPMV RNA translation, or a general proteolytic degradation of CPMV proteins (Ponz *et al.*, 1987). The last possibility seems very unlikely in view of our results on CPMV replication in two different hosts. Although in *C. amaranticolor* the virus-encoded non-structural proteins could hardly be detected, as a result of proteolytic degradation, the amount of virus-encoded structural proteins accumulating in *C. amaranticolor* and also the level of replicase activity *in vitro* were of the same order of magnitude as those in cowpea plants. This suggests that high turnover levels do not necessarily result in lower virus yields.

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REFERENCES

ARGOS, P. KAMER, G., NICKLIN, M. J. H. & WIMMER, E. (1984). Similarity in gene organization and homology between proteins of animal picornaviruses and plant comovirus suggest a common ancestry of these virus families. *Nucleic Acids Research* **12**, 7251–7276.

CHU, P. W. G. & WESTAWAY, E. G. (1987). Characterization of Kunjin virus RNA-dependent RNA polymerase: reinitiation *in vitro*. *Virology* **157**, 330–337.

DORSSERS, L., ZABEL, P., VAN DER MEER, J. & VAN KAMMEN, A. (1982). Purification of a host-encoded RNA-polymerase from cowpea mosaic virus infected cowpea leaves. *Virology* **116**, 236–249.

DORSSERS, L., VAN DER MEER, J., VAN KAMMEN, A. & ZABEL, P. (1983). The cowpea mosaic virus RNA replicase complex and the host-encoded RNA-dependent RNA polymerase template complex are functionally different. *Virology* **125**, 155–174.

DORSSERS, L., VAN DER KROL, S., VAN DER MEER, J., VAN KAMMEN, A. & ZABEL, P. (1984). Purification of cowpea mosaic virus RNA replication complex: identification of a virus-encoded 110,000 dalton polypeptide responsible for RNA chain elongation. *Proceedings of the National Academy of Sciences, U.S.A.* **81**, 1951–1955.

EASTWELL, K. C., KIEFER, M. C. & BRUENING, G. (1983). Immunity of cowpeas to cowpea mosaic virus. In *Plant Molecular Biology, UCLA Symposia on Molecular and Cellular Biology. New Series*, vol. 12, pp. 201–211. Edited by R. B. Goldberg. New York: Alan R. Liss.

EGGEN, R. & VAN KAMMEN, A. (1988). RNA replication in comoviruses. In *RNA Genetics*, vol. 1, pp. 49–69. Edited by P. Ahlquist, J. Holland & E. Domingo. Boca Raton: CRC Press.

FRANSSEN, H., GOLDBACH, R., BROEKHUYSEN, M., MOERMAN, M. & VAN KAMMEN, A. (1982). Expression of middle-component RNA of cowpea mosaic virus: in vitro generation of a precursor to both capsid proteins by a bottom-component RNA-encoded protease from infected cells. *Journal of Virology* **41**, 8–17.

FRANSSEN, H., LEUNISSEN, J., GOLDBACH, R., LOMONOSSOFF, G. P. & ZIMMERN, D. (1984). Homologous sequences in non-structural proteins from cowpea mosaic virus and picornaviruses. *EMBO Journal* **3**, 855–861.

GOLDBACH, R. W. (1986). Molecular evolution of plant RNA viruses. *Annual Review of Phytopathology* **24**, 289–310.

GOLDBACH, R. (1987). Genome similarities between plant and animal RNA viruses. *Microbiological Sciences* **4**, 197–202.

GOLDBACH, R. & VAN KAMMEN, A. (1985). Structure, replication and expression of the bipartite genome of cowpea mosaic virus. In *Molecular Plant Virology*, vol. 2, pp. 83–120. Edited by J. W. Davies. Boca Raton: CRC Press.

HALL, T. C., MILLER, M. A. & BUJARSKI, J. J. (1982). Enzymes involved in the replication of plant viral RNAs. In *Advances in Plant Pathology*, vol. 1, pp. 179–211. Edited by D. Ingram & P. Williams. New York & London: Academic Press.

JASPARS, E. M. J., GILL, D. S. & SYMONS, R. H. (1985). Viral RNA synthesis by a particulate fraction from cucumber seedlings infected with cucumber mosaic virus. *Virology* **144**, 410–425.

KOCH, F. & KOCH, G. (editors) (1985). Replication of the viral RNA. In *The Molecular Biology of Poliovirus*, pp. 203–222. Wien & New York: Springer-Verlag.

KUHN, R. J. & WIMMER, E. (1987). The replication of picornaviruses. In *The Molecular Biology of the Positive Strand RNA Viruses*, pp. 17–51. Edited by D. J. Rowlands, M. A. Mayo & B. W. J. Mahy. Orlando & London: Academic Press.

McMASTER, G. K. & CARMICHAEL, G. C. (1977). Analysis of a single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proceedings of the National Academy of Sciences, U.S.A.* **74**, 4835–4838.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory.

MORROW, C. D., LUBINSKI, J., HOCHO, J., GIBBONS, G. F. & DASGUPTA, A. (1985). Purification of a soluble template-dependent rhinovirus RNA polymerase and its dependence on a host cell protein for viral RNA synthesis. *Journal of Virology* **53**, 266–272.

MOUCHÈS, C., BOVÉ, C. & BOVÉ, J. M. (1974). Turnip yellow mosaic virus RNA replicase: partial purification of the enzyme from the solubilized enzyme-template complex. *Virology* **58**, 409–423.

PONZ, F., GLASCOCK, C. B. & BRUENING, G. (1987). An inhibitor of polyprotein processing with the characteristics of a natural virus resistance factor. *Molecular Plant–Microbe Interactions* **1**, 25–31.

RICHARDS, O. C., MARTIN, S. C., JENSE, H. G. & EHRENFELD, E. (1984). Structure of poliovirus replicative intermediate RNA. *Journal of Molecular Biology* **173**, 325–340.

SHANKS, M., LOMONOSSOFF, G. P. & EVANS, D. (1985). Double-stranded, replicative form RNA molecules of cowpea mosaic virus are not infectious. *Journal of General Virology* **66**, 925–930.

TAUTZ, D. & RENZ, M. (1983). An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. *Analytical Biochemistry* **132**, 14–19.

VAN DER MEER, J., DORSSERS, L. & ZABEL, P. (1983). Antibody-linked polymerase assay on protein blots: a novel method for identifying polymerase following SDS-polyacrylamide gel electrophoresis. *EMBO Journal* **2**, 233–237.

VAN DER MEER, J., DORSSERS, L., VAN KAMMEN, A. & ZABEL, P. (1984). The RNA-dependent RNA polymerase of cowpea is not involved in cowpea mosaic virus RNA replication: immunological evidence. *Virology* **132**, 413–425.

VAN KAMMEN, A. & EGGEN, H. I. L. (1986). The replication of cowpea mosaic virus. *BioEssays* **5**, 261–266.

VAN KAMMEN, A., WELLINK, J., GOLDBACH, R., EGGEN, R. & VOS, P. (1987). The use of full-length DNA copies in the study of the expression and replication of the RNA genome of cowpea mosaic virus. In *Plant Molecular Biology*, pp. 433–442. Edited by D. von Wettstein & N.-H. Chua. New York & London: Plenum Press.

WATANABE, Y. & OKADA, Y. (1986). *In vitro* viral RNA synthesis by a subcellular fraction of TMV-inoculated tobacco protoplasts. *Virology* **149**, 64–73.

WELLINK, J., JAEGLE, M. & GOLDBACH, R. (1987). Detection of a novel protein encoded by the bottom component RNA of cowpea mosaic virus, using antibodies raised against a synthetic peptide. *Journal of Virology* **61**, 236–238.

WELLINK, J., VAN LENT, J. & GOLDBACH, R. (1988). Detection of viral proteins in cytopathic structures in cowpea protoplasts infected with cowpea mosaic virus. *Journal of General Virology* **69**, 751–755.

YOUNG, N. D. & ZAITLIN, M. (1986). An analysis of tobacco mosaic virus replicative structures synthesized *in vitro*. *Plant Molecular Biology* **6**, 455–465.

ZABEL, P., WEENEN-SWAANS, H. & VAN KAMMEN, A. (1974). *In vitro* replication of cowpea mosaic virus RNA. I. Isolation and properties of the membrane bound replicase. *Journal of Virology* **14**, 1049–1055.

ZABEL, P., DORSSERS, L., WERNARS, K. & VAN KAMMEN, A. (1981). Terminal uridylyl transferases of *Vigna unguiculata*: purification and characterization of an enzyme catalyzing the addition of a single UMP residue to the 3'-end of an RNA primer. *Nucleic Acids Research* **9**, 2433–2453.

ZABEL, P., MOERMAN, M., VAN STRAATEN, F., GOLDBACH, R. & VAN KAMMEN, A. (1982). Antibodies against the genome-linked protein VPg of cowpea mosaic virus recognize a 60,000-dalton precursor polypeptide. *Journal of Virology* **41**, 1083–1088.

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CHAPTER 4

High-level synthesis of cowpea mosaic virus RNA polymerase and protease in *Escherichia coli*

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High-level synthesis of cowpea mosaic virus RNA polymerase and protease in *Escherichia coli*

(Recombinant DNA; immunoblotting; plasmid constructs; poliovirus; comoviruses)

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SUMMARY

An expression system for the production of polymerase proteins of cowpea mosaic virus (CPMV) in *Escherichia coli* cells is described. High-level synthesis of proteins containing protease and polymerase moieties (110-kDa protein) and polymerase alone (87-kDa protein) were obtained from cells containing different plasmid constructions. Precursor and processed forms of CPMV proteins were detected by immunoblotting with antisera directed against 170-kDa precursor polyprotein and 24-kDa viral protease. Crude lysates and supernatant fractions of the lysates from *E. coli* cells harboring the various plasmid constructions were analysed for poly(A)-oligo(U) polymerase activity and found to be negative for CPMV activity under conditions where similar expression systems for the production of poliovirus RNA polymerase activity were positive. Thus, conditions for CPMV RNA replication may indeed be different from those for poliovirus even though the genomic organization of these viruses is similar.

INTRODUCTION

Cowpea mosaic virus (CPMV), a type member of the comoviruses, has a bipartite, plus-sense RNA genome; both RNA molecules (B- and M-RNA) carry a small protein, VPg, covalently attached to their 5′ ends and poly(A) at their 3′ ends (Daubert et al., 1978; Stanley et al., 1978; El Manna and Bruening, 1973; Steele and Frist, 1978). Expression of the genomic segments of CPMV results in the synthesis of polyproteins which are subsequently cleaved by a viral protease into functional proteins (Vos et al., 1988b). A summary of the cleavage products observed for the larger genomic segment is shown in Fig. 1. The genome structure, gene organization and protein processing of CPMV are strik-

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Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); CPMV, cowpea mosaic virus; IAA, indoleacrylic acid; IgG, immunoglobulin G; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; VPg, viral protein genome-linked; wt, wild type.

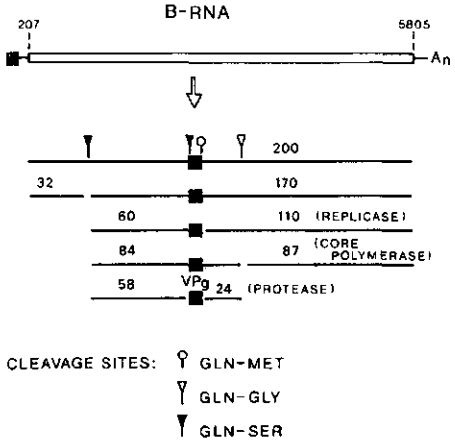

Fig. 1. Map of B-RNA of CPMV and of its protein products. B-RNA contains a single open reading frame represented by the open bar; the positions of the translational start and stop codons are indicated. VPg is indicated by a black square and all other protein sequences by single lines. The sizes (in kDa) of the initial polyprotein and processing proteins are shown along with the proposed cleavage sites.

ingly similar to those of picornaviruses (Eggen and Van Kammen, 1988; Franssen et al., 1984), except that the CPMV genome consists of two RNA molecules and that of picornaviruses of only one.

Definition of the mode of RNA replication of comoviruses has remained elusive. Purified replication complexes from CPMV-infected leaves contain a 110-kDa polypeptide as the sole detectable viral protein (Dorssers et al., 1984). At least two host proteins are intimately associated with this complex, but their role in viral RNA replication, if any, remains obscure. Thus, the 110-kDa viral protein, which includes 24-kDa protease and 87-kDa polymerase sequences (Fig. 1), has been suggested to be the active polymerase in CPMV RNA replication (Eggen and Van Kammen, 1988). This differs drastically from the situation with poliovirus, a prototype of the picornaviruses, where only the cleaved-off polymerase sequence has polymerase activity and the sequence comparable to the 110-kDa of CPMV, 3 CD-protein, has none (Van Dyke and Flanegan, 1980). Since the genomes of both poliovirus and CPMV have VPg at their 5′ terminus, VPg (or a precursor form of VPg) has been implicated in RNA strand initiation (Eggen and Van Kammen, 1988; Semler et al., 1988). Thereafter the 110-kDa protein in CPMV or the 52-kDa polymerase in poliovirus may elongate the RNA strands. CPMV infection in plants is also associated with a dramatic stimulation of a host RNA polymerase (130 kDa) activity (Van der Meer et al., 1984). The role of this enzyme in CPMV RNA replication in vivo is unknown.

A template-dependent CPMV polymerase has never been isolated from infected plant material, but plant virus studies have demonstrated such an activity for brome mosaic virus (Miller and Hall, 1983; Quadt et al., 1988) and alfalfa mosaic virus (Houwing and Jaspars, 1986), which have tripartite genomes, and for turnip yellow mosaic virus (Mouches et al., 1974), which has a monopartite genome. The terminal structures on the genomes of these viruses (capped 5′ ends and tRNA-like 3′ ends) differ from those of CPMV and exhibit various modes of genome expression (Goldbach and Wellink, 1988). Thus they may employ entirely different replication strategies. Utilization of infected plants as a source of CPMV RNA polymerase is complicated by additional host activities, such as a 130-kDa host RNA polymerase (Dorssers et al., 1983) and a terminal transferase activity (Zabel et al., 1981) and also by the elimination of polymerase activity observed when tightly bound template is removed from crude replication complexes (R.E., unpublished results). Therefore, the prokaryotic expression system of *E. coli* seemed an appropriate candidate to express the 110-kDa and 87-kDa proteins of CPMV (Fig. 1), and to examine possible elongation activity associated with these proteins. This RNA-synthetic activity has been demonstrated successfully for polioviral polymerase expressed in *E. coli* (Rothstein et al., 1988; Morrow et al., 1987).

Such an expression system may generate a template-dependent activity due to CPMV sequences which are not associated with membranes, as seen in crude replication complexes from CPMV-infected leaves, and would be more amenable to definitive study of the RNA replication process. This paper describes our attempts to generate sizable levels of 110-kDa and 87-kDa polypeptides and to examine associated polymerase activities.

MATERIALS AND METHODS

(a) Enzymes, plasmids, strains and other materials

Restriction endonucleases, *Sma*I linkers, PolIk, DNA ligase, T4 polynucleotide kinase, agarose (electrophoresis grade), and MBN were from Bethesda Research Laboratories, Boehringer-Mannheim Biochemicals, or New England Biolabs and were used as prescribed by the supplier. Lysozyme, RNaseA, Pronase, and indoleacrylic acid were from Sigma Chemical Co. Nitrocellulose BA85 sheets were from Schleicher & Schuell. Plasmid vector, pATH-3, was provided by Dr. Carol Dieckmann, University of Arizona. Host strains used for construction and isolation of vectors were *E. coli* JM109 and RZ1032.

(b) Construction of plasmids

(1) pATH(Del)-120

A *Nru*I-*Cla*I fragment, encompassing 35 kDa of *trpE* sequences, was deleted from the expression vector, pATH-3, and a *Sma*I linker was added at the *Nru*I site to facilitate fusion of foreign sequences in the vector downstream from the *trp* promoter. This vector still retains the correct reading frame. All CPMV sequences were derived from cDNAs whose transcripts are infectious. Specifically for the present construction CPMV sequences were derived from pTB108 (Vos et al., 1988), including a 133-bp *Rsa*I-*Kpn*I fragment (nt 3001–3134 in the B-cDNA; Lomonossoff and Shanks, 1983) and a *Kpn*I-*Cla*I fragment (nt 3134 to the 3′ terminus of the B-RNA-encoded sequences). These fragments were ligated to the modified *Sma*I-digested vector to generate pATH(Del)-120 (see Fig. 2). This construct encodes 2 kDa of the N-terminal TrpE sequences fused to CPMV sequences starting 46 nt upstream from VPg, through VPg, the 24-kDa protease, and the 87-kDa polymerase.

(2) pEXC-3C-120*

Plasmid pEXC-3D (Richards et al., 1987) was digested with *Bgl*II, the ends blunted by filling in, *Sma*I linker added and digested with *Sma*I followed by *Sal*I to obtain a 3200-bp fragment. This fragment contains a *trp* promoter and the DNA code for the N-terminal 54 aa of poliovirus 3C protease to which may be fused foreign DNA for continued expression. In this case the 133-bp *Rsa*I-*Kpn*I fragment (nt 3001–3134 in B-cDNA) from pTB108 (Vos et al., 1988a) and the 3500-bp *Kpn*I-*Sal*I fragment (nt 3134 to a point beyond the 3′ terminus of B-cDNA sequences) from pTB208 (a B-cDNA, unpublished) were inserted downstream from the poliovirus sequences to generate pEXC-3C*-120 (see Fig. 2). This construct contains 162 bp encoding the N terminus of poliovirus 3C protease fused to CPMV sequences starting 46 nt upstream from VPg, and continuing through the 24-kDa protease gene and the 87-kDa polymerase gene.

(3) pEXC-110

An *Sst*I-*Bam*HI fragment (nt 2301–3857 in the B-cDNA) from pTB1GBgl (R.E., J. Verver, J. Wellink, A. de Jong, R. G. and A.V.K., manuscript in preparation) was inserted into M13mp19 and the chimeric DNA underwent three cycles of growth in *E. coli* RZ1032 in the presence of uridine (0.25 μg/ml) for the random insertion of U residues, as described by Kunkel (1985). Single-stranded DNA was isolated and hybridized with a phosphorylated oligo (24-mer), spanning the normal VPg/24-kDa cleavage site and containing a single nt modification at the nt −1 position (A → T) with respect to the sequence upstream from the 24-kDa protease. Closure of the DNA to a full duplex, transformation of *E. coli* and the efficient selection of mutant DNA (newly created *Nde*I site) were performed as described (Kunkel, 1985). Then the unique 109-bp *Nde*I-*Sph*I fragment from the chimeric M13 DNA was isolated after filling-in the *Nde*I site. This fragment was mixed with a 3480-bp *Hin*dIII-*Bam*HI fragment (*Hin*dIII site blunted with MBN) from pEXC-3C*-120 and a 2844-bp *Sph*I-*Bgl*II fragment from pTB1GBgl (R.E., J. Verver, J. Wellink, A. de Jong, R.G. and A.v.K., manuscript in preparation), ligated and the mixture was used to generate pEXC-110 (see Fig. 2). pEXC-110 encodes 24-kDa protease and 87-kDa polymerase of CPMV without additional coding cowpea sequences.

(4) Insertion of mutant sites into clones

(i) Modification of the 24-kDa protease–87-kDa polymerase cleavage site

The *Sst*I-*Bam*HI fragment (nt 2301–3857 in B-cDNA) was isolated from pTB1Bgl (R.E., J.

Verver, J. Wellink, A. de Jong, R.G. and A.v.K., manuscript in preparation) inserted into M13mp18, and submitted to mutagenesis selection as described above and by Kunkel (1985) using a phosphorylated oligo. Hybridization with this oligo leads to modification at −4 (G → C) and +3 (T → G) with respect to the start of the code for the 87-kDa gene. Partial *Bgl*I digestion, blunting with the exonuclease activity of PolIk, and closure with DNA ligase produced a plasmid with a new *Sma*I site. A 723-bp *Kpn*I-*Bam*HI fragment containing the mutation was isolated and exchanged with the corresponding 723-bp fragment from pATH (Del)-120, pEXC-3C*-120, and pEXC-110 to create a mutant site in each of these constructs, where the wt glutamine-glycine cleavage site is removed and replaced with arginine (1 aa deleted).

