CHARACTERIZATION OF THE VIRAL PROTEINS INVOLVED IN THE RNA REPLICATION OF COWPEA MOSAIC VIRUS

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Voor mijn ouders

Stellingen

1. De virale eiwitten die betrokken zijn bij de RNA replicatie van cowpea mosaic virus worden niet allemaal door het B-RNA gecodeerd.

   Dit proefschrift.

2. Positieve-strengs RNA virussen maken voor hun RNA replicatie gebruik van eiwitten die in de gastheercel betrokken zijn bij de translatie.

   Blumenthal en Carmichael (1979). Annu. Rev. Biochem. 48, 525-548.
   Quadt et al. (1993). Proc. Natl. Acad. Sci. USA 90, 1498-1502.
   Janda en Ahlquist (1993). Cell 72, 961-970.
   Andino et al. (1993). EMBO J. 12, 3587-3598.

3. Het bestuderen van het copiëren van een RNA matrijs *in vitro* door het poliovirus polymerase, waarbij gebruik gemaakt wordt van oligo(U) als primer, zal niet leiden tot meer inzicht in het RNA replicatie mechanisme van poliovirus.

4. De argumenten die Waldherr et al. geven, rechtvaardigen niet de titel van hun ingezonden brief.

   Waldherr et al. (1993). *MRS6-yeast homologue of the choroideremia gene.* Nature Genetics 3, 193-194.

5. De aanwezigheid van het P1 eiwit van cauliflower mosaic virus in tubulaire structuren van protoplasten die door dit virus geïnfecteerd zijn, maakt het onwaarschijnlijk dat de RNA-bindende eigenschappen van dit eiwit iets met virustransport te maken hebben.

   Citovsky et al. (1991). Proc. Natl. Acad. Sci. USA 88, 2476-2480.
   Perbal et al. (1993). Virology 195, 281-285.

6. Genen die betrokken zijn bij erfelijke aandoeningen bij de mens zullen in de toekomst vaker in de computer worden gevonden dan in het laboratorium.

7. Het nieuwe biljet van honderd gulden geeft treffend weer dat het kabinet Lubbers-3 niet in staat is de kosten te drukken.

8. De frequentie van helicoptervluchten boven het St. Radboud ziekenhuis in Nijmegen doet vermoeden dat spoedeisende medische ingrepen met name op mooie zomerse dagen vereist zijn.

9. Achteraf is het vaak alsof je een voorgevoel hebt gehad.

10. Een permanent is slechts tijdelijk.

11. Het dragen van werkkleding tijdens de lunch door artsen en co-assistenten is ergerniswekkend en medisch onverantwoord.

Stellingen behorende bij het proefschrift
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Chapter 1

General Introduction

I. INTRODUCTION

A large number of viruses that infect animals or plants possess a positive (+)-strand (messenger-sense) RNA genome. Due to the advances in molecular biology much has been learnt in the last decade about the structure, the molecular organization and expression of such viral genomes. Yet, replication of the viral RNA genomes is still a poorly understood event in the viral life-cycle. The understanding of the biochemistry of viral RNA replication would be considerably helped by the availability of an *in vitro* replicase system. Since more than 25 years *in vitro* replicase systems have been on hand for certain RNA phages and, especially in the case of Qβ, have provided detailed knowledge about the enzyme activities involved in phage RNA replication (Blumenthal and Carmichael, 1979). In contrast to the relative ease by which an *in vitro* replication system was obtained for these RNA phages, the isolation of RNA replicase complexes capable of replicating viral RNA *in vitro* appears to be an arduous task for eukaryotic RNA viruses and only recently this has been accomplished for the first time for cucumber mosaic virus (Hayes and Buck, 1990).

Replication of the genomic RNAs of single-stranded (+)-sense RNA viruses requires the formation of complementary (-)-strand RNA using genomic RNA as a template, whereupon progeny viral RNA can be synthesized using (-)-strand RNA template. The RNA replicase activity is catalyzed by a viral RNA-dependent RNA polymerase (RdRp) and proceeds in a 5' to 3' direction. Consequently, initiation of RNA synthesis has to occur at the 3' end of the template RNA. Initiation of RNA synthesis is a crucial step in the replication cycle as both the 3' ends of (+)- and (-)-strand RNA must be specifically recognized by the replicase among the abundant cellular RNAs. As the genomic RNAs of viruses of distinct groups have different terminal structures there will probably be different mechanisms of initiation of RNA synthesis. Up to now very little is known about the process of initiation of viral RNA synthesis. Upon their synthesis progeny RNA strands are released from their template RNA, a process that might be dependent on the unwinding activity of an RNA helicase. In addition, RNA helicase activity may also be required for unwinding of secondary structures in the template RNA. Accordingly, the RdRp is associated with other proteins, encoded by the virus and/or host, to constitute a structure that specifically recognizes and replicates the viral RNA and is therefore termed RNA replication complex.

The aim of the investigations described in this thesis has been to gain more insight into the biochemical properties of the proteins involved in the replication of the genomic RNAs of the plant comovirus cowpea mosaic virus (CPMV; for reviews see Goldbach and van Kammen, 1985; Eggen and van Kammen, 1988). For a better understanding of the complexity of the replication mechanism of CPMV and to fully appreciate the specific problems involved in its study, in the following a comparison will be made first with replication of the genome of other (+)-strand RNA viruses. Then I will discuss the previous approaches undertaken to elucidate the mode of RNA replication of CPMV (Dorssers, 1983; Eggen, 1989).