(ii) Insertion of lacZ *sequence into polymerase gene*

To inactivate polymerase activity, a 141-bp *Sau*3A fragment from M13mp19 (from the *Bam*HI site in the multiple cloning site to the *Pvu*I site at nt 6409) was inserted into the *Bam*HI site (nt 3857 in B-cDNA) of pEXC-110, without disturbing the reading frame. Confirmation of the proper orientation of the insert in the cowpea sequence was established by restriction analysis.

(c) Expression of CPMV sequences and preparation of extracts

Overnight cultures of *E. coli* JM109, harboring the various expression vectors, were grown in M9 medium, supplemented with 0.5% Casamino acids and thiamine (10 μg/ml), or in LB medium (Maniatis et al., 1982) at 37°C. Additional supplements were 50 μg Ap/ml and 20 μg tryptophan/ml. These cultures were diluted ten- or twelve-fold supplemented M9 medium or LB medium containing 50 μg Ap/ml and either tryptophan (40 μg/ml; uninduced) or IAA (5 μg/ml; induced). Growth continued at 37°C for 2 h or until an A_{595} of 0.8 to 1.0 was attained.

For most immunoblots, total cell lysates were obtained by collection of cells by centrifugation, washed twice with 10 mM Na · phosphate pH 7.0, and boiled in sample buffer (5% glycerol, 2.5% β-mercaptoethanol, 1.5% SDS, 0.005% bromophenol blue). For cell lysates in which polymerase activity was to be measured, cells were collected by centrifugation, washed twice with 10 mM HEPES pH 8.0, and resuspended in the same buffer at 1/10 the original growth volume. Cell suspensions, on ice, were sonicated with a 20 Kc Branson sonifier in four 30-s bursts separated by 2-min intervals for cooling. Sometimes these cell sonicates were centrifuged at 10000 rev./min in a Sorvall SS-34 rotor at 2°C for 10 min, to separate into supernatant and pellet fractions for assay. Pellets were resuspended in the same volume of 10 mM HEPES pH 8.0.

(d) Immunoblot analysis of expressed proteins

Samples from bacterial cultures were fractionated on SDS–polyacrylamide gels (10%) and these gels were analysed by immunoblot analysis (Burnette, 1981; Towbin et al., 1979), using nitrocellulose BA85 sheets. To visualize CPMV sequences, the nitrocellulose sheets were treated sequentially with rabbit anti-24-kDa serum or preadsorbed anti-170-kDa serum and then with [^{125}I]protein A. Anti-170-kDa serum was pretreated with a sonicate of *E. coli* cells to eliminate host-reactive species. All antisera were mixed at appropriate dilutions in RIA buffer (Zabel et al., 1982). Some nitrocellulose sheets were developed by an alternative detection system to observe immunoreactive proteins. Rather than [^{125}I]protein A, the antirabbit IgG-alkaline phosphatase conjugate from Promega Biotec was used (Blake et al., 1984).

(e) Polymerase assays

The poly(A)-oligo(U) polymerase assay system was used, basically as initially described by Flanegan and Baltimore (1977). Reaction mixtures (50 μl) contained 50 mM HEPES pH 8.0/500 μM ATP or a mixture of 500 μM ATP, GTP, and CTP/12 μM UTP/3 mM Mg · acetate/60 μM $ZnCl_2$; 3 μg/ml of actinomycin D; 1 μg poly(A); 0.34 μg oligo(U), and [^{3}H]UTP (2×10^6 cpm/assay). Usually 10 μl of extract was used for assay at 30°C and aliquots were removed at zero time and after 30 min for acid-precipitable material. Each sample was also assayed for primer dependence and in the absence of template and primer. Precipitates were collected on Whatman GF/C filter discs and discs were counted in Insta-Fluor (Packard) scintillation fluid.

RESULTS AND DISCUSSION

(a) Immunoblot analysis of CPMV products by anti-24-kDa serum

Various CPMV constructions were made using full-length B-cDNA clones, from which infectious transcripts were obtained, by insertion of specific sequences into expression plasmids to provide a means for expression of 110-kDa (protease plus polymerase) and 87-kDa (polymerase) proteins (Fig. 2). It was presumed that either the generated 24-kDa protease or some fusion product containing 24-kDa sequences would be active in the host cells to generate these desired products from primary translation products. Initial observations indicated that the various plasmids induced different amounts of 110-kDa and 87-kDa protein; this prompted our attempts to maximize their expression by examination of several plasmid constructions. Since experience has shown that cleavage between the 24-kDa and 87-kDa protein sequences was very efficient in these expression systems, a mutant site was inserted into each construction to prevent this cleavage event and, hopefully, force expression of increased levels of 110-kDa protein, the putative active polymerase for CPMV (Dorssers et al., 1984). Fig. 3 illustrates the presumed polypeptide products that would be generated upon expression from these plasmids in *E. coli* cells. Thus, pEXC-110 would be expected to generate both the 110-kDa and 87-kDa proteins whereas its mutant form could only generate 110-kDa product (Fig. 3A). Since both of these constructions initiate with the N-terminal methionine of

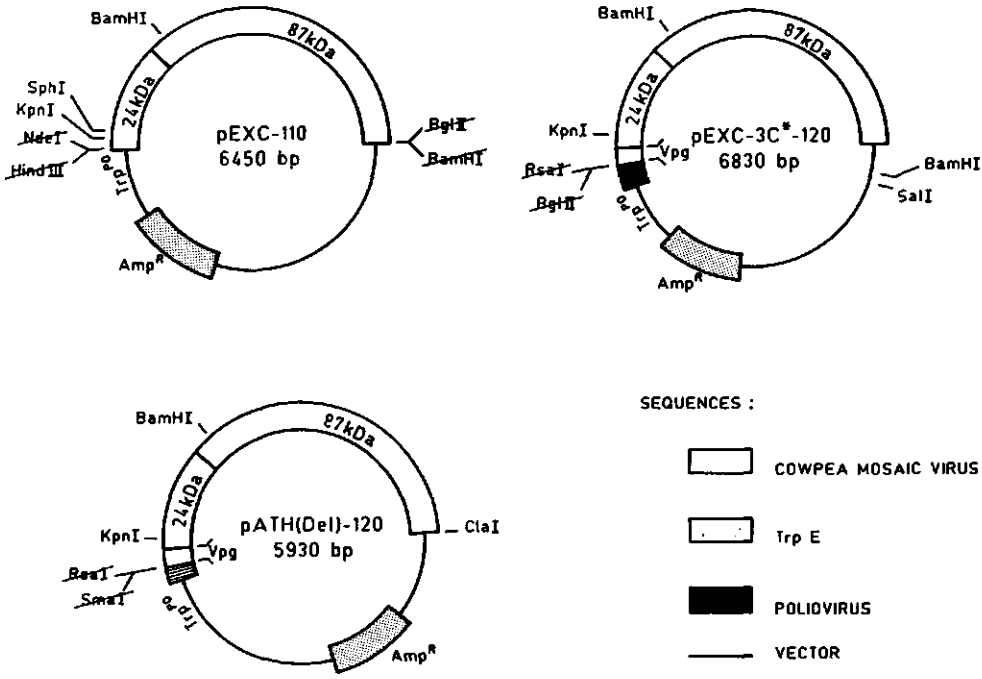

Fig. 2. Plasmids constructed for the synthesis of CPMV sequences. Plasmid construction is described in MATERIALS AND METHODS, section b. Synthesis of CPMV sequences is under control of a tryptophan promoter either to form fusion proteins (pEXC-3C*-120 or pATH(Del)-120) or a polyprotein with CPMV sequences only (pEXC-110). Termination signals are provided by the natural termination codes in the 87-kDa gene. Growth of these plasmids in *E. coli* is under Ap selection. Restriction sites for exchange of gene fragments, mentioned in section b, are indicated. The 3C* in pEXC-3C*-120 refers to a truncated portion of the poliovirus 3C protease gene.

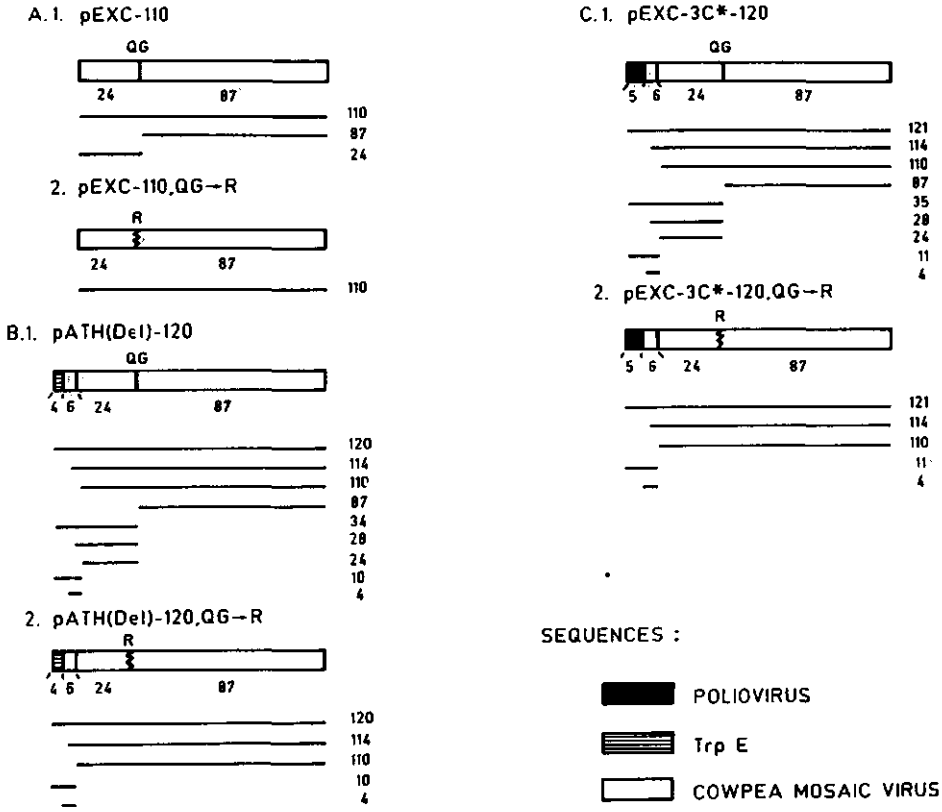

Fig. 3. Pertinent gene segments in expression plasmids and their expected translation-processing products. Bars indicate regions of plasmids containing CPMV sequences and lines depict both primary and processed translation products from the gene segments. Protein sizes given in kDa. QG → R denotes plasmids which have acquired a mutation at the junction of the 24-kDa protease/87-kDa polymerase genes by mutating the sequence coding for the glutamine/glycine cleavage site into a single arginine codon, described in MATERIALS AND METHODS, section b.

the 24-kDa protease (Wellink et al., 1986), the translation products do not contain extra amino acids. The primary translation products of pATH(Del)-120 and its mutant included an N-terminal *trpE* sequence of 17 aa fused to CPMV sequences starting 16 aa upstream from the VPg code (Fig. 3B). The plasmid pEXC-3C*-120 initiates with expression of the N-terminal 54 aa of poliovirus 3C protease fused directly to CPMV sequences, again originating 16 aa upstream from the VPg code (Fig. 3C). All constructions utilized the natural stop signals at the end of the 87-kDa cistron.

Expression from the plasmid constructions of Fig. 2 is shown in Fig. 4, in the presence or absence of the inducer IAA. Plasmid pEXC-110 and its mutant, QG → R, gave the best production of the 110-kDa protein (Fig. 4, lanes 2–5). Also a strongly reactive 24-kDa protease and a less pronounced 35-kDa band were seen for the wt construct (lane 2) and an alternate cleavage product, 30-kDa (lane 4), for the mutant. Good induction of expression from the wt plasmid was seen (lanes 2 and 3), but induction was much less pronounced for the mutant (lanes 4 and 5). Additionally, both the wt and mutant plasmids showed another immunoreactive species of about 95-kDa which is distinct from 87-kDa poly-

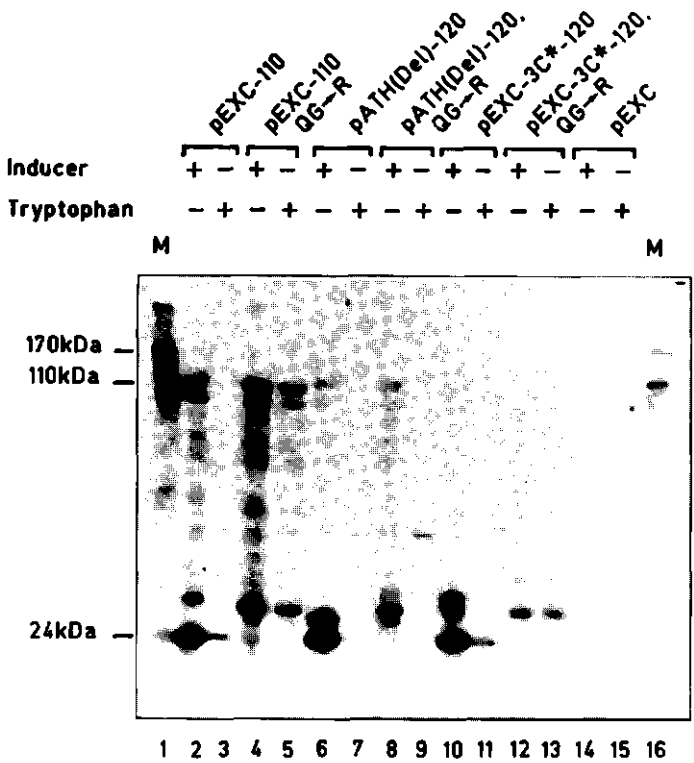

Fig. 4. Immunoblot analysis of proteins synthesized in *E. coli* containing plasmids depicted in Fig. 2 and immunoreactive with anti-24-kDa serum. Overnight cultures of bacteria, grown in M9 medium, were diluted ten-fold into fresh M9 medium and grown at 37°C for 60 min. Either inducer (5 μg IAA/ml) or tryptophan (40 μg/ml) was added and growth continued at 37°C for an other 120 min. Cells were collected, washed, and boiled in sample buffer for 5 min. Total protein was fractionated in a 10% polyacrylamide–0.1% SDS gel. Western immunoblot analysis was performed, using anti-24-kDa serum for the primary antibody and [^{125}I]protein A as the secondary probe. The appropriate cell lysates are indicated at the top of the figure; pEXC refers to an expression plasmid which has poliovirus 3C protease insertion only, M denotes lanes containing extracts from CPMV-infected cowpea leaves and illustrate the migration of 170-kDa, 110-kDa, and 24-kDa CPMV proteins.

merase. This protein may result from an internal initiation site at nt 3210, as described by Garcia et al. (1987).

Plasmid pATH(Del)-120 and its mutant displayed detectable 110-kDa protein only after induction (Fig. 4, lanes 6–9). Even more pronounced was the formation of 24-kDa protease and an alternate cleavage product, 28 kDa (lane 6), from the wt construct and alternate cleavage products, 28 kDa and 30 kDa, from the mutant.

Plasmid pEXC-3C*-120 displayed a barely detectable 110-kDa protein only after induction (Fig. 4, lanes 10 and 11). This construct also showed a prominent 24-kDa protease and a doublet of 30–35 kDa. The mutant (lanes 12 and 13) only showed a less distinct band at about 28 kDa.

All of these immunoreactive species were absent in a plasmid without CPMV sequences, pEXC (Fig. 4, lanes 14 and 15). Thus, the species observed in the other constructions were attributable to expression of CPMV sequences. A comparable accumulation of 110-kDa protein was observed from both mutants and wt constructions. A possible explanation for this observation could be a restricted production of

110-kDa protein due to (1) absolute depletion of tryptophan levels in the cell during induction and (2) the fact that only certain levels of the 110-kDa protein were tolerated before it became toxic to the cell.

The best induction was observed when overnight growth of *E. coli* harboring these plasmids was in M9 medium followed by dilution and induction in M9 medium. The induction was uniformly more pronounced with the wt constructs compared to the mutant (QG → R) constructs. Additional growth regimens included overnight LB medium diluted into LB medium for induction, overnight LB medium diluted into M9 medium, and overnight LB medium and overnight M9 medium, alone (not shown). A further point of interest is that the mutant 110-kDa protein, with only a single aa deletion and an aa change (arginine) has an electrophoretic mobility distinctly higher than its wt counterpart (Fig. 4, lanes 2–9).

(b) Immunoblot analysis of CPMV products by anti-170-kDa serum

Immunoblotting a similar 0.1% SDS–10% polyacrylamide gel, which fractionated synthesized proteins, with anti-170-kDa serum gave the results shown in Fig. 5. The advantage of this blot is that it detects 87-kDa protein (polymerase) as well as the 110-kDa and 24-kDa (weak immunoreactivity) proteins. Both the wt and mutant pEXC-110 expressed 110-kDa protein, but 87-kDa protein and 24-kDa protease were only detected in wt pEXC-110 (lanes 2–5). This was expected as 24-kDa protease cannot

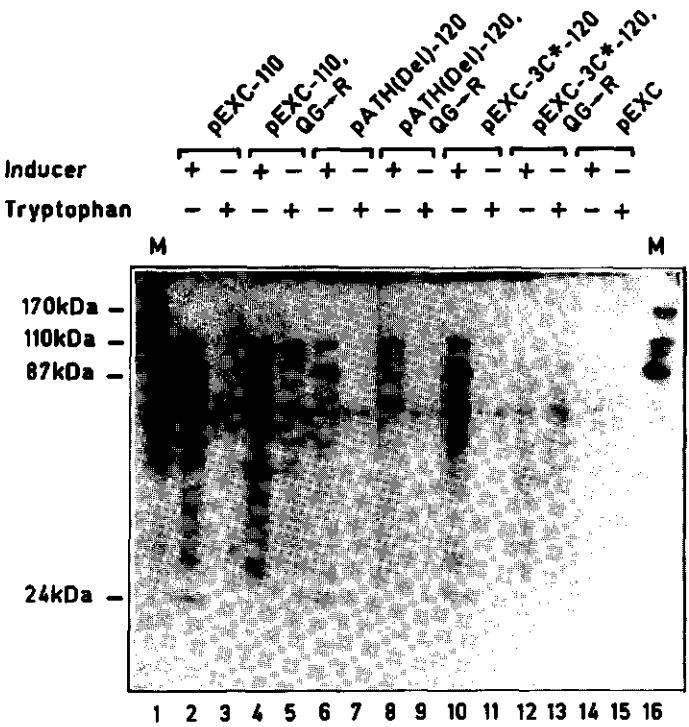

Fig. 5. Immunoblot analysis of proteins synthesized in *E. coli* containing plasmids depicted in Fig. 2 and immunoreactive with anti-170-kDa serum. Bacterial growth conditions, samples and gel are identical to those described in Fig. 4. Western immunoblot analysis was performed, using anti-170 kDa serum for the primary antibody and [^{125}I]protein A as the secondary probe. Cell lysates used are indicated at the top of the figure. Markers similar to those in Fig. 4 are indicated on the right.

cleave after elimination of the glutamine/glycine cleavage site. Induction was good, but not as pronounced with pEXC-110, QG → R. Plasmid pATH(Del)-120 and its QG → R mutant gave similar results but expression from these plasmids was lower (lanes 6–9). Induction of the wt and mutant pATH(Del)-120 was clearly demonstrated. Expression from pEXC-3C*-120 made some 110-kDa protein but most of it was rapidly cleaved to 87-kDa polymerase (lane 10). This situation was distinctly different from expression of pEXC-110 (lane 2). Expression from pEXC-3C*-120, QG → R (lanes 12 and 13) resulted neither in detectable 87-kDa nor in 110-kDa protein. Expression of pEXC alone was also examined for immunoreactive species (Fig. 5, lanes 14 and 15) and detectable species were absent, confirming that the species observed in other constructions were due to expression of CPMV sequences.

Conclusions based on the data from the immunoblots were that production of 110-kDa protein, relative to the production of 87-kDa protein, was most efficient from pEXC-110 and absolute production of 87-kDa protein was equally efficient from pEXC-110 and pEXC-3C*-120. Both of these plasmids gave very efficient cleavage to a 24-kDa protease. Thus, favorable expression systems have been demonstrated for production of substantial amounts of proteins to study their role in replication of CPMV RNA. Furthermore, the 24-kDa protease, or a fusion form containing 24-kDa sequences, was active in *E. coli* in recognition of the natural cleavage sites of CPMV precursor proteins.

(c) Cellular distribution of synthesized CPMV proteins

It was of immediate interest whether the 110-kDa and 87-kDa proteins synthesized in *E. coli* cells resided in the soluble or insoluble portions of crude cell lysates. This would give a clear indication of the course of purification of these proteins that could be followed. Thus, crude sonicates of induced cells were centrifuged at 10000 × *g* for 10 min to separate the lysate into supernatant and pellet fractions. These samples were fractionated in an SDS–polyacrylamide gel and immunoblotted anti-170-kDa serum to examine the partition of 110-kDa and 87-kDa species (Fig. 6). With pEXC-110, more 110-kDa and 87-kDa proteins were found in the pellet than in the supernatant (lanes 3 and 4). The plasmid pEXC-3C*-120 accumulated very little 110-kDa protein (lanes 8 to 10; top band is the uncleaved fusion protein), but efficiently cleaved a sizable portion of the precursor to 87-kDa protein (mostly in the pellet). Plasmid pEXC-3D showed no CPMV-reactive species, as expected (lanes 11 to 13). Expression of these plasmids led to considerable breakdown of immunoreactive protein and most of these products accumulated in the pellet fractions (lanes 4, 7 and 10).

(d) Synthesized enzymatic activity

The mixtures of synthesized CPMV proteins in crude sonicates, from cells harboring the plasmids described above, were examined for RNA polymerase activity in a poly(A)-oligo(U) assay system. Supernatant and pellet fractions derived from the crude sonicates were also assayed. The expression systems examined were confined to cultures grown overnight in LB medium or M9 medium and then diluted to M9 medium with inducer. Based on silver-stained proteins in an SDS–polyacrylamide gel, identical amounts of the specific CPMV proteins and poliovirus polymerase were used in the RNA polymerase assays.

Under conditions where poliovirus RNA polymerase activity was clearly demonstrable, i.e., expression of polymerase from pEXC-3D (Rothstein et al., 1988), no observable CPMV polymerase activity, above control levels, was observed (Table I). Thus, pEXC-3D showed an activity dependent upon the presence of exogenous template and primer, but still exhibited a low-level activity with template alone. On the other hand, those plasmids which synthesized immunoreactive 110-kDa and 87-kDa proteins (pEXC-110 and pEXC-3C*-120) showed no activity above the level of pEXC-3D in the presence of exogenous template alone. In fact, when assays were done in the presence of exogenous template and primer, even lower levels of this activity were observed. As further control and to ensure that cells bearing CPMV sequences were not expressing a terminal transferase activity, assays were performed on extracts from cells expressing no CPMV sequences (pEXC) or from cells bearing plasmids containing an insertion of a 141-bp segment of the

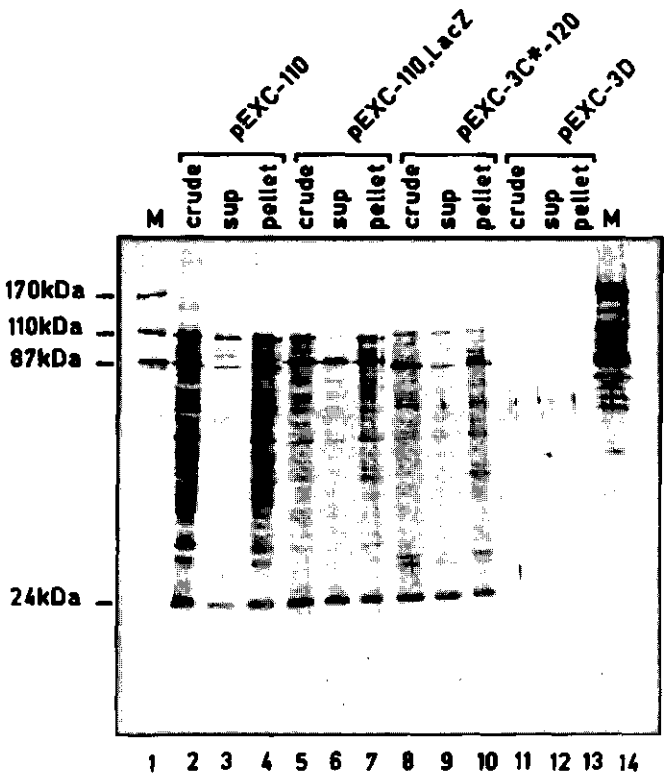

Fig. 6. Distribution of proteins synthesized in *E. coli* containing plasmids depicted in Fig. 2 and immunoreactive with anti-170-kDa serum. Bacterial growth conditions were as described in Fig. 4 except dilution of overnight culture was twelve-fold and only growth in the presence of inducer was examined, growth with inducer lasted 3 h. Crude cell lysates were obtained by sonication. Aliquots of these crude lysates were fractionated into supernatant and pellet fractions (see MATERIALS AND METHODS, section c). All samples were boiled in sample buffer (comparable cell equivalents) and fractionated as in Fig. 4. Western immunoblot analysis was performed, using anti-170-kDa serum for the primary antibody and anti-rabbit alkaline phosphatase conjugate for the secondary antibody detection system. The appropriate cell fractions are indicated at the top of the figure. M denotes protein markers identical to those used in Fig. 4.

lacZ gene in the CPMV 87-kDa coding region (pEXC-110, *lacZ*). In the latter case, retention of the reading frame in CPMV sequences downstream from the insertion was confirmed by immunoblot analysis (Fig. 6, lanes 5–7); modified 110-kDa and 87-kDa proteins were observed which were slightly larger than their wt counterparts (Fig. 6, lanes 2–4). Both of these control extracts exhibited a similar low level of activity (Table I). Thus, we concluded that this low-level activity, particularly that found with exogenous template alone (terminal transferase activity), was not due to synthesis of CPMV sequences. Furthermore, under conditions where extracts of pEXC-3D were active in poliovirus polymerizing activity, addition of extracts which produced CPMV sequences had no dramatic inhibitory effect on poliovirus activity (not shown). Thus extracts derived from cells producing CPMV sequences did not contain an inhibitor of polymerase activity. No detectable activity of any kind was found in the pellet fraction (Table I). This observation has been depicted for pEXC-110, but it is also true for any sonicate.

Some observations should be reported with

TABLE I

Poly(A)-oligo(U) assay of *Escherichia coli* sonicates

Extract[a]	cpm [^{3}H]UMP incorporated[b]		
	Template + primer	Template alone	No template or primer
pEXC-3D	46500	6300	3900
pEXC-110	4800	6900	2550
pEXC-3C*-120	5200	5850	2950
pEXC	8000	8400	6400
pEXC-110, *lacZ*	3800	5150	1600
pEXC-110, crude sonicate	3700	5700	2500
pEXC-110, sup	5900	5900	2100
pEXC-110, pellet	400	300	300

[a] Synthesis was in M9 medium in the presence of inducer. Lysates were prepared from *E. coli* JM109, containing the respective plasmids by sonication and supernatant fractions obtained by centrifugation as described in MATERIALS AND METHODS, section c. Pellet fractions were resuspended in a volume of buffer equal to that of the supernatant.

[b] Assays were performed as described in MATERIALS AND METHODS, section e.

respect to the assay system and detectable activity. The assay is extremely sensitive to variations in energy source, Mg^{2+} levels, NH_4^+ supplements, the presence of spermidine, and actinomycin D levels (not shown). Often a dramatic stimulation of a host activity was observed, presumably due to a lower 'effective' Mg^{2+} concentration. Indications are that Mg^{2+} in the crude sonicates may effectively contribute to apparent polymerase activity. Assays were routinely performed with (*i*) template + primer, (*ii*) template alone, or (*iii*) neither template nor primer. We suggest that meaningful poly(A)-oligo(U) assays with *E. coli* extracts require these assays to avoid misleading results.

(e) Conclusions

A 110-kDa protein (protease + polymerase sequences of CPMV) was synthesized particularly well from plasmids pEXC-110 and pEXC-110, QG → R. The best synthesis of 87-kDa protein (polymerase) was observed from pEXC-3C*-120; this protein was found predominantly in the pellet fraction of cell lysates. Yet no observable polymerase activity was found in lysates from cells harboring any plasmid constructs. Interestingly, poliovirus, which has a genomic organization similar to CPMV, has been shown to possess RNA polymerase activity upon synthesis of polymerase sequences (52-kDa) in an *E. coli* expression system (Rothstein et al., 1988; Morrow et al., 1987). On the other hand, synthesis of similar sequences from CPMV (87-kDa) in similar expression systems did not yield a comparable activity. Hence the 87-kDa polypeptide from CPMV did not appear sufficient for polymerase activity. The fact that we have not been able to assign an activity to the 110-kDa or 87-Kda proteins, specifically, still does not preclude that this observation may be due to: (1) incorrect protein folding during synthesis in *E. coli* (2) lack of glycosylation or other post-translational modifications; (3) a need for some supplementary protein for activity (either viral or host protein); (4) a need for a membranous environment; or (5) a need for a polyprotein from which the polymerase is simultaneously cleaved and incorporated into an active replication complex. In addition one may argue that the assay itself was not appropriate in that CPMV polymerase does not have a poly(A)-oligo(U) polymerase activity per se but may require a different template and/or primer.

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REFERENCES

Blake, M.S., Johnston, K.H., Russell-Jones, G.J. and Gotschlich, E.C.: A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. 136 (1984) 175–179.

Burnette, W.M.: 'Western blotting': electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radio-iodinated protein A. Anal. Biochem. 112 (1981) 195–203.

Daubert, S.D., Bruening, G. and Najarian, R.C.: Protein bound to the genome RNAs of cowpea mosaic virus. Eur. J. Biochem. 92 (1978) 45–51.

Dorssers, L., Van der Meer, J., Van Kammen, A. and Zabel, P.: The cowpea mosaic virus RNA replication complex and the host-encoded RNA-dependent RNA polymerase-template complex are functionally different. Virology 125 (1983) 155–174.

Dorssers, L., Van der Krol, S., Van der Meer, J., Van Kammen, A. and Zabel, P.: Purification of cowpea mosaic virus RNA replication complex: identification of a virus-encoded 110000 dalton polypeptide respousible for RNA chain elongation. Proc. Natl. Acad. Sci. USA 81 (1984) 1951–1955.

Eggen, R. and Van Kammen, A.: RNA replication in comoviruses. In Domingo, E., Holland, J.J. and Ahlquist, P. (Eds.), RNA Genetics, Vol. I, RNA-Directed Virus Replication. CRC Press, Boca Raton, FL, 1988, pp. 49–69.

El Manna, M.M. and Bruening, G.: Polyadenylate sequences in the ribonucleic acids of cowpea mosaic virus. Virology 56 (1973) 198–206.

Flanegan, J.B. and Baltimore, D.: A poliovirus-specific primer-dependent RNA polymerase able to copy poly(A). Proc. Natl. Acad. Sci. USA 74 (1977) 3677–3680.

Franssen, H., Leunisen, J., Goldbach, R., Lomonossoff, G. and Zimmern, D.: Homologous sequences in non-structural proteins from cowpea mosaic virus and picornaviruses. EMBO J. 3 (1984) 855–861.

Garcia, J.A., Schrijvers, L., Tan, A., Vos, P., Wellink, J. and Goldbach, R.: Proteolytic activity of the cowpea mosaic virus encoded 24K protein synthesized in *Escherichia coli*. Virology 159 (1987) 67–75.

Goldbach, R. and Wellink, J.: Evolution of plus-strand RNA viruses. Intervirology 29 (1988) 260–267.

Houwing, C.J. and Jaspars, E.M.J.: Coat protein blocks the in vitro transcription of the virion RNAs of alfalfa mosaic virus. FEBS Lett. 209 (1986) 284–288.

Kunkel, T.A.: Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82 (1985) 488–492.

Lomonossoff, G.P. and Shanks, M.: The nucleotide sequence of cowpea mosaic virus B RNA. EMBO J. 2 (1983) 2253–2258.

Maniatis, T., Fritsch, E.F. and Sambrook, T.: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

Miller, W.A. and Hall, T.C.: Use of micrococcal nuclease in the purification of highly template-dependent RNA-dependent RNA polymerase from brome mosaic virus-infected barley. Virology 125 (1983) 236–241.

Morrow, C.D., Warren, B. and Lentz, M.R.: Expression of enzymatically active poliovirus RNA-dependent RNA polymerase in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 84 (1987) 6050–6054.

Mouches, C., Bove, C. and Bove, J.M.: Turnip yellow mosaic virus-RNA replicase: partial purification of the enzyme from the solubilized enzyme-template complex. Virology 58 (1974) 409–423.

Quadt, R., Verbeek, H.J.M. and Jaspars, E.M.J.: Involvement of a non-structural protein in the RNA synthesis of brome mosaic virus. Virology 165 (1988) 256–261.

Richards, O.C., Ivanoff, L.A., Bienkowska-Szewczyk, K., Butt, B., Petteway, S.R., Rothstein, M.A. and Ehrenfeld, E.: Formation of poliovirus RNA polymerase 3D in *Escherichia coli* by cleavage of fusion proteins expressed from cloned viral cDNA. Virology 161 (1987) 348–356.

Rothstein, M.A., Richards, O.C., Amin, C. and Ehrenfeld, E.: Enzymatic activity of poliovirus RNA polymerase synthesized in *Escherichia coli* from viral cDNA. Virology 164 (1988) 301–308.

Semler, B.L., Kuhn, R.J. and Wimmer, E.: Replication of the poliovirus genome. In Domingo, E., Holland, J.J. and Ahlquist, P. (Eds.), RNA Genetics, Vol. I, RNA-Directed Virus Replication. CRC Press, Boca Raton, FL, 1988, pp. 23–48.

Stanley, J., Rottier, P., Davies, J.W., Zabel, P. and Van Kammen, A.: A protein linked to the 5′ termini of both RNA components of the cowpea mosaic virus genome. Nucleic Acids Res. 5 (1978) 4505–4522.

Steele, K.P. and Frist, R.H.: Characterization of the 3′ termini of the RNAs of cowpea mosaic virus. J. Virol. 26 (1978) 243–248.

Towbin, H., Staehelin, T. and Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.

Van der Meer, J. Dorssers, L., Van Kammen, A. and Zabel, P.: The RNA-dependent RNA polymerase of cowpea is not involved in cowpea mosaic virus RNA replication: immunological evidence. Virology 132 (1984) 413–425.

Van Dyke, T.A. and Flanegan, J.B.: Identification of poliovirus polypeptide P63 as a soluble RNA polymerase. J. Virol. 35 (1980) 732–740.

Vos, P., Jaegle, M., Wellink, J., Verver, J., Eggen, R., Van Kammen, A. and Goldbach, R.: Infectious RNA transcripts derived from full-length DNA copies of the genomic RNAs of cowpea mosaic virus. Virology 165 (1988a) 33–41.

Vos, P., Verver, J., Jaegle, M., Wellink, J., van Kammen, A. and Goldbach, R.: Two viral proteins involved in the proteolytic processing of the cowpea mosaic virus polyproteins. Nucleic Acids Res. 16 (1988b) 1967–1985.

Wellink, J., Rezelman, G., Goldbach, R. and Beyreuther, K.: Determination of the proteolytic processing role in the polyprotein encoded by the bottom-component RNA of cowpea mosaic virus. J. Virol. 59 (1986) 50–58.

Zabel, P., Dorssers, L., Wernars, K. and Van Kammen, A.: Terminal uridylyl transferase of *Vigna unguiculata*: purification and characterization of an enzyme catalyzing the addition of a single UMP residue to the 3′ end of an RNA primer. Nucleic Acids Res. 9 (1981) 2433–2453.

Zabel, P., Moerman, M., Van Straaten, F., Goldbach, R. and Van Kammen, A.: Antibodies against the genome-linked protein VPg of cowpea mosaic virus recognize a 60000-dalton precursor polypeptide. J. Virol. 41 (1982) 1083–1088.

CHAPTER 5

Improvements of the infectivity of *in vitro* transcripts from cloned cowpea mosaic virus cDNA: impact of terminal nucleotide sequences

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Submitted

SUMMARY

Full length DNA copies of both B- and M-RNA of cowpea mosaic virus (CPMV) were constructed behind a T7 promoter. By trimming the promoter sequence B- and M-RNA like transcripts with varying numbers of additional non-viral sequences at the 5' end were obtained upon transcription with T7 RNA polymerase. The infectivity of the transcripts in cowpea protoplasts was greatly affected by only a few extra non-viral nucleotides at the 5' end. The addition of about four hundred non-viral nucleotides at the 3' end did not have any effect.

Optimal infection was obtained when transcripts had only 1 extra G-residue at the 5'-end. Using such transcripts, in 40% of the cowpea protoplasts a replication and expression of B-RNA like transcripts was observed and in 10% of the protoplasts both B- and M-RNA like transcripts multiplied. Moreover, cowpea plants could also be infected with these transcripts. Sequence analysis showed that the 5' and 3' termini of the transcripts were completely restored during their replication in plants, including poly(A) tails of variable length.

Swopping experiments have been used to identify an influential point mutation in the coding region for the viral polymerase of a non-infectious B-transcript. This experiment demonstrates the potential of the optimized infection system for future analysis of virus encoded functions.

INTRODUCTION

Cowpea mosaic virus (CPMV), type member of the comoviruses, has a genome consisting of two plus sense RNAs, denoted B- and M-RNA. B-RNA contains the information for viral RNA replication, whereas M-RNA encodes two capsid proteins and one or more functions required for cell-to-cell transport in plants (for a review see Goldbach and van Kammen, 1985).

Both genomic RNAs have a small protein (VPg) covalently linked to their 5' ends and a poly A tail of variable length, at their 3' end (Jaegle et al., 1987; Ahlquist et al., 1979). The genetic information of the RNAs is expressed by the production of polyproteins, from which the mature proteins are generated by proteolytic processing (Wellink et al., 1986, 1987, 1987[a]).

To some of the viral proteins, both intermediate and ultimate cleavage products, specific functions have been assigned, based for the greater part on detailed in vitro studies of the expression strategy (Verver et al., 1987; Vos et al., 1988[a]) and the viral RNA replication (for review see Eggen and Van Kammen, 1988; Figure 1). However, in vivo studies of the molecular biology of the virus have been hampered, due to the lacking of well defined mutants of CPMV.

Recently full-length DNA copies of B- and M-RNA have been constructed and cloned downstream of a phage T7 promoter. The cloned DNA copies can be transcribed in vitro into RNA molecules which are infectious upon inoculation of cowpea protoplasts (Vos et al., 1988). The infectivity of such transcripts was very low and did neither allow direct infection of cowpea plants nor was the system suitable to study the effects of mutations in the RNAs. These transcripts synthesized in vitro were devoid of VPg but had, instead, two additional guanosine residues at the 5' end and four to five non-viral nucleotides at the 3' end. As it is known from other systems that non-viral extensions at the transcript termini, in particular at the 5' ends, may strongly influence the infectivity (Dawson et al., 1986; van der Werf et al., 1986; Janda et al., 1987; Rice et al., 1987; Shaklee et al., 1988; Ziegler-Graff et al., 1988), we have now examined whether the infectivity of the RNA transcripts is improved by removal of the additional

nucleotides from 5' end by trimming the T7 promoter sequence.