II. POSITIVE-STRAND RNA VIRUSES CAN BE DIVIDED IN SUPERGROUPS

The (+)-sense RNA viruses differ in characteristics like capsid morphology, serology, organization and expression of the genome and a variety of biological features and are subdivided into subgroups on basis of these differences. Despite the diversity among (+)-sense RNA viruses, computer-aided sequence comparisons of the viral encoded proteins have revealed remarkable homologies among the non-structural proteins of both plant and animal (+)-sense RNA viruses.

Recognition of conserved sequence motifs has led to the 'supergroup' concept, which places a large number of eukaryotic (+)-sense RNA viruses into two supergroups; i.e. the picorna-like viruses and the Sindbis- or alpha-like viruses (Goldbach 1990; Strauss and Strauss, 1988; Goldbach et al., 1991; King et al., 1991).

The picorna-like supergroup comprises the animal picornaviruses and plant viruses of the como-, nepo-, bymo- and potyvirus groups. The structure, organization and expression of the genome of the viruses belonging to this supergroup are rather similar irrespective of their genome being monopartite (picorna- and potyviruses) or bipartite (como-, bymo- and nepoviruses). The genomic RNAs have a small viral protein covalently linked to their 5' ends (VPg; viral protein, genome linked) and a 3' terminal poly(A) tail. For each virus translation of the genomic RNAs yields large polyproteins, which are processed into smaller proteins by action of viral proteases. Moreover, significant sequence homology exists between the corresponding non-structural proteins encoded by these viruses and the arrangement of the genes for these proteins on the genetic map is strikingly similar. This is best exemplified by comparing poliovirus (a picornavirus) with CPMV, which reveals that the poliovirus 2C, 3B, 3C and 3D proteins and the analogous CPMV proteins, 58K, VPg, 24K and 87K, are encoded in the same order on the genome (Franssen et al., 1984a; Argos et al., 1984). The corresponding proteins have significant homology in amino acid sequence and probably perform similar functions in viral RNA replication.

The plant viruses belonging to the alpha-like supergroup are all related to the animal Alphaviridae, of which Sindbis virus is the best-characterized member. Although, the structure and expression of the genome of the various members of this supergroup is more diverse than for viruses of the picorna-like group, they all specify a number of proteins exhibiting significant sequence homology, encoded by a similarly ordered gene set. Viruses of the alpha-like supergroup have capped RNA genomes and produce one or more subgenomic mRNAs for expression of some of their genes. On the other hand, the 3' terminus of the RNAs is rather variable and may be either a poly(A) tail, a tRNA-like structure or devoid of any apparent structure.

For both the picorna-like and the alpha-like supergroup viruses the non-structural proteins with conserved amino acid domains have been shown or implied to be involved in viral RNA replication. In the next part of this paragraph I shall first discuss some of the characteristics of these proteins and then briefly review RNA replication for selected members of each supergroup.

III. CONSERVED DOMAINS WITHIN VIRAL REPLICATIVE PROTEINS

The RNA-dependent RNA polymerases (RdRp's) of animal viruses, plant viruses and bacteriophages have characteristic conserved sequence motifs in their amino acid sequence (Kamer and Argos, 1984; Poch et al., 1989; Koonin, 1991). One prominent block is S/GTXXXTXXXNT/S (in which X may be any amino acid) followed 21 to 52 amino acids downstream by a second block consisting of a highly conserved GDD sequence embedded in a stretch of hydrophobic amino acid residues (Kamer and Argos, 1984; Franssen et al., 1984a). The GDD consensus sequence is supposed to be at or near the catalytic site of the polymerase molecule. Indeed, single amino acid substitutions at the glycine in the GDD sequence of the viral RNA polymerases of Qβ and poliovirus resulted in partial or complete loss of polymerase activity (Inokuchi and Hirashima, 1987; Jablonski et al., 1991).

A second group of proteins conserved among picorna-like and alpha-like viruses contain a GXXXGKS/T nucleotide binding (NTP)-motif (Walker et al., 1982; Gorbalenya et al., 1985).

Computer-aided sequence comparison between the NTP-motif-containing proteins of (+)-strand RNA viruses has led to their classification in three major groups; the NTP-binding proteins of alpha-like viruses, picorna-like picorna- and comoviruses and picorna-like poty- and bymoviruses (Gorbalenya et al., 1988, 1989; Hodgman, 1988). Strikingly, the NTP-binding proteins of alpha-like viruses and picorna-like poty- and bymoviruses bear resemblance to helicases that are related to eukaryotic translation initiation factor eIF-4A, whereas NTP-binding proteins of the picorna-like picorna- and comoviruses appear to be related to SV40 large T-antigen, a protein containing RNA and DNA helicase activity (Gorbalenya and Koonin, 1989; Gorbalenya et al., 1990; Lain et al., 1989). The suggestion arising from these sequence comparisons have led Lain et al. (1990, 1991) to test viral proteins for helicase activity and indeed they were able to show that the cilindric inclusion protein of plum pox (poty)virus contains a RNA helicase activity dependent on the hydrolysis of ATP to ADP.