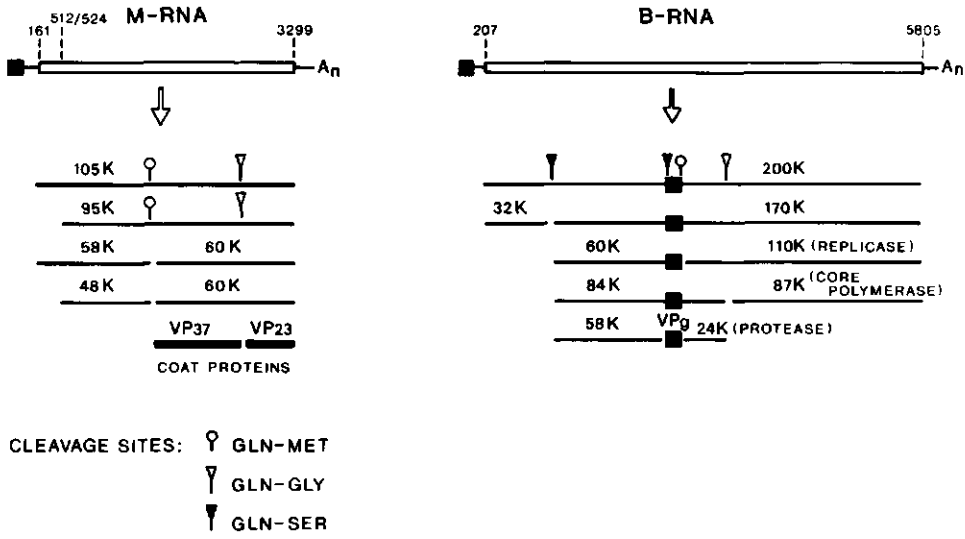

Figure 1:
Expression and genetic organization of the RNAs of CPMV. Both RNAs contain single open reading frames represented by the open bar on which the positions of the start and stop codons are shown. Vpg is indicated by a black box, other proteins by single lines and the cleavage sites by o, glutamine-methionine, ▽, glutamine-glycine and ▼, glutamine-serine.

Here we report that using transcripts with only 1 extra G at their 5' ends and an optimized inoculation procedure, expression of B-RNA transcripts is observed in 40% of the inoculated protoplasts while expression of both B- and M-RNA transcripts is detected in 10% of the inoculated protoplasts. In addition these transcripts could be used for direct infection of cowpea plants which allowed us to analyse the progeny virus derived from B- and M-RNA transcripts after multiplication in plants.
The improved system is suitable to analyse the effects of specific mutations on the multiplication mechanism of CPMV in vivo.

MATERIALS AND METHODS

DNA techniques

Plasmid DNA was isolated by the method of Birnboim and Doly (1979). All enzymes were purchased from Gibco BRL or Pharmacia and used as described by the manufacturers. DNA fragments were isolated from agarose gels by freeze-squeezing (Tautz and Renz, 1983) or by elution from polyacrylamide gels (Maxam and Gilbert, 1977).
Standard techniques were used to join DNA fragments and to transform the DNA into competent Escherichia coli HB101, JM109 or DH5α cells (Maniatis et al., 1982; Hanahan, D., 1985; Yanisch-Perron et al., 1985). Oligodeoxy-nucleotides were synthesized using β-cyanoethyl phosphoramidites and a Cyclone DNA synthesizer (Bioresearch, Inc.).
Oligonucleotide-directed mutagenesis was performed essentially as described by Vos et al. (1988) using the procedure for the efficient selection of mutated DNA developed by Kunkel et al. (1985).
Linkers were inserted as described by Lathe et al. (1984).
Recombinant DNA molecules were screened by mapping with restriction enzymes or by nucleotide sequence analysis, using single-stranded or double-stranded DNA (Sanger et al., 1977; Korneluk et al., 1985).

Construction of modified cDNA clones (see also Figure 2)

The full-length cDNA clones of B-RNA, pTB3 and pTB108 and the full-length cDNA of M-RNA, pTM207 described previously (Verver et al., 1987; Vos et al., 1988) were used as starting material for the construction of modifications of the 5' end 3' terminal structures of the cDNAs and for exchanging of cDNA fragments.
pTB3 has 35 nucleotides between the T7 transcription initiation site and the 5' end of the viral cDNA. In pTB108 and pTM207 there are 2 G-residues between the T7 transcription initiation site and the 5' end of the BcDNA and M-cDNA respectively.

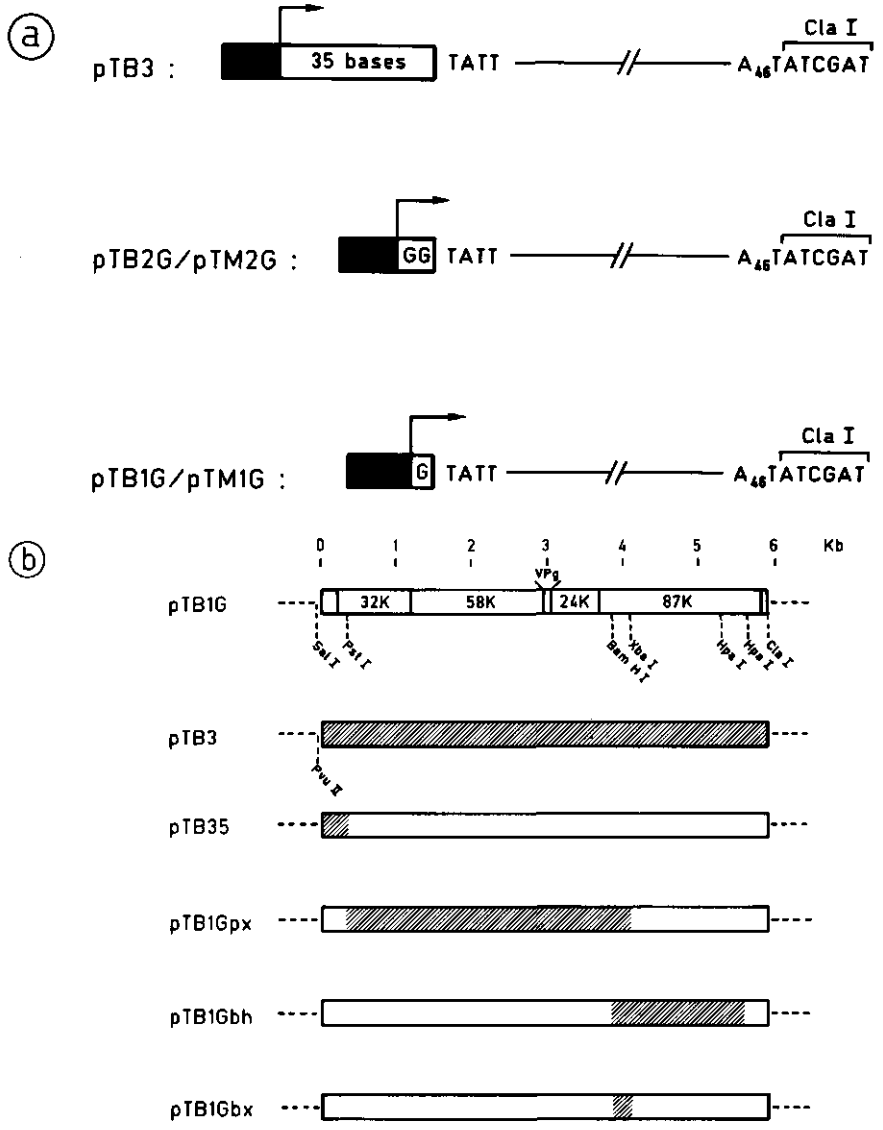

Figure 2:
Schematic representation of the B- and McDNA clones (a) Organization surrounding the transcription initiation sites in plasmids pTB3, pTB2G/pTM2G and pTB1G/pTM1G. The cDNAs, with TATT at the 5' end and 46 A residues at the 3' end is indicated by an interrupted single line. The black box represents the T7 RNA polymerase promoter sequence along with the initiation and direction of transcription marked by an arrow. (b)Genomic organization: the cDNAs are indicated by open bars, plasmid sequences by dotted lines. The derivation of the plasmid sequence of each plasmid is shown by the same shading.

(a) pTB2G and pTM2G

In pTB108 and pTM207 a MluI-SalI-MluI linker (ACGCGTCGACGCGT) was inserted into a PvuII site (34 nucleotides upstream from the viral cDNAs), resulting in clones pTB2G and pTM2G, with a unique SalI site. Upon transcription of these plasmids RNAs are produced that have 2 extra G-residues at the 5' end.

(b) pTB35

This plasmid was constructed by exchanging the PstI-ClaI fragment (position 346 to the 3' end of the BcDNA) in clone pTB3 for the corresponding fragment of clone pTB2G. The nucleotide sequence between residues 0 to 346 of the BcDNAs in pTB3 and pTB1G, were checked and found to be identical (data not shown). Transcription of pTB35 results in RNA that has 35 extra nucleotides at the 5' end.

(c) pTB1G and pTB0G

These plasmids were constructed to produce B-RNA transcripts with only 1 or no extra nucleotide respectively at the 5' end. For the construction of pTB1G and pTB0G a 393 bp SalI-PstI fragment of pTB2G (nucleotides -44 to 346) was inserted into SalI and PstI digested M13 mp19 RF. Single-stranded recombinant phage DNA and an oligonucleotide with the sequence CTCACTATAGTATTAAAATC (for pTB1G) or CTCACTATATATTAAAATC (for pTB0G) were used for site-directed mutagenesis. The mutated fragments were subsequently transferred from the M13 clones to the plasmid pTB2G by exchanging the normal for the modified SalI-PstI fragments.

(d) pTM1G

For the construction of pTM1G the same oligonucleotide as described for pTB1G and recombinant M13mp19 DNA in which a 319 bp SalI-HindIII fragment of pTM2G (nucleotides -44 to 272) was cloned, were used. The mutation was introduced in pTM2G by the exchange of a 226 bp SalI-BglII fragment (nucleotides -44 to 189 of the McDNA) from the M13 clone, containing the mutated sequence, into plasmid pTM2G. Transcripts produced from pTM1G had 1 extra G residue at their 5' end.

(e) pTB1GBgl

The oligonucleotide with the sequence AAAAAAAGATCTACCTGCG was used to modify the ClaI site at the 3' end of the BcDNA into a BglII site by site directed mutagenesis, resulting in clone pTB1GBgl.

(f) pTB1GA0

pTB1GA0 was constructed by the introduction of a 440 bp BamHI-BglII fragment of plasmid pJII2, containing the assembly origin of the vulgare strain of tobacco mosaic virus (Sleat et al., 1986) into the BglII digested pTB1GBgl. (pJII2 was kindly provided by Dr. T.M.A. Wilson). pTB1GA0 transcripts were packaged in vitro into pseudo-vivion particles with coat proteins of the TMV vulgare strain (kindly provided by Dr. B. Verduin) as described by Rochon et al. (1986). In short, an oligomer preparation of coat proteins, freshly diluted to 1 mg/ml, was incubated with 40 µg/ml transcript RNA in 0.1 M sodium pyrophosphate, pH 7.3, 2mM EDTA at room temperature for 18 hr. Constituted rod-like particles were purified from the reaction mixture by centrifugation at 45.000 rpm in a 75 Ti rotor for 3 hr.

(g) pTB1Gpx and pTB1Gbh

These plasmids were derivatives of pTB1G in which a 3632 bp PstI-XhoI (position 346 to 3978 of the B cDNA) or a 1804 bp BamHI-HpaI fragment (position 3857 to 5661 of the B cDNA), respectively, were exchanged with the corresponding fragments of pTB3. In pTB1Gbx the BamHI-XhoI fragment in pTB1G (position 3857-3978) was replaced by the same fragment of pTB3.

In vitro transcription and translation

Recombinant plasmids were linearized with restriction enzymes followed by purification of the DNA by phenol extraction and ethanol precipitation. The resulting linear DNA templates were used for the production of run off transcripts. Reaction mixtures containing 40 mM Tris-HCl pH 8.0, 20 mM $MgCl_2$, 10 mM DDT, 100 µg/ml BSA, 1 mM of each four NTP's, 50 µg/ml template DNA and 1000 units/ml T7 RNA polymerase were incubated at 37°C for 30 minutes. For capping of transcripts the GTP concentration was lowered to 0.2 mM

while 2 mM m^7GpppG cap precursor was added. After transcription the DNA was degraded (Vos et al., 1984) and transcript recovered from the reaction mixture by precipitation overnight at 4°C in 2M LiCl.
To verify the intactness of the produced clones, the transcripts were translated in rabbit reticulocyte lysate, and the in vitro translation products analysed on 12,5 % SDS-polyacrylamide gels as previously described by Vos et al., 1988.

Inoculation of cowpea protoplasts and detection of viral RNA expression

Cowpea (Vigna unguiculata L.) mesophyll protoplasts were prepared from 10-day-old primary leaves. To 2.5 x 10^6 protoplasts in 150 µl 0.6 M mannitol, cooled on ice, 50 µg RNA transcript or 1 µg CPMV RNA in 25 µl ice-cold water was added, immediately followed by 0.5 ml ice-cold solution of 40% (w/v) poly-ethyleneglycol (MW 6000) and 5 mM calciumchloride. The suspension was gently mixed for 10 seconds and diluted with 4.5 ml of a 0.6 M mannitol solution containing 1 mM calcium-chloride, kept at room temperature. After incubation for 30 minutes at room temperature, the protoplasts were washed and incubated as described by Rezelman et al. (1980). Expression of in vitro transcripts or viral RNA in the inoculated protoplasts was determined by the indirect fluorescent antibody technique using antiserum raised against the B-RNA encoded 24K protein for protoplasts infected with alone B-RNA transcripts and antiserum raised against purified virus for protoplasts infected with a mixture of B- and M-RNA transcripts (Vos et al. 1988).

Inoculation of cowpea plants and purification of virus and viral RNA

Primary leaves of 10-day-old cowpea plants were inoculated with a mixture of 10 µg transcripts from B- and M-cDNA clones, 1 µg CPMV RNA or with a crude extract prepared from protoplasts inoculated with transcript RNA or CPMV and grown as described previously (Vos et al., 1988). Primary or secundary leaves were harvested at day 4 or 5 after inoculation and used directly for virus isolation. Viral RNA was extracted from CPMV as

described (Vos et al., 1988).

Analysis of transcripts and virion RNAs

The 5' termini of M-transcripts and M-RNA isolated from CPMV-RNA or transcript-RNA infected leaves were determined by the dideoxy chain-terminating method essentially as described by Johanningmeier et al. (1987) using 2 µg transcript RNA or 4 µg CPMV. A synthetic oligodeoxynucleotide primer that initiated cDNA synthesis at nucleotide 155 of the M-RNA was hybridized to the RNA.
The length of poly A tails of transcript and progeny RNA was determined using B-RNA isolated from agarose gel. One µg RNA was incubated with 5 units RNase T1 and 40 g RNase A in 0.3 M sodium-chloride, 0.03 M sodium-citrate for 30 minutes at 37°C, followed by a phenol extraction and an ethanol precipitation. The resulting polyadenylyl stretches were labelled at their 5' end with (γ-^{32}P)-ATP in the presence of T4 polynucleotide kinase and analyzed on a 6% sequencing gel (Maniatis et al., 1982).

Immunological methods

Immunoprecipitations of ^{35}S-labelled proteins from the cytoplasmic fractions of protoplast were performed as described by Wellink et al. (1986). Immunoblots of proteins from protoplasts or leave fractions, separated in 12.5% SDS-polyacrylamide gels were prepared and probed with antisera and ^{125}I-protein A as described by Zabel et al. (1982).

RESULTS

Optimalization of the protoplast inoculation procedure

Compared to the procedure previously used for the inoculation of cowpea protoplasts with RNA transcripts and viral RNA (Vos et al., 1988), the number of protoplasts, the amount of RNA and the volume of solutions added to the inoculation mixture were cut down by half. In addition, the protoplasts, the RNA and the poly-ethylene glycol solutions were all cooled on ice. These modifications increased the percentage of infected protoplasts five times i.e. now, 10% instead of 2% of the protoplasts became infected upon inoculation with capped B-transcripts having two extra G residues at the 5' end (Table I, pTB2Gcap). As an alternative for the inoculation procedure with poly ethylene-glycol, electroporation was tried. Under optimum conditions we obtained however neither higher infection levels nor could the concentration of RNA-transcripts or viral RNA be decreased to achieve the same maximum infection percentage as obtained with the poly-ethylene glycol method (data not shown).

Effect of 5' extensions on the infectivity of in vitro transcripts

Full-length cDNA copies of B- and M-RNA of CPMV were provided with a T7 RNA polymerase promoter so that different numbers of non-viral nucleotides were present between the transcription initiation site and the first nucleotide of the viral DNA copy (see Table 1). Upon runoff transcription of linearized plasmid DNA, transcripts were produced with either 35 nucleotides from the polylinker (pTB35), two G-residues (pTB2G, pTM2G), or only one extra G-residue (pTB1G, pTM1G) at their 5' end and 5 non-viral nucleotides at their 3' end. Except for the different 5' ends, the cDNA clones selected for the comparison of the infectivity of transcripts were identical. The efficiency of *in vitro* transcription was not influenced by the sequence alterations in the T7 transcriptional initiation region. The transcription efficiency was also not decreased when capped transcripts were produced

TABLE I. Infectivity of CPMV RNA and RNA transcripts.

VIRUS/CLONE	RNA OR TRANSCRIPT CHARACTERIZATION	% PROTOPLASTS PRODUCING	
		B-RNA ENCODED PROTEINS[a]	M-RNA ENCODED PROTEINS[b]
CPMV RNA	VPg-UAUU------A_{50-140}	20	20
pTB35	X_{35}-UAUU------A_{46}UAUCG	0.2	-
pTB2G	GG-UAUU------A_{46}UAUCG	5	-
pTB2G-cap	m^7GpppGG-UAUU------A_{46}UAUCG	10	-
pTB1G	G-UAUU------A_{46}UAUCG	25	-
pTB1G-cap	m^7GpppG-UAUU------A_{46}UAUCG	40	-
pTB1G-AO	G-UAUU------$A_{46}X_{440}$	25	-
pTB1G + pTM2G	GG-UAUU------A_{46}UAUCG	25	0.3
pTB1G + pTM1G	G-UAUU------A_{46}UAUCG	25	10

Detection of viral RNA expression in cowpea protoplasts by the indirect fluorescent antibody technique (Vos *et al.*, 1988). Antisera were used raised against the B-RNA encoded 24K protein (a) or the virus particle (b).

from pTB35 and pTB2G, but with clone pTB1G the yield of capped transcripts was 10% of that with clone pTB2G.

In addition, a cDNA clone (pTB0G) was designed to produce transcripts lacking any extra nucleotides at the 5' end. Removal of the residual G-residue from the transcription initiation site of T7 RNA polymerase (Dunn and Studier, 1983), appeared however, to block transcription completely and no transcripts could be recovered.

The effect of different non-viral extensions on the specific infectivity of the transcripts was analyzed in cowpea protoplasts (see Table I). Fifty µg of RNA transcripts from each of the constructs were used to inoculate 2.5 x 10^6 cowpea protoplasts. Fourty hours after inoculation the inoculated protoplasts were examined for the presence of virus-encoded proteins using the indirect immunofluorescent antibody technique. Antibodies directed against the B-RNA encoded 24K protein were used to detect the production of B-RNA (Vos *et al.*, 1988).

If protoplasts were inoculated with pTB35 transcripts, 0.2% of the protoplasts were infected. This percentage was increased to 5% when pTB2G-transcripts were used and even to 25% with pTB1G-transcripts. The percentage of infected protoplasts generally doubled when the transcripts were capped and reached a maximum of 40% with capped pTB1G-transcripts. Upon inoculation with a mixture of B- and M-RNA transcripts the number of protoplasts that became infected with M-RNA transcripts was determined by immunofluorescence using antibodies raised against CPMV particles. This number was much lower due to the dependence of M-RNA replication on B-RNA (Vos *et al.*, 1988). The strong effect of extra nucleotides at the 5' end of transcripts was again clear. Using M-RNA transcripts with only one extra G residue at the 5' end the infectivity was about 30 times higher than with 2 extra G's at the 5' end (pTM1G versus pTM2G, see Table I). The percentage of protoplasts in which infection with B-RNA could be detected with antibodies against the 24K protein was the same upon inoculation with a mixture of B-RNA and M-RNA transcripts as with B-RNA transcripts alone.