In addition to the conserved sequence motifs in a RdRp and a putative helicase, other conserved amino acid sequences are found in proteins shared among viruses within each of the supergroups. The alpha-like viruses encode a protein that exhibits an RNA methyltransferase activity, required for capping of the genomic viral RNAs (Mi et al., 1989; Dunigan and Zaitlin, 1990). The picorna-like viruses lack this protein but instead encode VPg, which is present at the 5' ends of newly synthesized RNA strands of both (+)- and (-)-sense polarity. VPg is supposed to be involved in initiation of RNA synthesis, although its precise role in this is still unclear (Takeda et al., 1986; Tobin et al., 1989). Like other proteins encoded by the picorna-like viruses, VPg is released by proteolytic processing from the viral primary translation products. For proteolytic processing of their polyproteins, proteases are encoded by all picorna-like viruses.

IV. REPLICATION OF THE GENOME OF POSITIVE-STRAND RNA VIRUSES

A. Genomic RNA replication of alpha-like viruses

For several animal viruses, such as Sindbis virus and Semliki Forest virus (Strauss and Strauss, 1986), and plant viruses, such as tobacco mosaic virus (TMV; Young and Zaitlin, 1987) belonging to the alpha-like supergroup so-called template-independent RdRp preparations have been obtained from infected cells. Upon the isolation of template-independent enzyme preparations from virus-infected cells the RdRps are found to be associated with template viral RNA and membrane structures. These RdRps are capable of elongating nascent (+)-strand RNA, the synthesis of which has been initiated *in vivo*, but are unable to use exogenous RNA templates to show RNA polymerase activity. Template-independent RdRp preparations can not be used for the study of steps in viral RNA replication, like (-)-strand synthesis and initiation of RNA synthesis, but they have been proven useful for identification the proteins catalyzing elongation of (+)-strand RNA.

For some alpha-like plant viruses, such as turnip yellow mosaic virus (TYMV; Mouches et al., 1974), brome mosaic virus (BMV; Miller and Hall, 1983), alfalfa mosaic virus (AlMV; Houwing and Jaspars, 1986), RdRp preparations are available which require addition of an RNA template for replicase activity. These RdRp preparations, referred to as template-dependent, were obtained by releasing the enzymatic activity from cellular membranes using non-ionic detergents like Lubrol, dodecyl-β-D-maltoside and Nonidet P-40, followed by removal of endogenous template RNA with micrococcal nuclease. The template-dependent RdRps of the bromoviruses, BMV (Miller and Hall, 1983; Quadt et al., 1988) and cowpea chlorotic mottle virus (CCMV; Miller and Hall, 1984) and the

ilarvirus AlMV (Quadt et al., 1991) show strong preference for their own genomic RNAs or of related viruses. Minus-strand RNAs are poor templates for such purified bromoviral RdRps, but the RdRp of BMV is able to produce subgenomic messenger RNA 4 by internal initiation on (-)-strand RNA 3 (Miller et al., 1985). The BMV template-dependent RdRp preparation has been used extensively to define the sequences of the tRNA-like structure at the 3' end of the viral RNAs that are important for the *in vitro* synthesis of (-)-strand RNA and for the identification of the subgenomic promoter for the synthesis of (+)-strand subgenomic RNA 4 (Dreher and Hall, 1988a, b; Marsh et al., 1988; French and Ahlquist, 1988). Analysis of the protein content of the BMV RdRp revealed the presence of the viral non-structural proteins P1 (methyltransferase and helicase domains) and P2 (polymerase domain) and several host-encoded proteins (Bujarski et al., 1982; Quadt et al., 1988). The AlMV RdRp contains in addition to the viral P1 and P2 proteins the coat protein, which has been implicated as a regulatory factor in AlMV RNA synthesis (Houwing and Jaspars, 1987; Quadt et al., 1991).

Thus far, the only well characterized *in vitro* RNA replication complex of an eukaryotic RNA virus has been described by Hayes and Buck (1990) for cucumber mosaic virus (CMV). They reported the purification of a RdRp preparation that is completely dependent on addition of CMV RNA template for activity, and that catalyzes the synthesis of both full-length (+)- and (-)-strand RNA and subgenomic RNA 4. No replicase activity was observed using the exogenous genomic RNAs of TMV, tomato bushy stunt virus and red clover mosaic virus. The CMV replicase was shown to contain the viral proteins P1 and P2 and a host (tobacco) protein of 50K, which all three seemed to be required for RNA synthesis. The identity of the 50K host protein has not yet been solved. A less-purified CMV RdRp preparation has been described by Quadt et al. (1991), which is capable of using the genomic RNAs of viruses of different taxonomic groups; i.e. CMV, AlMV, BMV and TMV as a template. The activity in their RNA polymerase preparation was inhibited by addition of ribosomal RNA, but not by tRNA, mRNA or several viral RNAs. This finding led the authors to hypothesize that the inhibition of polymerase activity was caused by the binding of a ribosomal protein involved in RNA replication to ribosomal RNAs. Interestingly, the involvement of a ribosomal protein in viral RNA replication is precedented by Qβ (Blumenthal and Carmichael, 1979).

B. Genomic RNA replication of picorna-like viruses

The mode of viral RNA replication for members of the picorna-like supergroup has been studied in greatest detail for human poliovirus and the plant cowpea mosaic virus. A distinct property of viruses of the picorna-like supergroup which distinguishes them from the alpha-viruses is the presence of VPg covalently-bound to the 5' ends of both (+)- and (-)-strand RNA. Therefore, any model for picorna-like viral RNA replication must account for the occurance of VPg at the 5' end of each progeny RNA molecule.

Poliovirus RNA synthesis is associated with membranes and, consequently, the crude membrane fraction of poliovirus-infected cells has been used extensively for the study of poliovirus RNA replication. The *in vitro* RNA synthesizing activity in such membrane fractions, also called crude replication complexes, is capable of elongating nascent RNAs the synthesis of which had been initiated *in vivo* and which already had VPg at their 5' ends. Treatment of these membrane complexes with detergent successfully dislodged the polymerase activity from the membranes, but did not free the enzyme from endogenous RNA (Lundquist et al., 1974). Both (+)- and (-)-strand

RNA products could be synthesized by this solubilized activity, but initiation of RNA synthesis did not seem to occur as addition of exogenous template had no effect on the activity (Lundquist and Maizel, 1978). These results demonstrate that a membranous environment is not prerequisite for the RNA-elongating activity in poliovirus RNA replication.

The purification of a template-dependent poliovirus RdRp activity was helped along by the assay developed by Flanegan and Baltimore (1977), that demonstrates that the poliovirus polymerase is capable of copying an exogenous poly(A) template with oligo(U) annealed to it as a primer. This polymerase assay allowed the definitive assignment of poliovirus protein $3D^{pol}$ as the ezyme that catalyzes RNA elongation in poliovirus RNA replication (Van Dyke and Flanegan, 1980). The oligo(U)-primed poliovirus polymerase activity is also able to transcribe CPMV RNA and β globin mRNA and is thus not specific for poliovirus RNA (Tuschall et al., 1982). Furthermore, despite the usefulness of this polymerase assay in the characterization of the RNA-elongating activity of $3D^{pol}$, this assay does not reflect the mechanism of *in vivo* initiation of poliovirus RNA synthesis as free oligo(U) is not found in poliovirus-infected cells.

In attempts to accomplish faithful RNA initiation *in vitro*, host factors were isolated from uninfected HeLa cells, which allowed RNA synthesis on viral RNA template in the absence of an oligo(U) primer (Dasgupta et al., 1980; Morrow et al., 1985; Andrews and Baltimore, 1986). One of these host factors was shown to contain terminal uridylyl transferase activity, capable of adding uridine residues to the 3' end of poliovirus RNA (Andrews and Baltimore, 1986). This finding led to the proposal of a mechanism of initiation of (-)-strand RNA synthesis, which included elongation of the poly(A) tail with uridines followed by hybridization of the added uridine residues onto the poly(A) tail. The hairpin primer thus generated could then be elongated by $3D^{pol}$ into products up to twice the length of the viral genome. It was subsequently claimed that addition of VPg to the newly synthesized (-)-strand RNA could occur by a self-catalyzed cleavage-linkage of VPg at the dimer junction (Tobin et al., 1989), but this was never substantiated by any observations *in vivo*. Moreover, if such model may appear plausible for the synthesis of VPg-linked (-)-strand RNA, it does not give a satisfactory explanation for the initiation of (+)-strand RNA synthesis. The involvement of host factors like terminal uridylyl transferase in the initiation of poliovirus RNA replication has suffered from the observation that snap-back structures in the template RNA formed by a non-specific endonuclease activity in the host factor preparations serve as a primer in RNA synthesis (Lubinski et al. 1986; Hey et al., 1987). Another model for initiation of poliovirus RNA synthesis proposes a primer function for uridylylated VPg. It has been shown that in crude membrane preparations of poliovirus-infected cells uridylylation of VPg (or a precursor) can be achieved, resulting in the *in vitro* synthesis of VPg-pU and VPg-pUpU (Takegami et al., 1983). Preformed VPg-pUpU could be elongated into longer products (Takeda et al., 1986), suggesting that uridylylated VPg is the precursor to elongation. However, also for this model definitive proof remains to be awaited.

A different approach to study poliovirus RNA replication makes use of infectious RNA copies that can be obtained from the cloned poliovirus cDNA sequence. Studies with transcripts of *in vitro* mutagenized poliovirus cDNAs have shown that RNAs that are unable to produce viral non-structural proteins are not co-replicated in HeLa cells by co-inoculated wild type (wt) poliovirus (Bernstein et al., 1986; Hagino-Yamagishi and Nomoto, 1989). These results are taken for evidence that translation and replication of poliovirus RNA are tightly linked and/or that at least one of the non-structural proteins is cis-acting in replication.

From the data described above it will be clear that there is still much indistinctness about the mode of polioviral RNA replication and especially the initiation of RNA synthesis. Given the analogies found

among CPMV and poliovirus, the problems involved in the study of CPMV RNA replication are largely similar to these described above. On the other hand, a clearly distinguishing property of CPMV with respect to poliovirus is that multiplication of CPMV RNA requires replication of two genomic RNA molecules. Furthermore, the fact that CPMV is a plant virus rises some additional problems in the purification of an *in vitro* replication system, as will be seen in the next section.