Effect of 3' extensions on the infectivity of in vitro transcripts

Having established that highest infectivity was obtained if only 1 extra G was present at the 5' end, clone pTB1G was used to determine the effect of 3' terminal extra nucleotides.
Two different full-length B-cDNA clones were constructed from which after linearization run off transcripts were produced with 4 (pTB1G) or 440 (pTB1GA0) extra non-viral nucleotides beyond the poly(A) tail. With both transcripts 25% of the protoplasts became infected in different experiments indicating that extra nucleotides at the 3' end have no deleterious effect on the infectivity of the transcripts.
The extra nucleotides at the 3' end of the transcripts produced from pTB1GA0 contained the assembly origin of TMV-RNA. Such recombinant RNA molecules can in principle be assembled *in vitro* using TMV coat proteins into ribonuclease-resistant rod-like particles consisting of B-RNA transcripts packaged in a TMV protein coat (Sleat *et al.*, 1986).
With low efficiency rod-like structures of the expected length could be constituted *in vitro* (data not shown). Only in 0.1 % of the protoplasts, inoculated with such pseudo virion particles, replication and expression of the B-transcripts was observed and this approach was therefore not further pursued.

Inoculation of cowpea plants and analysis of the progeny virus

In an earlier report on the infectivity of transcripts from full-length copies of CPMV RNA, transcripts with 2 additional G residues at their 5' end were not able to infect plants when inoculated directly on to the primary leaves of cowpea plants (Vos *et al.*, 1988). Using a mixture of B- and M-RNA transcripts with only one extra G at the 5' end as inoculum cowpea plants could now be infected and one week afte inoculation of the primary leaves of cowpea, both primary and secundary leaves showed the typical symptoms for infection with CPMV. The progeny virus was purified from the infected plants and the genomic RNAs were isolated from the viral particles to determinate the structure of the 5' end 3' terminal ends.

Figure 3:
Autoradiogram of a 6% acrylamide 7M urea thin sequence gel showing the poly(A) tail length distribution of B-RNA transcripts (lane 1), B-RNA isolated from transcript infected leaves (lane 2) or natural B-RNA (lane 3).

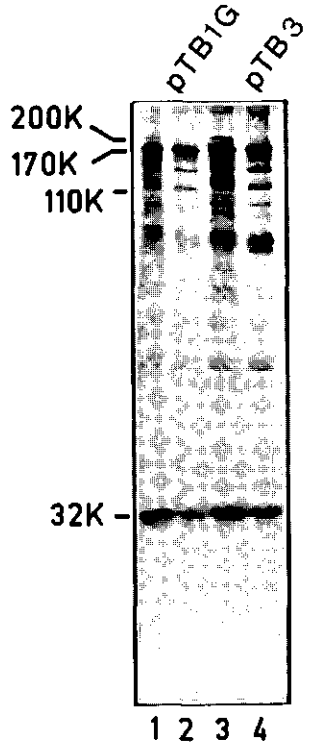

Figure 4:
Autoradiogram showing the in vitro translation products of pTB1G transcripts (lanes 1 and 2) and pTB3 (lanes 3 and 4). The translation products are shown after 1 hour (lanes 1 and 3) and 20 hours (lanes 2 and 4) incubation in reticulocyte lysate. To the left the positions of the relevant B-RNA encoded proteins are indicated. The proteins were analysed on a 10% SDS-polyacrylamide gel.

Direct RNA sequence analysis using an M-RNA specific primer showed that the 5' ends of M-RNA recovered from transcript- and virion RNA-infected leaves were identical (data not shown). However, the extra G at the 5' end of inoculum transcript RNA did not longer occur at he 5' end of the progeny RNA. Although the 5' end of the progeny RNA of the B-transcripts was not examined, it was expected that also in that case the extra G will not be found. Analysis of the 3' end showed that the poly A stretch as it occurred in B-RNA transcripts was elongated during replication in vivo (Fig. 3, lanes 1 and 2). The poly(A) length distribution was similar to the poly(A) tail of wildtype viral B-RNA (Fig. 3, lane 3).

Characterization of non-infectious pTB3 transcripts

The optimized infection system now allows of the in vivo analysis of specifically mutated transcripts. In a first attempt we tried to identify putative mutations in the previous described clone pTB3 (Verver et al., 1987), from which non-viable transcripts are synthesized, by swopping of fragments. If pTB3 transcripts with 35 non viral nucleotides at the 5' end, were translated in vitro in reticulocyte lysate, the B-RNA encoded translation product with a molecular weight of 170 kD migrated slightly slower in a 10 % SDS-poly-acrylamide gel as compared with the in vitro translation products from CPMV-RNA (Figure 4, lane 1 and 2). This difference might indicate one or more amino acid substitutions in the 170 kD protein, due to mutations in the coding region of the pTB3 transcript.
By exchanging 5' and 3' parts of pTB1G and pTB3, the chimeric clones pTB1Gpx, with the 5' part of pTB3 and the 3' part of pTB1G, and pTB1Gbh, with the 5' part of pTB1G and the 3' part of pTB3 were constructed. Transcription of these clones and subsequent translation of the transcripts showed that both hybrid clones contained the putative mutation. Since both clones had a 121 nucleotide BamHI-XhoI fragment in common, spanning residues 3857 to 3978, the mutation should reside in this region. To test this assumption, the small BamHI-XhoI fragment in pTB1G was replaced with the corresponding fragment of pTB3, resulting in clone pTB1Gbx. Translation of transcripts derived from plasmid pTB1Gbx showed again the anomalous migra-

tion of the 87K polypeptide containing proteins. By sequence analysis of the small BamHI-XhoI fragment the mutation in pTB3 has finally been identified an U to C transition at position 3940 of the RNA (data not shown). On protein level the point mutation results in the replacement of a leucine by a proline at position 90 in the amino acid sequence of the 87K protein. The infectivity of B-RNA transcripts from pTB1Gbx with one extra G at the 5' end and the U → C mutation of pTB3 was three times reduced when compared with pTB1G transcripts. The single point mutation, combined with the negative effects of the extra 35 nucleotides at the 5' end probably is the reason that the infectivity of pTB3 transcripts is below the level of detection.

DISCUSSION

In this paper it is reported how the system of producing *in vitro* infections transcripts from full-length cDNA clones of CPMV-RNAs has been improved. Previously, using B- and M-RNA-transcripts with two extra G-residues at the 5' end, at most 1 % of the inoculated cowpea protoplasts were infected with B-RNA transcripts and only 0.05 % of the protoplasts with both B- and M-RNA transcripts (Vos *et al.*, 1988). Both by a modification of the inoculation procedure and by the removal of one further G residue from the transcripts, leaving only 1 extra G residue at its 5' end the infectivity of the transcripts greatly increased. The changes resulted in a final system in which 25% of the protoplasts can routinely be infected with B-RNA transcripts alone whereas 10% of the protoplasts can be infected with B- and M-RNA transcripts at the same time. Our experiments confirm the importance of 5' terminal structure of transcripts with respect to their infectivity (Dawson *et al.*, 1986; Van der Werf *et al.*, 1986; Janda *et al.*, 1987; Rice *et al.*, 1987; Shaklee *et al.*, 1988; Vos *et al.*, 1988; Ziegler-Graff *et al.*, 1988). In contrast to that what has been found for the 5' end of transcripts, extensions at the 3' end in general do not show adverse effects on the transcript infectivity (Meshi *et al.*, 1986; Van der Werf *et al.*, 1986; Janda *et al.*, 1987; Shaklee *et al.*, 1988; Simon and

Howell, 1987). The importance of the 3' non-coding region, including the poly(A) tail, in the viral RNA replication is currently subject of investigation.

Although the level of infection has now been greatly improved, the specific infectivity of the transcripts (1,25 x 10^4 infected protoplasts/µg B-transcript) is still 40 times lower than that of natural CPMV RNA (5 x 10^5 infected protoplasts/µg B-RNA), tested under the same conditions. Compared with viral RNA, the in vitro transcripts are lacking the protein VPg at their 5' end, but instead had one additional G residue.
The infectivity of the in vitro transcripts proves that a covalently linked VPg is not required for establishing a CPMV infection, but its presence apparantly has a considerable benificial effect. Perhaps the presence of VPg protects the viral RNA against nucleolytic degradation. The importance of protection of the 5' end of the RNA is substantiated by the two times higher infectivity of capped transcripts as compared to transcripts without caps. At the other hand the relatively low specific infectivity of the transcripts may be caused by the presence of the extra G-residue at the 5' end which may disturb the replication process. If this G residue is transcribed into the 3' end of the minus-strand RNA, the presence of the extra C residue may have an adverse effect on the function of the minus strand as a template for positive strand RNA synthesis. The 5' extension does not reduce the infectivity as a result of translation effects since the translation efficiency of transcripts is not decreased in reticulocyte lysate (Vos et al., 1988[a]).
The 5' ends of the transcripts were restored in cowpea plants, an observation that has been described more frequently for infectious transcripts (Dasmahapatra et al., 1986; Dawson et al., 1986; Van der Werf, et al., 1986; Janda et al., 1987; Shaklee, 1988; Ziegler-Graff et al., 1988). It is not known at which moment the extra non viral nucleotide is removed. If indeed the transcripts are exposed to exonucleolytic attack in the cells perhaps only the transcripts which have lost the extra G participate in the replication of the RNA. This could also contribute to the low specific infectivity of the transcripts. Alternatively the extra G is removed if the viral polymerase terminates during minus strand RNA synthesis or initiates

during plus strand RNA synthesis with the ultimate 3' and 5' viral nucleotide respectively.

Characterization of CPMV RF molecules has revealed the presence of a 5' poly(U) stretch in minus strand RNA which implied that the poly(A) tails of the plus strand RNA was templated (Lomonossoff et al., 1985). Our observation that the poly(A) tail of the transcripts was elongated during a replication cycle in plants excludes that the poly(A) tail is exactly copied from the poly (U) stretch in the minus strand RNA. The extension could be the consequence of a "slipping" RNA polymerase (Chamberlin and Berg, 1964) on a poly U template. This implies that during copying of the uridylic acid sequence the A to U base pairing is interupted so that unpaired U residues are exposed again and serve repeatedly as template for the growing of the poly(A) chain. Another possibility for the elongation of the poly(A) tail is that it is extended by some terminal nucleotidyl transferase activity.

It has frequently been observed that transcripts from independent full-length cDNA clones are not equally infectious or not infectious at all (Ahlquist et al., 1984; Dawson et al., 1986; Meshi et al., 1986; Rice et al., 1987; Vos et al., 1988). By exchanging segments of the wildtype clone with cDNA of a clone from which non-infectious transcripts were synthesized we were able to define precisely the sequence change in clone pTB3 which influences the infectivity of the transcripts. An U to C transition at position 3939 of the B-transcript RNA resulted in a leucine to a proline change in the 87K protein, which is thought to be the core viral RNA dependent RNA polymerase (Eggen and Van Kammen, 1988). The amino acid change can affect the conformation of this protein, as indicated by the altered electrophoretic mobility, and apparently has a deleterious effect on its activity.

The optimized infection system now is suitable to identify mutations in interesting deviant phenotypes of CPMV concerning symptom expression (De Jager and Wesseling, 1981). Alternatively, specific CPMV mutants can now be created in vitro of which the effect on expression, RNA replication and transport of the virus can be tested in vivo.

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REFERENCES

- Ahlquist, P., and Kaesberg, P. (1979). Determination of the length distribution of poly(A) at the 3'-terminus of the virion RNAs of EMC virus, poliovirus, rhinovirus, RAV-61 and CPMV and of mouse globin mRNA. Nucleic Acids Res. 7, 1195-1204.
- Ahlquist, P., French, R., Janda, M., and Loesch-Fries, L.S. (1984). Multicomponent RNA plant virus infection derived from cloned viral cDNA. Proc. Natl. Acad. Sci. USA 81, 7066-7070.
- Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7, 1513-1523.
- Chamberlin, M., and Berg, P. (1964). Mechanism of RNA polymerase action: characterization of the DNA-dependent synthesis of polyadenylic acid. J. Mol. Biol. 8, 708-726.
- Dasmahapatra, B., Dasgupta, R., Saunders, K., Selling, B., Gallagher, T., and Kaesberg, P. (1986). Infectious RNA derived by transcription from cloned cDNA copies of the genomic RNA of an insect virus. Proc. Natl. Acad. Sci. USA 83, 63-66.
- Davanloo, P., Rosenberg, A.H., Dunn, J.J., and Studier, F.W. (1984). Cloning and expression of the gene for bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA 81, 2035-2039.

- Dawson, W.O., Beck, D.L., Knorr, D.A., and Grantham, G.L. (1986). cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts. Proc. Natl. Acad. Sci. USA 83, 1832-1836.
- De Jager, C.P., and Wesseling, J.B.M. (1981). Spontaneous mutations in cowpea mosaic virus overcoming resistance due to hypersensitivity in cowpea. Physiol. Plant Path. 19, 347-358.
- Dunn, J.J., and Studier, F.W. (1983). Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. J. Mol. Biol. 166, 477-535.
- Eggen, R., and van Kammen, A. (1988). RNA replication in comoviruses. In "RNA Genetics" (P. Ahlquist, J. Holland and E. Domingo, eds.), vol. I, pp. 49-69. CRC Press, Boca Raton, Florida.
- Garcia, J.., Schrijvers, L., Tan, A., Vos, P., Wellink, J., and Goldbach, R. (1987). Proteolytic activity of the cowpea mosaic virus encoded 24K protein synthesized in E. coli. Virology 159, 67-75.
- Goldbach, R., Rezelman, G., and van Kammen, A. (1980). Independent replication and expression of B-component RNA of cowpea mosaic virus. Nature (London) 286, 297-300.
- Goldbach, R., and van Kammen, A. (1985). Structure, replication and expression of the bipartite genome of Cowpea Mosaic Virus. In "Molecular Plant Virology" (Davis, J.W. ed.), vol. II, chapter 3. CRC Press, Boca Raton, Florida.
- Hanahan, D. (1985). Techniques for transformation of E. coli. In "DNA cloning, a practical approach" (D.M. Glover, ed.), vol. I, pp. 109-135. IRL Press, Oxford.
- Ishikawa, M., Meshi, T., Motoyoshi, F., Takamatsu, N., and Okada, Y. (1986). In vitro mutagenesis of the putative replicase gene of tobacco mosaic virus. Nucleic Acids Res. 14. 8291-8305.
- Jaegle, M., Wellink, J., and Goldbach, R. (1987). The genome-linked protein of cowpea mosaic virus is bound into the 5'-terminus of B- and M-RNA by a phosphodiester linkage to serine. J. Gen. Virol. 68, 627-632.
- Janda,, M., French, R., and Ahlquist, P. (1987). High efficiency T7 polymerase synthesis of infectious RNA from cloned brome mosaic virus cDNA and effects of 5' extensions on transcript infectivity. Virology 158, 259-262.

- Johanningmeier, U., Bodner, U., and Wildner, G.F. (1987). A new mutation in the gene coding for the herbicide-binding protein in Chlamydomonas. FEBS Lett 211, 221-224.
- Korneluk, R.G., Quan, F., and Gravel, R.A. (1985). Rapid and reliable dideoxy sequencing of double-stranded DNA. Gene 40, 317-323.
- Kunkel, T.A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82, 488-492.
- Lathe, R., Kieny, M.P., Skory, S., and Lecocq, J.P. (1984). Linker tailing: unphosphorylated linker oligonucleotides for joining DNA ter mini. DNA 3, 173-182.
- Lomonossoff, G., Shanks, M., and Evans, D. (1985). The structure of cowpea mosaic virus replicative form RNA. Virology 144, 351-362.
- Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Maxam, A.M., and Gilbert, W. (1977). A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA 74: 560-564.
- Meshi, T., Ishakawa, M., Motoyoshi, F., Semba, K., and Okada, Y. (1986). In vitro transcription of infectious RNAs from full-length cDNAs of tobacco mosaic virus. Proc. Natl. Acad. Sci. USA 83, 5043-5047.
- Rezelman, G., Goldbach, R., and van Kammen, A. (1980). Expression of bottom component RNA of cowpea mosaic virus in cowpea protoplats. J. Virol. 36, 366-373.
- Rice, C.M., Levis, R., Strauss, J.H., and Huang, H.V. (1987). Production of infectious RNA transcripts from sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker and in vitro mutagenesis to generate defined mutants. J. Virol. 61, 3809-3819.
- Rochon, D., Kelly, R., and Siegel, A. (1986). Encapsidation of 18S rRNA by tobacco mosaic virus coat protein. Virology 150, 140-148.
- Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.
- Shaklee, P.N., Miglietta, J.J., Palmenberg, A.C., and Kaesberg, P. (1988). Infectious positive- and negative-strand transcript RNAs from bacteriophage Q_β cDNA clones. Virology 163, 209-213.

- Simon, A.E., and Howell, S.H. (1987). Synthesis in vitro of infectious RNA copies of the virulent satellite of turnip crinkle virus. Virology 156, 146-152.
- Sleat, D.E., Turner, P.C., Fiuck, J.T., Butler, P.J.G., and Wilson, T.M.A. (1986). Packaging of recombinant RNA molecules into pseudovirus particles directed by the origin of assembly sequence from tobacco mosaic virus RNA. Virology 155, 299-308.
- Tautz, D., and Renz, M. (1983). An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. Anal. Biochem. 132, 14-19.
- Verver, J., Goldbach, R., Garcia, J.A., and Vos, P. (1987). In vitro expression of a full-length DNA copy of cowpea osaic virus B-RNA: identification of the B-RNA encoded 24-Kd protein as a viral protease. EMBO J. 6, 549-554.
- Vos, P., Verver, J., van Wezenbeek, P., van Kammen, A., and Goldbach, R. (1984). Study of the genetic organisation of a plant viral RNA genome by in vitro expression of a full-length DNA copy. EMBO J. 3, 3049-3053.
- Vos, P., Jaegle, M., Wellink, J., Verver, J., Eggen, R. van Kammen, A., and Goldbach, R. (1988). Infectious RNA transcripts derived from full-length DNA copies of the genomic RNAs of cowpea mosaic virus. Virology 166, 33-41.
- Vos, P., Verver, J., Jaegle, M., Wellink, J., van Kammen, A. and Goldbach, R. (1988[a]). Two viral proteins involved in the proteolytic processing of the cowpea mosaic virus polyproteins. Nucleic Acids Res. 16, 1967-1985.
- Wellink, J., Rezelman, G., Goldbach, R., and Beyreuther, K. (1986). Determination of the sites of proteolytic processing in the polyprotein encoded by the B-RNA of cowpea mosaic virus. J. Virol. 59, 50-58.
- Wellink, J., Jaegle, M., and Goldbach, R. (1987). Detection of a novel protein encoded by the bottom component RNA of cowpea moaic virus, using antibodies raised against a synthetic peptide. J. Virol. 61, 236-238.
- Wellink, J., Jaegle M., Prinz, H., van Kammen, A., and Goldbach, R. (1987[a]). Expression of the middle component RNA of cowpea mosaic virus in vivo. J. Gen. Virol. 68, 2577-2585.

- Van der Werf, S., Bradley, J., Wimmer, E., Studier, F.W., and Dunn, J.J. (1986). Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. Proc. Natl. Acad. Sci. USA 83, 2330-2334.
- Yamisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUc19 vectors. Gene 33, 103-119.
- Zabel, P., Moerman, M., van Straaten, F., Goldbach, R., and van Kammen, A. (1982). Antibodies against the genome-linked protein VPg of cowpea mosaic virus recognize a 60,000-dalton precursor polypeptide. J. Virol. 41, 1083-1088.
- Ziegler-Graff, V., Bouzoubaa, S., Jupin, I., Guilley, H., Jonard, G., and Richards, K., (1988). Biologically active transcripts of beet necrotic yellow vein virus RNA-3 and RNA-4. J. Gen. Virol. 69, 2347-2357.

CHAPTER 6

Analysis of sequences involved in cowpea mosaic virus RNA replication using site specific mutants

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Submitted

SUMMARY

Using a full-length cDNA clone of cowpea mosaic virus (CPMV) B-RNA from which infectious transcripts can be generated, we have examined if - and how a stretch of 11 nucleotides, UUUUAUUAAAA, comprising the nucleotides 5883 to 5893 in the 3' non-coding region of B-RNA, affects viral RNA replication. This stretch is not only present in B-RNA but also in M-RNA and represents the 7 nucleotides preceding the poly(A)-tail and the first 4 A residues of the poly(A)-tail. By replacing the A residue at position 5887 by a T, an Aha III restriction site was introduced in the cDNA which allowed the construction of a series of mutants in this region. The effect of the mutations on the replication of B-RNA transcripts derived from the mutated clones was tested in cowpea protoplasts as well as in cowpea plants.