V. REPLICATION OF THE GENOMIC RNAs OF CPMV

A. Structure and expression of the genome of CPMV

Cowpea mosaic virus, the type member of the plant comoviruses, has a genome consisting of two single-stranded RNA molecules each encapsidated in icosahedral particles (for reviews see Goldbach and van Kammen, 1985; Eggen and van Kammen, 1988). The two nucleoprotein particles, designated B and M component, have identical capsids composed of 60 copies of each of two different coat proteins but are distinguished by their nucleic acid content. B component contains a single RNA molecule (B-RNA) with a molecular weight of 2.04×10^6 and M component a RNA molecule (M-RNA) with a molecular weight of 1.22×10^6. Both RNAs have a small protein VPg covalently linked to their 5' ends, a poly(A) tail at their 3' ends and are of positive-sense polarity, which means that they can serve as messenger RNA (Fig. 1). Successful infection of cowpea plants by CPMV requires expression of both B- and M-RNA, however, a clear division of functions can be recognized in the execution of individual steps in the infection process. Thus the proteins encoded by M-RNA are indispensable for viral cell-to-cell transport (Wellink and van Kammen, 1989), while B-RNA supplies all the viral functions that are required for RNA replication (Goldbach et al., 1980).

The complete nucleotide sequence of both RNA molecules of CPMV has been elucidated (Lomonossoff and Shanks, 1983; van Wezenbeek et al., 1983). Extensive sequence homology was found in the 5' and 3' non coding regions of B- and M-RNA. Especially striking is a stretch of 11 nucleotides, UUUUAUUAAAA, in the 3' ends of both viral RNAs. This sequence is complementary to a stretch of 11 bases in the 5' ends of the viral RNAs, allowing one G-U base pairing, indicating that all the (+)- and (-)-strand RNAs have a very similar stretch of nucleotide sequences at their 3' ends. Therefore, this sequence may represent a recognition sequence for the viral RNA replicase. Indeed, *in vitro* mutagenesis of this sequence in the 3' end of B-RNA severely reduced virus infectivity (Eggen et al., 1989a).

Full-length cDNA clones of both viral RNAs have been obtained from which infectious RNAs can be generated by *in vitro* transcription (Vos et al., 1988a; Eggen et al., 1989b; Holness et al., 1989). These transcript RNAs and the availability of CPMV-specific antibodies has allowed a detailed analysis of the synthesis and proteolytic processing of CPMV proteins *in vitro* (rabbit reticulocyte lysates) as well as *in vivo* (Goldbach and van Kammen, 1985; Wellink et al., 1986, 1987; Vos et al., 1988b; Rezelman et al., 1989). Thus, the exact cleavage sites within the CPMV polyproteins have been mapped and identified as either a glutamine-methionine (Q/M), glutamine-glycine (Q/G) or glutamine-serine (Q/S) amino acid pair (FIG. 1). The B-RNA-encoded 24K protease was shown to be responsible for all these cleavages (Verver et al., 1987; Vos et al., 1988b). It should be emphasized that all the viral proteins described below, final cleavage products as well as precursors thereoff, have been detected in CPMV-infected plants and may therefore fulfil a function in the viral life cycle.

Translation of the single open reading frame of B-RNA results in the synthesis of a 200K

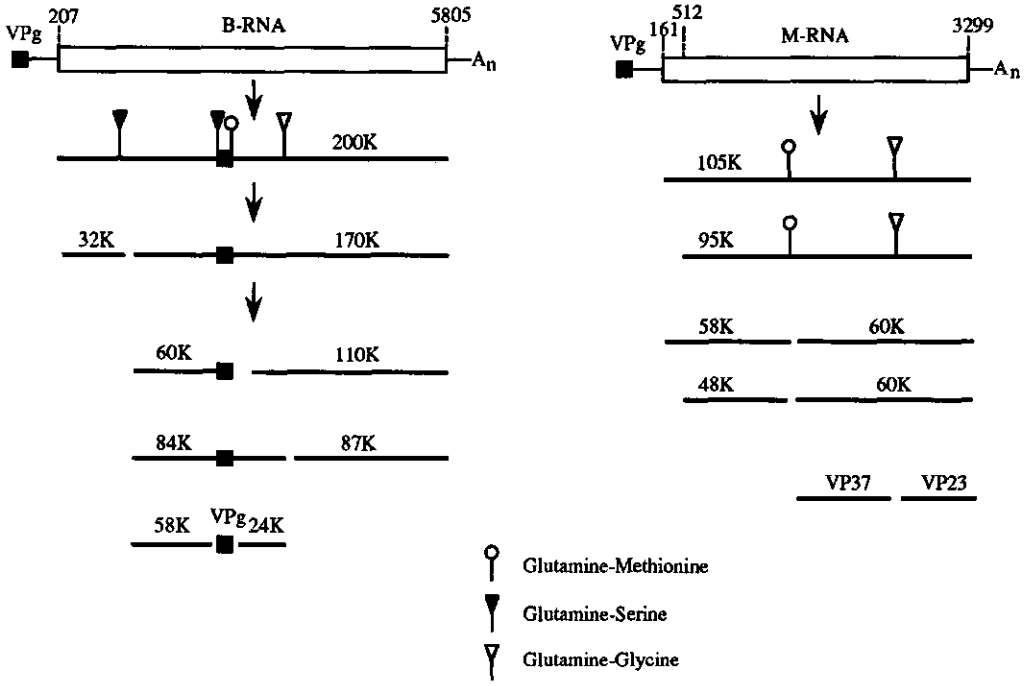

Figure 1. Genetic organization of CPMV RNAs and proteolytic processing of the primary translation products. The single open reading frame of both RNAs is represented by an open bar on which the positions of the start and stop codons are indicated. VPg is indicated by a black square, other proteins by a solid line. The position and the amino acid sequence of the proteolytic cleavage sites is indicated.

polyprotein, which is rapidly cleaved into the 32K and 170K proteins, sometimes already during its synthesis (Pelham, 1979, Franssen et al., 1984b). Further proteolytic processing of the 170K protein then occurs by three alternative pathways to give proteins of 1) 60K and 110K, 2) 84K and 87K, or 3) 58K and 112K (Rottier et al., 1980; Rezelman et al., 1980; Peters et al., 1992). The 60K, 84K, 110K and 112K proteins are further cleaved and the ultimate cleavage products of the 200K polyprotein are the 32K, 58K, VPg, 24K and 87K proteins.

Translation of M-RNA is initiated at two alternative start codons, yielding polyproteins of 105K and 95K, which have carboxy-coterminal ends (Holness et al., 1989; Verver et al., 1991). Primary cleavage of these polyproteins produces the 58K/48K proteins and a 60K protein. The 60K protein is the precursor of the two capsid proteins, VP37 and VP23, which are released by a second processing step.

The 24K protein encoded by B-RNA catalyzes all proteolytic cleavages, but it has been shown that the B-RNA encoded 32K protein has an important regulatory role in proteolytic processing of the B- and M-polyproteins. First, the 32K protein is required as a cofactor in the primary cleavage of the M-polyproteins (Vos et al., 1988b). Second, the 32K protein has an inhibitory effect on the processing of the 170K protein encoded by B-RNA (Peters et al., 1992). *In vitro* translation studies have revealed an interaction between the B-RNA encoded 32K and 58K proteins and it is probably

this protein-protein interaction that is important for the regulation of polyprotein processing (Franssen et al., 1984c; Peters et al., 1992).

B. Cellular location of CPMV RNA replication

CPMV infection is accompanied by the appearance of characteristic cytopathic structures in the cytoplasm of the cell (Assink et al., 1973; De Zoeten et al., 1974; Hibi et al., 1975). These cytopathic structures consist of large arrays of membranous vesicles surrounded by amorphous electron-dense material. CPMV B-RNA is able to replicate in cowpea protoplasts by itself, independently from M-RNA, and in such B-RNA-infected protoplasts proliferation of membranes and electron-dense material is also found (Rezelman et al., 1982). Hence, it appears that the induction of cytopathic structures is achieved by a B-RNA-encoded function and may turn out to be a prerequisite for viral RNA replication. Indeed, by autoradiography performed on sections of these cells and on isolated cytopathic structures De Zoeten et al. (1974) provided evidence that replication of CPMV RNA is associated with such membranous vesicles. It has been established that the electron-dense structures contain the bulk of the non-structural proteins encoded by CPMV B-RNA (Wellink et al., 1988), but it is not known whether these structures have like the membranous vesicles a specific function in viral RNA replication.

An indication as to how the membranous vesicles are involved in viral RNA replication may be found in the analogy with poliovirus. Membrane proliferation in poliovirus-infected cells commences with the formation of membrane protrusions at the rough endoplasmatic reticulum (rER), which eventually grow into the membranous vesicles (Tershak, 1984; Bienz et al., 1983). It has been found that the continuous phospholipid synthesis is required for efficient replication of poliovirus RNA (Guinea and Carasco, 1990). Furthermore, poliovirus RNA synthesis is strongly inhibited by Brefeldin A, which blocks vesicular protein transport from the ER to the Golgi apparatus (Maynell et al., 1992). Proteins of the P2 region of the poliovirus RNA-encoded polyprotein become associated with the rER soon after their synthesis (Bienz et al., 1983). Electron microscopy analysis has revealed that guanidine, an inhibitor of viral (+)-strand RNA synthesis, prevents replication complexes to become associated with the membrane vesicles by inhibiting the binding of protein 2C or a precursor thereoff to membranes (Bienz et al., 1987, 1990). Furthermore, it has been demonstrated that guanidine-resistant poliovirus mutants map to the highly conserved central domain of protein 2C, which has 30% sequence homology with the 58K protein of CPMV B-RNA (Pincus and Wimmer, 1986; Pincus et al., 1986). These results provide evidence that at least one of the funtions of protein 2C in viral RNA synthesis lies in the anchoring of replication complexes to the virus-induced membranes. By analogy, a similar function can be anticipated for the 58K protein of CPMV B-RNA.

C. Purification of CPMV replication complexes

Replication of CPMV RNA is associated with the vesicular membranes of the virus-induced cytopathic structures in infected cells and, accordingly, RNA polymerase activity has been detected in the crude membrane fraction of infected leaves (Zabel et al., 1974). It has further been demonstrated that the crude membrane fraction of CPMV-infected cowpea leaves actually harbors two functionally different RdRp activities; one that is encoded by the host and another that is virus-

specific (Dorssers et al., 1982, 1983). In the membrane fraction of uninfected cowpea leaves small amounts of (host) RdRp activity can be detected. In infected cowpea leaves this activity is increased at least 20-fold and represents more than 95% of the total RNA polymerase activity in the crude membrane fraction, masking almost completely the viral RdRp activity. The host RdRp can be stripped from the membranes by washing with a Mg^{2+} -deficient buffer, leaving the virus-specific RdRp activity associated with the membranes. The host RdRp, a monomeric protein of 130K, transcribes endogenous plant RNAs and viral RNAs into small (-)-sense RNA molecules (Dorssers et al., 1982; van der Meer et al., 1983). The physiological significance of the increase of the 130K host polymerase in CPMV-infected cells remains still unresolved. However, the increase of 130K polymerase is not essential for viral RNA replication as CPMV RNA replication in cowpea protoplasts is not accompanied by an increased production of 130K protein (van der Meer et al., 1984).