Only mutant transcripts with minor modifications appeared able to replicate in cowpea protoplasts and in plants, which indicates that the region indeed has a function in viral RNA replication. In addition the results support the existence of a supposed hairpin loop in this region. Those transcripts with deletions which will disturb the hairpin structure have decreased specific infectivities.

Mutant transcripts reversed stepwise to the wild-type sequence during replication in plants. This observation strengthens the idea that the stretch of 11 nucleotides has a function in viral RNA replication.

INTRODUCTION

The genome of cowpea mosaic virus (CPMV) consists of two positive-stranded RNA molecules, denoted B- and M-RNA, which possess a small protein, VPg, covalently linked at the 5'end, and a poly(A)-tail at the 3'end (for reviews see Goldbach and Van Kammen, 1985; Eggen and Van Kammen, 1988). Both genomic segments are expressed by the production of polyproteins which are cleaved into functional proteins by a virus encoded protease (Vos et al., 1988). The genetic information is functionally divided among M- and B-RNA, the former encoding the two capsid proteins and proteins presumably involved in cell-to-cell movement (Goldbach and Van Kammen, 1985) whereas the latter RNA encodes functions required for viral RNA replication (Eggen and Van Kammen, 1988). The research into the molecular mechanism of CPMV RNA replication has been impeded by the failure to purify a viral polymerase activity from infected leaves capable to accept exogenously added RNA templates (Dorssers et al., 1984). As an alternative approach to unravel the molecular mechanism of RNA replication, the construction of full-length cDNA clones, from which infectious transcripts can be obtained seems very promising (Vos et al., 1988; Eggen et al., 1989). Such clones enable the introduction of site specific mutations in the viral RNAs and the analysis of the effects of such mutations on the RNA replication process in vivo.

It is tempting to assume that crucial recognition signals for various viral and/or host proteins involved in viral RNA replication are located in the 5' and 3' terminal non-coding regions of the two RNAs. Indeed both the 5' and 3' termini of B- and M-RNA contain regions with high sequence homology (Fig. 1). Especially striking is a conserved stretch of 11 bases (UUUUAUUAAAA) in the 3'-ends of B- and M-RNA, consisting of the first 4 A residues of the poly(A)-tail and 7 nucleotides preceding this tail. The sequence is complementary, allowing 1 G-U pair, to an 11 nucleotide stretch found in the leader sequence of both RNAs. This implies that the plus-strand and the complementary minus-strand of both B- and M-RNA have a similar sequence of 11 nucleotides at the 3'end and, therefore this sequence might represent a signal with a function in viral RNA multiplication (Eggen

and Van Kammen, 1988).

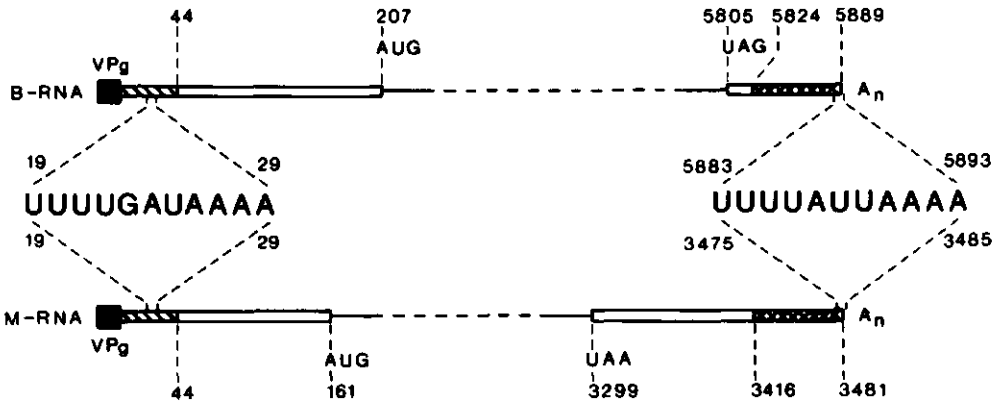

Figure 1:
Structural organization of the non-translated regions of the CPMV genome. The 5' and 3' non-translated regions in B- and M-RNA are represented as open bars. Within these bars two stretches with more than 80% nucleotide sequence homology between the RNAs are indicated as shaded areas (▨ 89%, ▩ 82%). The nucleotides 19 to 29, UUUUGAUAAAA, in the homologous parts of the 5' leader sequences of both RNAs are complementary to the first four A's of the poly(A) and the last seven nucleotides before the beginning of the poly(A)-tail.

Using a full-length cDNA clone from which infectious B transcripts can be generated (Eggen et al., 1989), we have examined the significance of this sequence by constructing a series of mutants in the 3' non-coding region of B-RNA transcripts, and analysing the multiplication of these mutants in cowpea protoplasts and cowpea plants. The results obtained suggest that the

conserved stretch is folded into a hairpin loop that may have a function in the viral RNA replication process.

MATERIALS AND METHODS

DNA techniques

Enzymes were purchased from Gibco-BRL, Pharmacia, Boehringer or New England Bio Labs and used as described by the suppliers. Standard recombinant DNA techniques for the isolation and ligation of DNA fragments and transformation of DNA into competent Escherichia coli JM109 or DH5α cells were performed according to Maniatis et al. (1982). Cloned viral cDNA molecules were analyzed with restriction enzymes or by nucleotide sequence analysis, using single-stranded or double-stranded DNA (Sanger et al., 1977; Korneluk et al., 1985). Oligodeoxynucleotides were synthesized using β-cyanoethyl phosphoramidites and a cyclone DNA synthesizer (Biosearch inc.). Oligonucleotide-directed mutagenesis was performed essentially as described by Vos et al. (1988) using the procedure for efficient selection of mutated DNA developed by Kunkel et al. (1985). Linkers were inserted as described by Lathe et al. (1984).

Construction of modified cDNA clones

A full-length cDNA clone of B-RNA, pTB1G, described previously (Eggen et al., 1989), was used for the construction of modified cDNA clones as depicted in Figure 2.

(a) Construction of pTB1G-Aha
For the construction of pTB1G-Aha a 2024 bp EcoRI-ClaI fragment from pTB1G (spanning from nucleotide 3865 to the 3'terminus of the B-RNA encoded sequence) was inserted into pBR322 DNA digested with EcoRI and ClaI.

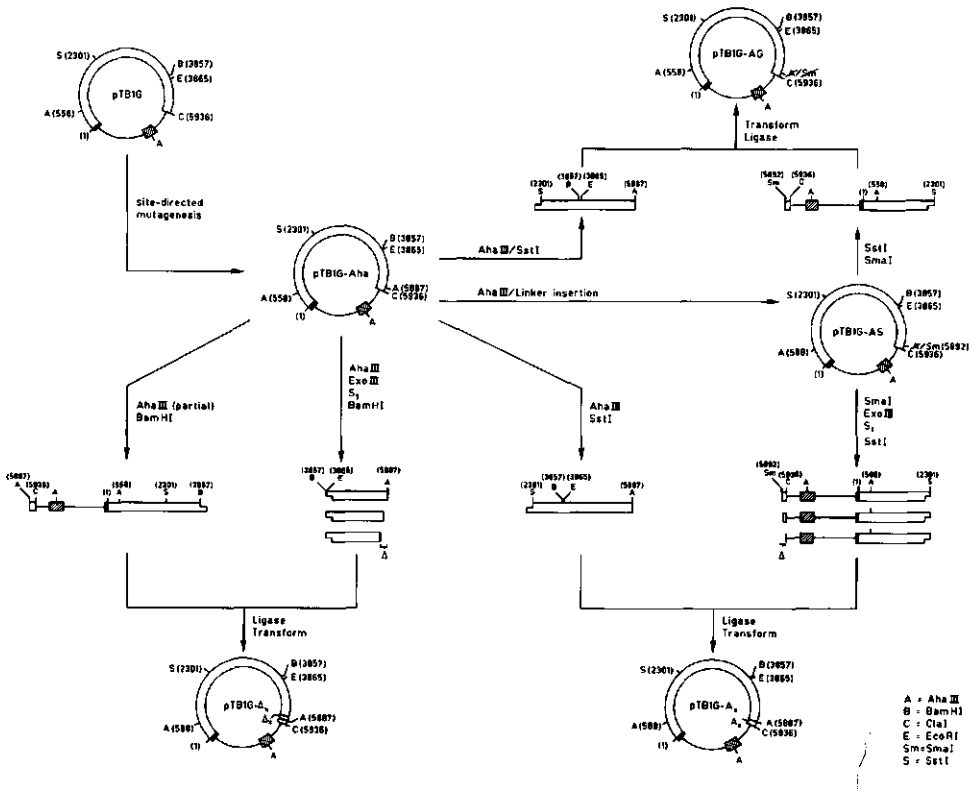

Figure 2:

Strategy for the construction of modified BcDNA clones. Open bars indicate regions of plasmids containing CPMV sequences and lines depict plasmid sequences. The solid box represents the T7 RNA polymerase promoter region, the dotted box cDNA regions with deletions and the hatched box the ampicilline resistance gene. For experimental details see Materials and Methods.

From the resulting plasmid the 2692 bp EcoRI-SalI fragment (nucleotide 3865 in the BcDNA to the SalI site beyond the 3' terminus of the B-cDNA sequence) was isolated and ligated to EcoRI and SalI digested M13mp$_{19}$ DNA. Single stranded recombinant phage DNA and an oligonucleotide with the sequence 5'ATGTGTTTTTTTTAAAAAA 3' were used for site directed mutagenesis.
The mutated fragment was transferred from the M13 clone to plasmid pTB1G by exchanging the wild-type for the modified 1951 bp XhoI-ClaI fragment (nucleotides 3978 to the 3'end of the BcDNA sequence), resulting in clone pTB1GAha. This plasmid has a newly created AhaIII restriction recognition sequence (TTTAAA in stead of ATTAAA) at position 5887 of the B-cDNA sequence, the A residues representing the first residues of the poly d(A) track.

(b) Construction of pTB1G-Δ_X
From plasmid pTB1G-Aha a series of mutant clones was constructed with deletions of variable length from the AhaIII-site at position 5887 of the BcDNA towards the 5' end.
Clone pTB1G-Aha was cut with AhaIII and the 3' termini were treated with Exonuclease III during different times of incubation. The resulting 5'extensions were removed with S_1 nuclease, thus creating blunt ends. The DNA was digested with BamHI (position 3857) and the fragment of about 2 kb (position 3857 to the trimmed AhaIII-site at position 5889 of the B-cDNA) was gel purified. A second portion of pTB1G-Aha was digested with BamHI and partially with AhaIII. The 6.1 kb AhaIII-BamHI fragment (containing the B-cDNA sequence from position 5890 to the 3'end, the vector sequences and the B-cDNA sequence from the 5'end to position 3857) was isolated from gel, and ligated to the treated 2kb fragments, resulting in plasmids pTB1G-Δ_X (X referring to a variable number of deleted nucleotides). The number of deleted nucleotides in each clone was precisely determined by dsDNA sequencing, using a specific oligonucleotide (5' AGAAGAGTTCAATTTCGC 3') that primed DNA synthesis at position 5792 of the BcDNA.

(c) Construction of pTB1G-AS
Plasmid pTB1G-AS was constructed by the insertion of a SmaI linker (CCCCCGGGGG) in the AhaIII-site at position 5887 of clone pTB1G-Aha.

(d) Construction of pTB1G-AG

For the construction of pTB1G-AG, the 3.6 kb SstI-SmaI fragment of clone pTB1G-AS (position 2301 to 5890 of the BcDNA) was removed and replaced by the corresponding 3.6 kb SstI-AhaIII fragment isolated from pTB1G-Aha DNA. The resulting construct had 5 G residues inserted in the AhaIII recognition sequence at position 5887 of the BcDNA.

(e) Construction of pTB1G-A_X

To obtain full-length BcDNA clones with shorter 3'- poly d(A) tracks pTB1G-AS DNA was digested with SmaI and the linearized DNA was trimmed with Exonuclease III (see section b), followed by a SstI digestion. DNA fragments of about 4.6 kb (spanning the BcDNA sequence from the modified SmaI site at position 5890 to the 3'end, the vector sequences and the BcDNA sequence from the 5'end to position 2301) were ligated to the 3.6 kb SstI-AhaIII fragment (position 2301 to 5887 of the BcDNA) derived from pTB1G-Aha DNA, resulting in plasmids pTB1G-A_X. The length of the remaining poly d(A) stretches was determined by dsDNA sequencing, using the oligonucleotide primer described in (b).

In vitro transcription

After linearization of recombinant plasmid DNA with the proper restriction enzyme, DNA was purified by phenol extraction and ethanol precipitation and used for the production of run off transcripts. In vitro transcription of templates with T_7 RNA polymerase and recovery of transcripts from reaction mixtures was as described previously (Verver et al., 1987).

Inoculation of cowpea protoplasts and detection of viral RNA replication

The preparation of cowpea protoplasts and their subsequent inoculation with CPMV-RNA or RNA transcripts was as described previously (Eggen et al., 1988; Rezelman et al., 1980). Multiplication of in vitro transcripts or viral RNA in the inoculated protoplasts was determined by the indirect fluorescent antibody technique, using antiserum raised against the B-RNA

encoded 24 k protein (Vos et al., 1988).

Inoculation of cowpea plants and recovery of progeny virus

Primary leaves of 10-day-old cowpea plants were inoculated with a mixture of 10 µg transcripts from B- and McDNA clones, 1 µg CPMV RNA or with a crude extract prepared from protoplasts inoculated with B- and M-RNA-transcripts or CPMV RNA, and grown as described previously (Vos et al., 1988). Usually primary or systematically infected leaves were harvested at day 4-5 or day 7-10 after inoculation, respectively, and used for virus isolation. Viral RNA was extracted from isolated virus as described by Vos et al. (1988).

Sequence analysis of transcripts and virion RNA's

To determine nucleotide sequences adjacent to the poly(A) tail of transcripts or virion RNA, single stranded cDNA, generated by oligo(dT) primed reverse transcription, was used as template in the dideoxy sequencing procedure. Two µg of B-RNA transcript or 4 µg of CPMV RNA was mixed with a 20 times excess of oligo(dT)12-18 in 12.5 µl of annealing buffer (50 mM Tris-HCl, pH 8.2, 60 mM NaCl, 10 mM DTT) and placed into 200 ml water of 68°C. After the mixture was cooled down in about 30 minutes to 35°C, 4.5 µl 5x reverse transcription buffer (250 mM Tris-HCl, pH 8.6, 30 mM $MgCl_2$, 500 mM NaCl, 50 mM DTT), 5 µl of a dNTP mixture (2 mM each) and 1 µl M-MLV reverse transcriptase (20 U/µl) were added. The reaction mixture was subsequently incubated for 5 minutes at room temperature, 15 minutes at 45°C and after the addition of 1 µl of the dNTP mixture, another 15 minutes at 45°C. The nucleic acids were purified by phenol extraction and ethanol precipitation and finally dissolved in 20 µl water. Five µl of this solution was used in dideoxy chain terminating reactions for double-stranded DNA as described by Korneluk et al. (1985) using a labelled oligonucleotide (5' AGAAGAGTTCAATTTCGC 3') that primed DNA synthesis at nucleotide 5792 of the BcDNA.

RESULTS

Construction of mutant BcDNA clones, and infectivity of its transcripts

To study the importance and possible role of the 3' non-translated region preceding the poly(A) tail and the poly (A) tail itself in CPMV RNA replication a series of mutations were introduced in a full-length cDNA clone of B-RNA, pTB1G (Eggen et al., 1989) as outlined in Figure 2.
The A residue at position 5887 of the viral cDNA clone pTB1G was substituted for a T residue by site directed mutagenesis which resulted in clone pTB1G-Aha containing a newly created AhaIII restriction recognition sequence (TTTAAA) just at the boundary of the 3' non-translated region and poly d(AT) track. The effect of this point mutation on the infectivity of pTB1G-Aha transcripts in cowpea protoplasts (table I) was that, compared with wild type (pTB1G) transcripts, the infectivity of pTB1G-Aha transcripts was reduced with 40%. Although the infectivity of the mutant transcript was decreased, its viability was more than enough to allow further mutagenesis and to analyze the effect of additional mutations on the replication of the resulting transcripts. For that purpose we used the newly created AhaIII-site at position 5887 to introduce different insertions and deletions. pTB1G-AS was constructed by the insertion of a SmaI-linker (CCCCCGGGGG) in the AhaIII-site. PTB1G-AS transcripts appeared to be non-infectious in cowpea protoplasts (table I). Transcripts lacking the poly(A) tail but having 5 non-viral C residues at their 3'end were produced from pTB1G-AS DNA, linearized with SmaI. These transcripts were also not infectious (table I).
By exchanging DNA fragments of pTB1G-Aha and pTB1G-AS, pTB1G-AG was produced (Figure 2). This plasmid contained 5 extra G residues within the AhaIII-site at position 5887 of the viral sequence. pTB1G-AG transcripts were, again, found to be not infectious in cowpea protoplasts (see table I). These results indicate that rigorous alterations in the sequence around position 5887 lead to complete abolishment of infectivity. The biological importance of the 3' non-translated region of B-RNA was further studied by deleting a small number of nucleotides in this region.

Table I

B-cDNA clone	Structure of 3' region of transcript	Infectivity (%)
pTB1G	- GUUUUUAUUA$_{46}$UAUCG	100
pTB1G-Aha	- GUUUUUUUUA$_{46}$UAUCG	60
pTB1G-Δ_1	- GUUUUUUU A$_{46}$UAUCG	60
pTB1G-Δ_2	- GUUUUUU A$_{46}$UAUCG	70
pTB1G-Δ_3	- GUUUUU A$_{46}$UAUCG	70-80
pTB1G-Δ_4	- GUUUU A$_{46}$UAUCG	20
pTB1G-Δ_7	- GU A$_{46}$UAUCG	0.5
pTB1G-Δ_8	- G A$_{46}$UAUCG	0
pTB1G-A28	- GUUUUUUUUA$_{28}$UAUCG	60
pTB1G-A18	- GUUUUUUUUA$_{18}$UAUCG	60
pTB1G-A4	- GUUUUUUUUA$_4$ UAUCG	1
pTB1G-AS	- GUUUUUUUUCCCCCGGGGGA$_{46}$UAUCG	0
pTB1G-AS x SmaI	- GUUUUUUUUCCCCCC	0
pTB1G-AG	- GUUUUUUUUGGGGGA$_{46}$UAUCG	0

Table I. Effect of mutations in the 3' region on transcript infectivity. $2.5.10^6$ cowpea protoplasts were inoculated with 50 µg RNA transcripts. Fourty hours after inoculation the protoplasts were examined for the presence of virus-encoded proteins using the indirect immunofluorescent antibody technique. The infectivity of pTB1G transcripts was arbitrarily set on 100%. This corresponds with 30% infected cowpea protoplasts.

Clones containing such deletions (Figure 2) were denoted pTB1GΔ_X, the subscript X representing the number of deleted nucleotides. RNA transcripts were produced from the respective cDNA clones and the infectivity of the transcripts tested in cowpea protoplasts (table I). pTB1G-Aha transcripts and transcripts from pTB1G-Δ_1, -Δ_2 and -Δ_3 were equally infectious but the infectivity decreased to 20% of that of pTB1G transcripts when 4 nucleotides were deleted (pTB1G-Δ_4 transcripts). Larger deletions had a further deleterious effect on the infectivity as shown by the very low infectivity of pTB1G-Δ_7 transcripts (0,5%) and the complete lack of infectivity of pTB1G-Δ_8 transcripts (Table I).

To study the importance of the poly(A) tail at the 3'end of CPMV RNA for infectivity, B-cDNA clones were constructed with a poly d(AT) track of variable length (pTB1G-A_X, see figure 2). From such clones in vitro transcripts were obtained with a poly (A) tail consisting of 46 (pTB1G-A_{46}), 28 (pTB1G-A_{28}), 19 (pTB1G-A_{19}) and 4 A residues (pTB1G-A_4). The infectivity of these transcripts in cowpea protoplasts is shown in table I. Compared with the transcripts from the starting clone pTB1G with a poly(A) tail of 46 residues, the infectivity of pTB1G-A_{28} and pTB1G-A_{19} transcripts is the same. However the infectivity of pTB1G-A_4 transcripts with a poly(A) tail of only 4 residues was 50 times lower than that of pTB1G transcripts.

Plant infection and progeny analysis

To examine the effect of mutations in the 3' non-coding region of B-RNA on the multiplication of the viral RNA in intact plants, inocula of B-RNA transcripts from wild-type clone pTB1G and from mutant clones pTB1G-Δ_2 and pTB1G-Δ_3 were, supplemented with M-RNA transcripts from pTM1G (Eggen *et al.*, 1989). Although it was possible to inoculate cowpea plants with these mixtures of transcripts directly, higher infectivities were obtained when first the mixed inoculum was used to infect protoplasts, in which the transcripts were multiplied and encapsidated, and then the plants were inoculated with the extract of the protoplasts.

When extracts from protoplasts infected with pTB1G (wild-type) or pTB1G-Δ_3

transcripts were used as inoculum, equally intense symptoms were detectable on the inoculated leaves after 3 days.
Moreover, the upper leaves showed the typical mosaic symptoms of a wild-type virus infection immediately upon appearance of these leaves. With extracts of protoplasts infected with pTB1G-Δ_2 transcripts as inoculum the symptom expression was less severe in inoculated leaves and, moreover, delayed in systemically infected leaves (symptoms appeared only after 7 days). When mixtures of pTB1G, pTB1G-Δ2 and pTB1G-Δ3 transcripts respectively with pTM1G transcripts in equal amounts and concentrations were used to infect cowpea plants directly, the development of symptoms was slower than with protoplast extracts as inoculum but among the mutants the differences in symptom expression were similar (data not shown). This result shows that the differences in symptom expression between mutants were the result of modified nucleotide sequences and not due to variations in protoplast extraction. When crude extracts of systemically infected leaves of pTB1G-plants, pTB1GΔ_2-plants or pTB1GΔ_3-plants were used as inoculum, there was no longer a variation in symptom development among the different mutants.

(i) Progeny analysis of pTB1G-Δ_2 transcripts

The amount of progeny virus that could be recovered from the primary leaves inoculated with pTB1G-Δ2 was only about 1/20 of the amount isolated after infection with pTB1G. From systemically infected leaves of the same plants however, pTB1G-Δ_2 progeny virus was obtained in amounts comparable to what was found in plants infected with pTB1G transcripts.
When the nucleotide sequence in the mutated region of the pTB1G-Δ2 progeny B-RNA from the inoculated leaves was determined, the result show that these sequences were equivocal (Fig. 3^b, Table II) and consisted of a mixture of the parental mutant sequence UUUUUAAAAAA (Fig. 3^a, Table II) and sequences in which at the positions 5887, 5888 and 5889 changes in the sequences were introduced. The sequence data indicated the sequence UUUUU$^{A}_{a}$AAAAA i.e. an equal signal of A and U residues at position 5887 and 5889 and mainly U and a weakly visible A (= a) signal at position 5888.

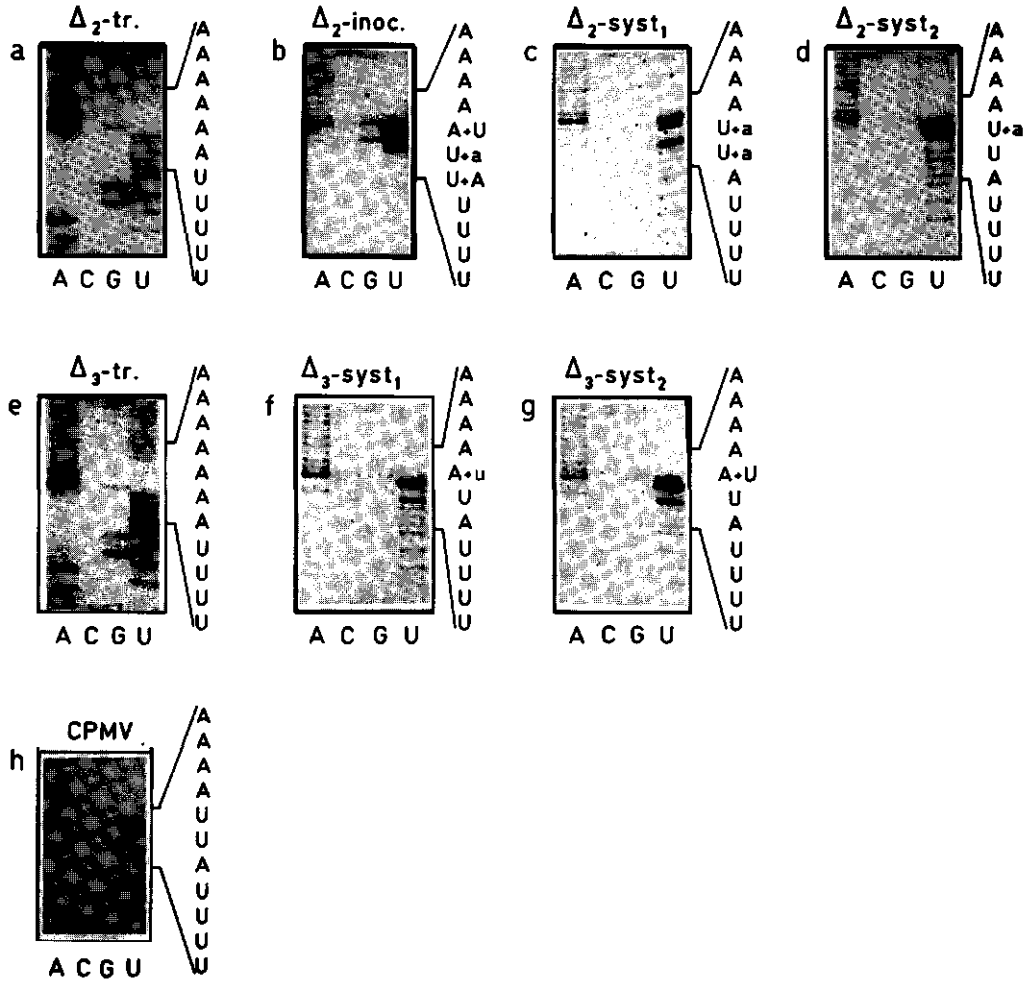

Figure 3:

Autoradiogram showing the RNA sequences around the border of the 3' non-coding region and the poly(A)-tail of wild-type B-RNA (CPMV) and transcripts from mutant clones pTB1G-Δ2 and pTB1G-Δ3 with their progenies. The detected sequences are shown along from 5' (bottom) to 3' (top). The small letters represent the nucleotides which are weakly observed in a mixture of nucleotides. Inoc., syst.1 and syst.2 indicate the progeny from the primary leaves, systemically infected leaves from the same plant and systemically infected leaves after a second passage respectively.

Table II

Origin of RNA		Structure of 3' region
Δ_2:	transcript	- U U U U U A A A A A A -
	inoculated leaf	- U U U U U U A A A A A -
	(first passage)	A a U
	systemically infected leaf	- U U U U A U U A A A A -
	(first passage)	a a
	systemically infected leaf	- U U U U A U U A A A A -
	(second passage)	a
Δ_3:	transcript	- U U U U A A A A A A A -
	systemically infected leaf	- U U U U A U A A A A A -
	(first passage)	u
	systemically infected leaf	- U U U U A U A A A A A -
	(second passage)	U
CPMV:		- U U U U A U U A A A A -

Table II. Sequence analysis of progeny virus derived from pTB1G-Δ2 and pTB1G-Δ3 transcripts after several replication cycles in cowpea plants.

This mixture of sequences indicates that reversion of the mutant sequence to the wild-type sequence UUUUAUUAAAA (Fig. 3^h, Table II) had occurred. The reversion to the wild-type sequence is even more pronounced in the progeny isolated from the systemically infected leaves for which the sequence UUUUA$^{aa}_{U}$AAAA was found (Fig. 3c, Table II), with consistently an A residue at position 5887 and preferentially U residues at positions 5888 and 5889. If the progeny virus from these systemically infected leaves was used as inoculum for a second plant infection, the nucleotide sequence of the progeny virus isolated from the systemically infected leaves after the second passage was UUUUAU$^{a}_{U}$AAAA (Fig. 3^d, Table II) and consisted almost entirely of wild-type sequence UUUUAUUAAAA (Fig. 3^h, Table II).

(ii) Progeny analysis of pTB1G-Δ_3 transcripts

The concentration of progeny virus in plants inoculated with pTB1G-Δ3 transcripts was the same as that in plants inoculated with pTB1G Sequence analysis of the pTB1G-Δ_3 progeny isolated from systemically infected leaves showed that the parental mutant sequence UUUUAAAAAAA, (Fig. 3^e, Table II) was not stable but changed during replication cycles in the cowpea plant so that a mixed sequence UUUUAU$^{A}_{U}$AAAA (Fig. 3^f, Table II) was found. Here an uridine has been introduced at position 5888 while at position 5889 most RNA molecules had an A residue but, some others an uridine. These nucleotide changes again show a reversion to the wild-type sequence UUUUAUUAAAA in this area like was found for Δ_2. After a second passage of the isolated pTB1G-Δ_3 progeny virus, the nucleotide changes towards the wild-type sequence became even more prominent (UUUUAU$^{A}_{U}$AAAA, Fig. 3^g, Table II), the U residue at position 5889 as found in the wild-type sequence now becoming clearly visible.

DISCUSSION

In this paper we have described the introduction of specific mutations in the 3' non-coding region of CPMV B-RNA using a full length viral cDNA clone from which infectious transcripts could be obtained. The data obtained show that some of the mutant transcripts are still able to replicate as well in cowpea protoplasts as in cowpea plants, and that has enabled us to examine the effect and stability of the mutations *in vivo*.

Since transcription starts at the 3' end of a template RNA molecule, a viral replicase should recognize the 3' termini of both positive and negative viral RNA strands. Therefore it is tempting to assume that conserved, 3'-terminal sequences in both B- and M-RNA have a crucial function in initial steps in this process (e.g. binding of the replicase) (Fig. 1).

The preserved sequence in the 3' non-coding region of both viral RNA's can be folded into a structure (see structure I, Figure 4), which may represent a signal function in the RNA replication process.

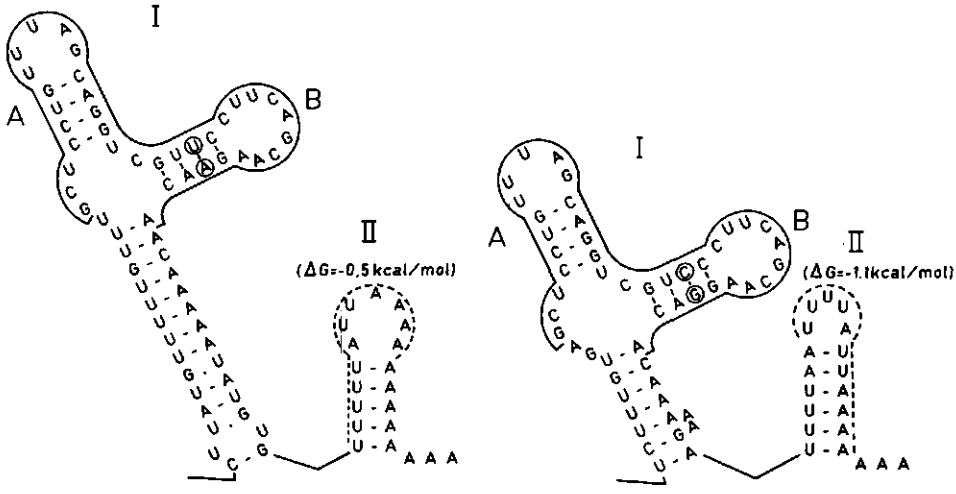

Figure 4: CPMV B-RNA CPMV M-RNA

Secondary structure predictions of the 3' regions of both B- and M-RNA consisting of the non-coding regions immediately preceding the poly(A)-tail and some residues of the poly(A)-tail. The solid line in I represents an

identical structure in the two RNAs allowing an U-A to C-G covariation in hairpin B (shown by open circles). The interrupted line in hairpin II represents a conserved stretch of 11 nucleotides in the 3' ends of B- and M-RNA which might represent a signal in viral RNA replication (see also Figure 1.). Thermodynamic parameters were calculated according to Freier et al. (1986).
A programme developed for the prediction of pseudoknots in RNA (J.P. Abrahams et al, in preparation) as well as the Zuker programme (Zuker, M. and Stiegler, P., 1981) were used.

The existence of this structure is supported both by a covariation of nucleotides in hairpin B of B- and M-RNA such that the base pairing potential is preserved (James et al., 1988) and by the fact that a similar structure can be deduced, with two covariations in hairpin B, from the nucleotide sequence of the M-RNA of another comovirus, red clover mottle virus (Shanks et al.,1986). Downstream of structure I in both CPMV-RNA's a stretch of 11 nucleotides (UUUUAUUAAAA), with an homologous counterpart in the 5' leader (see Fig.1), can be folded into a stem-loop structure (see Fig. 4, structure II), although the stability of this structure is very low (ΔG = -0,5 kcal/mol). The results of the experiments described in this paper unequivocally demonstrate the importance of the 11 nucleotide stretch and are in favour of the existence of the supposed hairpin structure.
The U to A substitution in B-RNA (UUUUAUUAAAA → UUUUUUUAAAA), which created a useful AhaIII restriction site decreased the infectivity of the transcript with 40%. This transversion might influence the stem-length in the hairpin (5 → 8 U-A basepairs), resulting in a more stable hairpin (ΔG = -3,2 kcal/mol). The insertion of a 10-mer SmaI linker in this AhaIII site resulted in non-viable transcripts. This linker indeed disturbes the primary structure and potentially changed the stability of the hairpin by lengthening the stem with 2 or 3 C-G basepairs. Also the presence of only 5 extra G-residues in this region of the transcript (mutant pTB1G-AG) had a dramatic effect on the infectivity, again indicating that a correct formation of the hairpin loop may be essential for infectivity of the RNA transcripts.

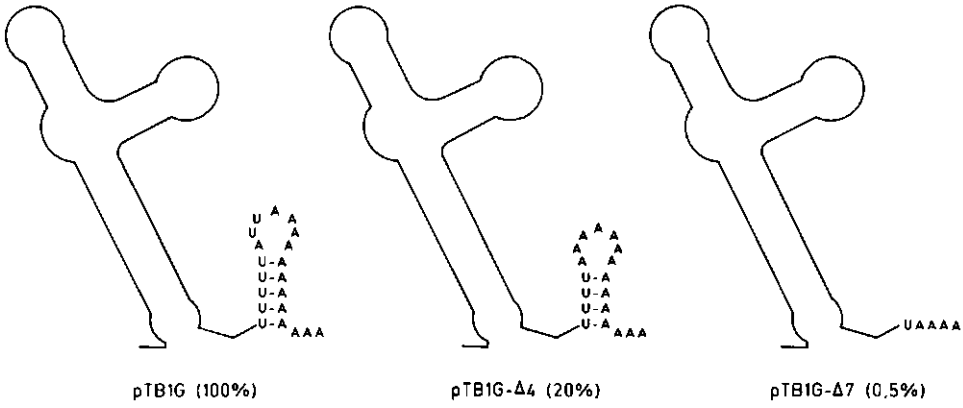

Figure 5:
Predicted effect of mutations on hairpin II (see Figure 4) formation in B-RNA transcripts. The effect of the mutations has been classified in three groups, shown along with percentages which indicate the specific infectivities of the transcripts (see also Table I). In pTB1G, which represents also pTB1G-Δ1, pTB1G-Δ2 and pTB1G-Δ3, the stem length is not disturbed. A further deletion (pTB1G-Δ4) diminishes the stem length, which is even more pronounced in the last group, (pTB1G-Δ7), representing also mutant pTB1G-Δ8. The solid line indicates structure I from Figure 4.

In addition, a decrease of infectivity was also observed when the pertinent part of the RNA was prevented to fold by shortening the poly(A)-tail to 4 residues.
It remains obscure whether the hairpin represents a recognition signal in RNA replication or that it stabilizes the RNA. The latter hypothesis seems less plausible since the poly(A) tail has been proposed to protect the RNA against degradation (Huez et al., 1983). Our experiments indicate that, besides this protective function, the poly(A)-tail of CPMV is also essential for viral RNA replication. For poliovirus RNA it was shown that removal of much of the poly(A)-tail reduces the infectivity of the RNA (Spector and Baltimore, 1974).

The experiments described in this paper show that a surprisingly short poly(A) stretch is sufficient to allow replication of the transcripts. Transcripts without a poly(A)-tail but with 5 C's at the 3' end are not infectious whereas transcripts with only 4 A residues and 5 non-viral nucleotides (UAUCG) are infectious, although at a low level.
During replication in cowpea plants the nucleotide sequence of the pTB1G-Δ_2 and pTB1G-Δ_3 deletion transcripts ultimately reversed to the wild-type sequence, which again supports the relevance of the specific nucleotide sequence adjacent to the 3'-poly(A)-tail in the virus multiplication. Especially the residues A and U on positions 5887 and 5889 respectively of the B-RNA are highly preferred as indicated by their rapid appearance in vivo in both deletion mutants.
It remains to be established whether these reversions are the result of selectively advantageous point mutations (Holland et al., 1982) or arise by a specific recombination with the homologous nucleotide stretch in wild-type M-RNA transcript, also present during infection. Transcripts from pTB1G-Δ_2 and pTB1G-Δ_3 differ only by 1 nucleotide (Δ_2 has an uridine on position 5887 where Δ_3 has an adenine). This difference may have influenced a possible hairpin structure such that the stem in Δ_2 transcripts is one U-A basepair longer. Systemic spreading of the progeny of pTB1G-Δ_2 transcripts is, when compared with the progeny of pTB1G-Δ_3 transcripts, significantly delayed. This suggests that the pertinent nucleotide sequence, correctly folded, has a function in the RNA replication. The less efficient multiplication of pTB1G-Δ_2 transcripts probably also explains that due to the higher selection pressure the nucleotide reversion to the wild-type sequence is observed more quickly in the pTB1G-Δ_2 progeny than in the pTB1G-Δ_3 progeny. In this paper we report that viable mutant transcripts can be produced from full-length cDNA clones of a plant viral genome equipped with a VPg and a poly (A) tail. The results illustrate the potential of these transcripts in unraveling crucial molecular mechanisms during virus multiplication, such as RNA replication, RNA encapsidation and virus transport.

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REFERENCES

- Dorssers, L., van der Krol, S., van der Meer, J., van Kammen, A., and Zabel, P. (1984). Purification of cowpea mosaic virus RNA replication complex: Identification of a virus-encoded 110,000-dalton polypeptide responsible for RNA chain elongation. Proc. Natl. Acad. Sci. USA 81, 1951-1955.
- Eggen, R., and van Kammen, A. (1988). RNA replication in comovirusses. In "RNA Genetics" (P. Ahlquist, J. Holland and E. Domingo, eds.), vol. I, pp. 49-69. CRC Press, Boca Raton, Florida.
- Eggen, R., Verver, J., Wellink, J., de Jong, A., Goldbach, R., and van Kammen, A. (1989). Improvements of the infectivity of in vitro transcripts from cloned cowpea mosaic virus cDNA: impact of terminal nucleotide sequences. Submitted for publication.
- Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Neilson, T., and Turner, D.H. (1986). Improved free-energy parameters for predictions of RNA duplex stability. Proc. Natl. Acad. Sci. USA 83, 9373-9377.
- Goldbach, R., and van Kammen, A. (1985). Structure, replication and expression of the bipartite genome of Cowpea Mosaic Virus. In "Molecular Plant Virology" (Davis, J.W. ed.), vol. II, chapter 3. CRC Press, Boca Raton, Florida.