The virus-specific RdRp from CPMV-infected cells is capable of fully elongating nascent viral RNA chains that have already been initiated *in vivo* and thus displays the features of a viral RNA replicase. In crude membrane fractions deprived of the host RdRp, the RNA products synthesized by the viral RdRp are all (+)-stranded and are predominantly found as replicative form (RF) RNA; i.e. double-stranded full length (+)- and (-)-stranded viral RNA (Dorssers et al., 1983). The CPMV RdRp has been freed from the membranes by treatment with Triton X-100. Subsequent Sepharose 2B chromatography and glycerol gradient centrifugation yielded a highly purified RdRp preparation still capable of elongating nascent RNA chains to full-length viral RNAs (Dorssers et al., 1984). This result demonstrates that a membranous environment is not prerequisite to the RNA-elongating activity of the CPMV polymerase, just as has been found for the poliovirus polymerase (Lundquist et al., 1974). Analysis of the protein composition of the purified RdRp revealed that the 110K protein encoded by B-RNA was the only detectable viral protein in the preparation. Moreover, it was found that the 110K protein cosedimented precisely with the RNA polymerase activity during purification. Two host-encoded proteins of 68K and 57K were also present in the highly purified RdRp prepraration, but it is not known whether these proteins are subunits or just contaminants of the viral RdRp. From these results it has been concluded that the B-RNA-encoded 110K protein, which consist of the 24K protease and the 87K (polymerase domain) protein, is the active viral RNA polymerase in CPMV RNA replication. This is in contrast with poliovirus where the protein corresponding to the 110K protein, 3CD, has no polymerase activity while the protein homologous to the 87K protein, 3D, is the active viral RNA polymerase (Van Dyke and Flanegan, 1980).

Initiation of RNA synthesis did not seem to occur in membrane fractions and purified RdRp preparations from CPMV infected plants (Dorssers et al., 1983, 1984; Eggen et al., 1988). Likewise, similar attempts to achieve initiation of poliovirus RNA synthesis *in vitro*, have met with little success. The RdRp of poliovirus, on the other hand, is capable of transcribing an exogenous poly(A) template if primed with oligo(U) (Flanegan and Baltimore, 1977). Thus far it has not been possible to isolate from infected plants a CPMV RdRp activity that is able to transcribe added template RNA.

D. Synthesis of the putative CPMV polymerase in *Escherichia coli*

For poliovirus, expression in *Escherichia coli* of the coding sequence for the viral polymerase, protein $3D^{pol}$, has proven an elegant strategy to study the polioviral RdRp. With this prokaryotic expression system it was possible to obtain relatively easy large quantities of an enzyme exhibiting

RNA-elongating activity in an assay using exogegenous poly(A)-template and oligo(U)-primer (Morrow et al., 1987; Rothstein et al., 1988). An additional advantage of the synthesis of the polioviral RNA polymerase in *E. coli* is the possibility to identify amino acids that are essential for polymerase activity by introducing mutations in the coding region and testing the effect specifically on RNA polymerase activity (Plotch et al., 1989; Burns et al., 1989; Jablonski et al., 1991). Such mutation analysis had not been feasible with the viral polymerase obtained from poliovirus-infected cells, given the impossibility to dissect the viral RNA polymerase activity from other processes required for viral infectivity, such as protein synthesis and proteolytic protein processing. The successes obtained with the synthesis of the poliovirus polymerase in *E. coli* has prompted Richards et al. (1989) to employ the same prokaryotic expression system for the synthesis of the CPMV RNA polymerase. The use of *E. coli* as a potential source of CPMV polymerase seemed especially promising as there is no interfering host polymerase activity as in cowpea.

Both the putative CPMV polymerase (110K) and the protein containing the conserved amino acid motifs found in all RNA polymerases (87K) were synthesized in *E. coli*, using the same expression vector as has been used to produce poliovirus polymerase (Richards et al., 1989). Indeed, these expression vectors synthesized proteins of 110K and 87K, that exactly comigrated with the authentic viral proteins from CPMV-infected cells and that were reactive with CPMV-specific antisera. Moreover, faithful proteolytic processing of the 110K protein occurred in *E. coli* as seen by the appearance of the 24K and 87K proteins. Apparently, the 24K protease domain of the 110K protein was biologically active. In contrast, no activity could be demonstrated for the 87K polymerase domain of the same 110K protein as tested in a poly(A)/oligo(U) polymerase assay. Neither was there any detectable RNA polymerase activity for the 87K protein produced independenly from the 24K protein.

Although these results may suggest that the polymerases of CPMV and poliovirus have different requirements for showing RNA-synthesizing activity, there are several other possible explanations for the lack of polymerase activity in these experiments: 1) The stability of CPMV proteins in E. coli may be low. Indeed, the 87K and 110K proteins were to a large extent degraded into numerous lower molecular weight products. 2) The CPMV proteins from *E. coli* may be incorrectly folded or may have aggregated into insoluble complexes. This possibility has to be taken into account since only a minor fraction of the 87K and 110K proteins remained in the supernatant upon centrifugation of *E. coli* extracts at 10,000 x g. 3) Essential post-translational modification of the CPMV proteins may not occur in *E. coli*. 4) The need for a larger precursor from which the CPMV polymerase is simultanously cleaved and incorporated into an active replication complex. 5) In contrast to the poliovirus polymerase, CPMV polymerase may require a supplementary protein for activity, which may be either encoded by the host or by CPMV B-RNA. 6) The CPMV polymerase does not have a poly(A)/oligo(U) polymerase activity, but may require another template and/or primer or may not be capable of accepting an exogenous template at all.