- Holland, J., Spindler, K., Horodyski, F., Graban, E., Nichol, S., and Van de Pol, S. (1982). Rapid evolution of RNA genomes. Science 215, 1577-1585.
- Huez, G., Cleuter, Y., Bruck, C., van Vloten-Doting, L., Goldbach, R., and Verduin, B. (1983). Translational stability of plant viral RNAs microinjected into living cells. Influence of a 3'-poly(A) segment. Eur. J. Biochem. 130, 205-209.
- Jaegle, M., Wellink, J., and Goldbach, R. (1987). The genome-linked protein of cowpea mosaic virus is bound into the 5'-terminus of B- and M-RNA by a phosphodiester linkage to serine. J. Gen. Virol. 68, 627-632.
- James, B.D., Olsen, G.J., Liu, J., and Pace, N.R. (1988). The secondary structure of Ribonuclease P RNA, the catalytic element of a ribonucleoprotein enzyme, Cell 52, 19-26.
- Korneluk, R.G., Quan, F., and Gravel, R.A. (1985). Rapid and reliable dideoxy sequencing of double-stranded DNA. Gene 40, 317-323.
- Kunkel, T.A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82, 488-492.
- Lathe, R., Kieny, M.P., Skory, S., and Lecocq, J.P. (1984). Linker tailing: unphosphorylated linker oligonucleotides for joining DNA termini DNA 3, 173-182.
- Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular cloning, a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbor, N.Y.
- Rezelman, G., Goldbach, R., and van Kammen, A. (1980). Expression of bottom component RNA of cowpea mosaic virus in cowpea protoplasts. J. Virol. 36, 366-373.
- Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.
- Shanks, M., Stanley, J., and Lomonossoff, G.P. (1986). The primary structure of red clover mottle virus middle component RNA. Virology 155, 697-706.
- Spector, D.H., and Baltimore, D. (1974). Requirement of 3'-terminal poly(adenylic acid) for the infectivity of poliovirus RNA. Proc. Natl. Acad. Sci. USA 71, 2983-2987.

- Verver, J., Goldbach, R., Garcia, J.A., and Vos, P. (1987). 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. The EMBO Journal 6, 549-554.
- Vos, P., Jaegle, M., Wellink, J., Verver, J., Eggen, R., van Kammen, A., and Goldbach, R. (1988). Infectious RNA transcripts derived from full-length DNA copies of the genomic RNAs of cowpea mosaic virus. Virology 166, 33-41.
- Vos, P., Verver, J., Jaegle, M., Wellink, J., van Kammen, A., and Goldbach, R. (1988a). Two viral proteins involved in the proteolytic processing of the cowpea mosaic virus polyproteins. Nucleic Acids Research 16, 1967-1985.
- Zuker, M., and Stiegler, P. (1981). Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Research 9, 133-148.

CHAPTER 7

Concluding remarks

CONCLUDING REMARKS

The aim of the studies described in this thesis was to gain more insight in the complex molecular mechanisms underlying the RNA replication of the cowpea mosaic virus genome.

Previously the replication of CPMV RNA has been examined extensively with crude membrane fractions prepared from CPMV infected cowpea leaves (Zabel, 1978; Dorssers, 1983). These studies resulted in the identification of a host-encoded RNA-dependent RNA polymerase (Mr 130K), with unknown biological function, and the virus-encoded replicase (Mr 110K). As integral constituent of a membrane bound replication complex (RCX) the viral replicase was only capable of elongating viral plus-sense RNA chains that have already been initiated *in vivo*. Furthermore, the *in vitro* activity was not representative for the *in vivo* situation in that no single-stranded (ss) progeny RNA production was observed. To resolve the mechanism of initiation of CPMV RNA synthesis and to examine the role of different virus-encoded proteins and specific genomic RNA sequences in this process a replication system that also accepts exogenously added RNA templates is required. Despite exhaustive attempts it has not yet been possible to reconstitute polymerase activity after the removal of the tightly bound template RNA from crude replication complexes isolated from CPMV infected cowpea leaves. In chapter 2 we have explained that CPMV RNA replication probably requires a primer and a template for the initiation of RNA synthesis. Based on this idea we have tried to reconstitute polymerase activity with a variety of mixtures of primers and templates. Wildtype CPMV-RNA and plus or minus-stranded transcript RNAs were tested as exogenously added templates. The primers used in the assays were oligo(U) or a short RNA stretch corresponding with the first 94 nucleotides of the viral B-RNA. In addition a mixture of poly(A) and oligo(U) as used in the research on RNA replication of poliovirus, has been tested in our system. Since the viral replicase activities, isolated from CPMV infected cowpea tissue, were low, incomplete and overshadowed by the strong host-encoded RNA-dependent RNA polymerase activity, the *in vitro* RNA replication experiments were difficult to perform and it was troublesome to interprete the results. In an attempt to overcome these problems the *in vitro* RNA replication assays and reconstitution experiments were extended to crude membrane fractions, prepared from another systemic host of CPMV, *Chenopodium amaranticolor*. Indeed the host-encoded RNA-dependent RNA polymerase activity was less prominent in this plant and it was shown that due to the greater stability of the CPMV-RCX in this host,

ss progeny RNA was produced. However, as a result of the more stable RCX too, it was very difficult to remove the endogenous RNA template and hence reconstitution of polymerase activity with exogenously added templates was not possible.
As an alternative approach to analyse the function of viral proteins in the CPMV RNA replication, we have started to examine the possibility of reconstituting a system for viral RNA synthesis from CPMV encoded proteins produced in Escherichia coli. For that purpose fragments of cDNA clones containing the coding regions of the B-RNA encoded 87K putative core polymerase and the 110K viral replicase were used to produce sizable levels of these proteins in E. coli. Such an expression system may generate a template-dependent activity due to CPMV proteins which are not associated with membranes and hence could be more amenable to study the RNA replication process. This RNA synthetic activity has been demonstrated successfully for polioviral polymerase expressed in E. coli. Although the expected CPMV encoded proteins were synthesized, they did not show any polymerase activity, contrasting with the highly active polio polymerase, simultaneously obtained in the same way. Since poliovirus and CPMV have analogous features of the genomic RNA and their expression strategy and show a comparable functional organization in their polyproteins, it has been assumed that both viruses probably would have a similar RNA replication mechanism. The results however contradict this idea and indicate that the analogy between CPMV and poliovirus can not be extended to homologous mechanistical aspects of the virus multiplication. This hypothesis is strengthened by the differences in the expression mechanisms (Wellink, 1988). Probably as a result of the split genome of CPMV, whereas poliovirus has a genome consisting of a single RNA molecule, gene expression will be differently regulated. This makes sense since for the production of progeny virus a single RNA molecule provided with a single VPg is required versus 60 copies of each of the capsid proteins. The divided genome of CPMV enables a regulated production of these proteins, whereas for poliovirus such regulation is not possible. Since the expression of virus-encoded proteins is needed for viral RNA replication, it is plausible to assume that differences in the regulation of gene expression may result in different RNA replication mechanisms for poliovirus and CPMV.
Although the 87K and 110K B-RNA-encoded proteins produced in E. coli appear to be not capable or not sufficient for polymerase activity, the expression system has shown to be very useful for the production of separate virusencoded proteins, among which the active viral protease.
Based on both the expression experiments and the inability to prepare a template-dependent replication system, it seems a plausible hypothesis that the

processing products 87K and 110K, already folded in a certain way, are not able to (re)initiate RNA replication on an added template, but that a larger precursor protein from which the polymerase is simultaneously cleaved and incorporated into an active RCX is needed. If this hypothesis is true it would be impossible to observe complementation between two mutant B-RNA molecules, each encoding only a part of the open reading frame such that the two mutant RNA molecules together produce the complete set of B-RNA encoded proteins. These experiments still have to be performed.

As polymerase activity is only expected upon binding of the proteins to a template and upon subsequent initiation of complementary RNA strand synthesis, it would be worthwhile to study these processes independently. Firstly, one can examine the binding between specific polymerase proteins, which may be isolated from the E. coli expression system, and templates, followed by reconstitution of a functional replication complex by the addition of suitable primers like specific oligonucleotides or VPg in various forms and membranes which can be isolated from plant or synthesized in vitro.

For the examination of specific nucleotide sequences involved in the viral RNA replication infectious transcripts produced from full-length DNA copies of B- and M-RNA have been exploited (Vos, 1987). Both the infection procedure and the specific infectivity of the transcripts were improved, resulting in a system suitable to introduce site specific mutations in the viral RNAs and to analyse the effects of such mutations on the RNA replication in vivo.

The potential of the infectious transcripts has been demonstrated by the analysis of genomic RNA sequences with putative RNA replication signals. By subtile modifications, a function in the RNA replication could be described to an homologous nucleotide-stretch which is present in the 3' region of both B- and M-RNA. The importance of these nucleotides in the viral RNA multiplication was strengthened by the reversion of the mutated stretches to the wildtype sequence in this area during replication cycles in vivo.

As the reversion was a stepwise process, the reversion probably occurs by the preferential multiplication of those RNA molecules which have advantageous point mutations in this nucleotide stretch. Alternatively, recombination with the homologous area in the wildtype M-RNA transcript could be the underlying mechanism for reversion. When B- and M-RNA transcripts with identical mutations in this region will be used as inoculum, one can discriminate between these two possibilities. Whether the nucleotide stretch indeed forms the supposed hairpin, which may protect the RNA against degradation or alternatively have a specific signal function in RNA replication or encapsidation, remains an interesting

question to be solved.
A major advantage for further studies on the RNA replication of CPMV is that this virus has a genome consisting of two RNA molecules. B-RNA can replicate independently and contains the genetic information needed for the RNA multiplication. M-RNA contains the information for the cell to cell transport of the virus and needs B-RNA encoded proteins for its multiplication. This dependency must be exploited in future experiments by modifying the M-RNA sequence at specific places while keeping the B-RNA encoded replication machinery intact. Such studies may considerably contribute to the fully understanding of the complex RNA replication mechanism of CPMV and related viruses.

Zabel, P. (1978). Thesis, Agricultural University, Wageningen, The Netherlands.
Dorssers, L. (1984). Thesis, Agricultural University, Wageningen, The Netherlands.
Vos, P. (1987). Thesis, Agricultural University, Wageningen, The Netherlands.
Wellink, J. (1987). Thesis, Agricultural University, Wageningen, The Netherlands.

CHAPTER 8

Samenvatting

SAMENVATTING

Virussen die parasiteren op planten, vormen een groep met een enorme variatie wat betreft vorm en grootte van het virusdeeltje, structuur van de moleculen die de genetische informatie bevatten (genoom) alsmede de manier waarop de genetische informatie omgezet wordt in functionele eiwitten (expressie). Binnen deze variabele groep vertegenwoordigen de virussen met een positief enkelstrengs RNA genoom, dat direct als boodschapper RNA kan dienen, de meerderheid en zijn moleculair genetisch het best bestudeerd. Informatie over de moleculaire mechanismen die ten grondslag liggen aan de vermenigvuldiging van virale RNAs (replicatie) is echter slechts spaarzaam aanwezig. Als basismechanisme wordt aangenomen dat de replicatie verloopt via de synthese van een complementaire negatieve RNA keten die vervolgens als matrijs dient voor de productie van positieve virale RNA moleculen. Voor deze RNA-vermenigvuldiging, die telkens start bij het 3' uiteinde van de matrijs, is de expressie van het virale genoom nodig. Aangezien zowel de terminale structuren van het genoom alsook de expressiemechanismen per virusgroep variëren, is het logisch te veronderstellen dat iedere groep zijn eigen specifieke manier heeft om het virale RNA te multipliceren. Deze veronderstelling wordt in detail bediscussieerd in hoofdstuk 2.
Het doel van het in dit proefschrift beschreven onderzoek is het verkrijgen van inzicht in de virale genen en genomische nucleotide sequenties die een rol spelen in het replicatiemechanisme van cowpea mosaic virus (CPMV) dat als gastheer de cowpea, Vigna unguiculata (kouseband) infecteert. Aangezien over de structuur van het genoom en de manier waarop de genetische informatie van dit virus tot expressie komt al veel bekend is, is CPMV een geschikt onderzoeksobject voor het onderzoek naar de virale RNA replicatiemechanismen. Het genoom van dit virus bestaat uit twee RNA moleculen (B- en M-RNA) met positieve polariteit, die elk afzonderlijk ingepakt in identieke eiwitmantels de virusdeeltjes vormen. De RNA moleculen worden gekenmerkt door een klein eiwit (VPg) aan het 5' uiteinde en een poly(A)-staart aan het 3' uiteinde en worden tot expressie gebracht door de productie van polyeiwitten die vervolgens door specifieke proteolyse gesplitst worden in functionele eiwitten. De genetische informatie is functioneel verdeeld over beide RNAs; het M-RNA codeert voor de manteleiwitten en eiwitten die betrokken zijn bij het transport van het virus door de plant terwijl het B-RNA de informatie bevat die essentieel is voor de virale RNA replicatie. In eerder onderzoek werden een door het gastheer gecodeerd RNA afhankelijk RNA polymerase, met nog steeds onbekende functie, en een door het virus gecodeerd, membraan

gebonden replicase geïdentificeerd. Uit deze studies bleek dat het virale replicase, geïntegreerd in een replicatiecomplex, niet in staat was om in een celvrij extract (in vitro) de synthese van nieuwe RNA ketens te initiëren.
Om het initiatiemechanisme van de virale RNA replicatie te bestuderen was het nodig een in vitro systeem te ontwikkelen dat toegevoegde RNA moleculen als matrijs zou kunnen gebruiken. Ondanks verwoede pogingen bleek het onmogelijk om replicase activiteit te reconstrueren nadat de oorspronkelijke RNA-matrijs uit het replicatiecomplex verwijderd was. Uit deze experimenten bleek ook dat in de cowpea-extracten, gemaakt van door CPMV geïnfecteerde planten, de synthese van het belangrijkste replicatie product, het enkelstrengs virale RNA, niet detecteerbaar was. Daarom werden de in vitro RNA replicatie experimenten uitgevoerd met ruwe membraan fracties afkomstig van een andere gastheer van CPMV: Chenopodium amaranticolor (ganzevoet) (Hoofdstuk 3). Het CPMV replicatiecomplex bleek in deze plant een stabielere conformatie te bezitten, hetgeen resulteerde in een detecteerbare productie van enkelstrengs genomisch CPMV-RNA. Deze stabiliteit van het replicatiecomplex echter veroorzaakte tevens dat de endogene matrijs moeilijk te verwijderen was en het replicatiecomplex niet meer toegankelijk bleek voor door ons aangeboden RNA moleculen.
Als een alternatieve manier om de functie van door het virus gecodeerde eiwitten in de RNA replicatie te bestuderen werd geprobeerd om RNA-afhankelijke RNA-polymerase activiteit te meten met behulp van eiwitten die afkomstig waren uit een heteroloog systeem. Daartoe werden fragmenten van DNA copieën, die de genetische informatie voor het virale replicase bevatten, tot expressie gebracht in de bacterie Escherichia coli. Ondanks het feit dat de verwachte virusspecifieke eiwitten gemaakt werden, was geen polymerase activiteit toe te wijzen aan deze CPMV eiwitten. Dit in tegenstelling tot het nauw verwante en op identieke manier verkregen polymerase van poliovirus, dat zeer efficiënt bleek te functioneren (Hoofdstuk 4). Mogelijk behoeft het polymerase van CPMV post-translationele modificaties, hetgeen bewerkstelligd zou kunnen worden door gebruik te maken van een eukaryotisch expressie systeem. Gebaseerd op de experimenten, beschreven in de hoofdstukken 3 en 4 zou het een aannemelijke hypothese kunnen zijn, dat het virale polymerase, zoals dat aanwezig is in het replicatiecomplex en geproduceerd wordt in E. coli de RNA synthese niet kan initiëren omdat het een, voor de verlangde activiteit, verkeerde structuur bezit. Wellicht dat voor dit proces de grotere poly-eiwitten nodig zijn die eerst een complex vormen met een RNA matrijs en vervolgens specifiek gekliefd worden tot het kleinere functionele polymerase (Hoofdstuk 2).
Aangezien de polymerase activiteit dan pas waarneembaar is als het eiwit en de

RNA matrijs elkaar herkennen, binden en vervolgens de complementaire RNA streng synthetiseren, zou het bestuderen van de afzonderlijke deelreacties een goede mogelijkheid zijn om het onderzoek in deze richting te vervolgen. De hiervoor benodigde, door het virus-gecodeerde, eiwitten zouden geproduceerd kunnen worden in het bacteriële expressie systeem.
Tijdens het onderzoek werden van zowel het M- als het B-RNA volledige DNA copieën gemaakt, waarvan infectieuze RNA transcripten konden worden verkregen. De lage specifieke infectieusiteit van deze eerste generatie transcripten liet echter de analyse van specifieke nucleotide sequenties in het RNA genoom, met een mogelijke signaal functie in de RNA replicatie, niet toe. Door enerzijds de infectie-procedure te optimaliseren en anderzijds transcripten te produceren met slechts een niet viraal G residu aan de 5' kant en 5 niet virale nucleotiden aan de 3' kant werd een systeem verkregen dat geschikt was om het effect van specifieke mutaties in het transcript op de RNA replicatie in vivo te bestuderen (Hoofdstuk 5). Het aanbrengen in het B-RNA, dat onafhankelijk van het M-RNA kan repliceren in geïsoleerde plantecellen, van subtiele mutaties in een reeks van 11 nucleotiden die aanwezig is in de 3' gebieden van zowel het B- als het M-RNA resulteerde in het toewijzen van een functie in de RNA replicatie aan deze sequentie. Het belang van deze sequentie werd versterkt doordat de gemuteerde sequentie tijdens de RNA replicatie in de plant reverteerde naar de wildtype sequentie in dit gebied. Het mechanisme dat ten grondslag ligt aan deze reversie zou een preferentiële vermenigvuldiging kunnen zijn van de RNA moleculen met een voordelige puntmutatie of een recombinatie met de homologe, onveranderde, sequentie in het M-RNA. Het is duidelijk dat deze vraag, evenals de onbekendheid van de specifieke functie van de 11 nucleotiden verder onderzoek behoeft.
De resultaten beschreven in dit proefschrift illustreren het potentieel van de opgedane kennis en vaardigheden in het ontrafelen van de moleculaire mechanismen die plaatsvinden tijdens de vermenigvuldiging van CPMV.
Verdere studies waarbij gebruik gemaakt zal worden van efficiënte heterologe expressie-systemen en op specifieke plaatsen gemuteerde RNA transcripten zullen een aanzienlijke bijdrage leveren in de kennis van het complexe RNA replicatiemechanisme van CPMV.

CURRICULUM VITAE

Rik Eggen werd op 22 oktober 1959 in Heerlen geboren, waar hij de lagere school (Don Santo school) en de middelbare school (Gymnasium β, Berdardinuscollege) doorliep. In 1978 begon hij met zijn studie biologie aan de Katholieke Universiteit te Nijmegen. In oktober 1981 werd het kandidaatsexamen behaald. Zijn doctoraalfase werd uitgevoerd op de afdelingen Chemische Cytologie (Prof.Dr. G. Borst-Pauwels; onderzoek naar de biochemische mechanismen achter de resistentie van gist tegen zware metalen), Microbiologie (Prof.Dr. G. Vogels; onderzoek naar de methanogenese van Methanosarcina barkeri met acetaat als koolstof- en energiebron) en Moleculaire Biologie (Prof.Dr. J.G.G. Schoenmakers en Prof.Dr. R.N. Konings; onderzoek naar de replicatie van de filamenteuze bacteriofaag Pf3). De studie biologie werd op 30 oktober 1984 afgesloten met het behalen van het doctoraalexamen.
Van 1 november 1984 tot en met 31 oktober 1988 was hij werkzaam op de afdeling Moleculaire Biologie van de Landbouwuniversiteit Wageningen (Prof.Dr. A. van Kammen), waar hij het in dit proefschrift beschreven, en door de "Nederlandse organisatie voor Wetenschappelijk Onderzoek" gesubsidieerde onderzoek verrichtte.
Vanaf 1 november 1988 is hij als wetenschappelijk medewerker verbonden aan de afdeling Microbiologie van de Landbouwuniversiteit Wageningen (Prof.Dr. A.J.B. Zehnder en Prof.Dr. W.M. de Vos) waar hij de genetica van Archaebacteriën bestudeert.