VI. SCOPE OF THE INVESTIGATIONS

In the next chapters of this thesis experiments are described undertaken to study the biochemical activities of the viral proteins that are involved in the replication of the genomic RNAs of CPMV. The main objective of these studies was to obtain CPMV polymerase activity that is capable of using exogenous (CPMV) RNA template. For this, the putative CPMV polymerase was produced in insect cells using the baculovirus expression vector system, which might alleviate the potential

shortcomings of the prokaryotic *E. coli* expression system. To address also the question whether polyprotein processing or additional B-RNA-encoded proteins are required for CPMV polymerase activity, individual as well as precursor proteins were synthesized in insect cells and extracts of these cells were assayed for RdRp activity (chapters 2 to 4). In these experiments, poliovirus polymerase was produced under the same conditions to have a positive control in the polymerase assays. While the poliovirus polymerase thus obtained was indeed capable of elongating oligo(U) annealed to a poly(A) template, it was not possible to demonsrate the same activity for the CPMV polymerase. In a subsequent experiment (chapter 5), synthesis of the proteins encoded by B-RNA was accomplished in cowpea protoplasts in order to investigate a possible requirement for host proteins in CPMV RNA replication. Although, the replication of M-RNA could be demonstrated in protoplasts in which the B-RNA open reading frame was expressed, it was not possible to obtain CPMV polymerase activity *in vitro* using extracts of these protoplasts supplemented with a template and/or primer. These experiments suggest that the CPMV polymerase is not able to use exogenous template RNA. In order to define the signals on the RNAs of CPMV by which they are recognized as templates for viral RNA synthesis, defined mutants of B- and M-RNA were tested for their ability to be replicated (chapter 6).

The experimental data described in this thesis have added valuable information to our concept of CPMV RNA replication, resulting in a model presented in chapter 7.

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Chapter 2

Expression of plant viral genes in animal cells: high-level synthesis of cowpea mosaic virus B-RNA-encoded proteins with baculovirus expression vectors

Expression of plant virus genes in animal cells: high-level synthesis of cowpea mosaic virus B-RNA-encoded proteins with baculovirus expression vectors

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The baculovirus expression system has been used to produce non-structural proteins encoded by bottom-component RNA (B-RNA) of cowpea mosaic virus (CPMV). For this, cDNAs containing the 60K, 87K, 110K and 170K protein coding sequences were each provided with an ATG start codon and the cDNA containing the 60K coding sequence with a TAA stop codon immediately downstream of the coding sequence. Recombinant baculoviruses were retrieved which harboured the modified B-cDNA sequences under the control of the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV). Upon infection of *Spodoptera frugiperda* cells with these recombinant baculoviruses, proteins were produced which were indistinguishable from the viral proteins found in CPMV-infected plants as judged by their migration in polyacrylamide gels and their reactivity with CPMV-specific antisera. Specific processing of CPMV polyproteins in cells infected with the 110K- and 170K-encoding baculovirus recombinants proved that the CPMV-encoded 24K protease activity contained in these polyproteins is active in these cells. Approximately 10% of the 110K protein was processed into 87K and 24K proteins and the 170K protein almost completely into the 110K, 87K, 84K, 60K and 24K polypeptides. In *S. frugiperda* cells infected by recombinant *Ac*NPVs harbouring the 87K or 110K coding sequences, the CPMV-specific proteins amounted to 10 to 20% of the total cellular protein content, whereas in cells infected by recombinants encoding the 60K and 170K polypeptides the amounts of CPMV-specific proteins synthesized were much lower. Northern blot analysis indicated that the low-level synthesis of the 60K and 170K polypeptides was not due to inferior transcription of the cloned genes but was probably the result of inefficient translation of the RNAs derived from these constructs. It is concluded that plant virus genes can be efficiently expressed in an animal cell expression system to yield proteins that are structurally and, in at least one case (24K protein), functionally identical to the authentic plant virus proteins.

Introduction

Cowpea mosaic virus (CPMV), the type member of the comoviruses, possesses a bipartite, plus-sense RNA genome. Both RNAs (denoted B- and M-RNA) carry a small protein VPg covalently linked to their 5′ terminus, a poly(A) tail at their 3′ terminus and encode a large polyprotein from which the functional proteins are generated through proteolytic processing by a viral protease (for reviews see Goldbach & van Kammen, 1985; Eggen & van Kammen, 1988). M-RNA encodes the two capsid proteins and protein(s) required for cell-to-cell transport (van Wezenbeek *et al.*, 1983; Wellink & van Kammen, 1989), whereas B-RNA encodes all viral functions necessary for viral RNA replication (Goldbach *et al.*, 1980; Eggen & van Kammen, 1988). Knowledge of the precise function of each of the B-RNA-encoded proteins in CPMV replication is, however, still very limited and partly based on amino acid homology of some CPMV proteins with those of the picornaviruses (Franssen *et al.*, 1984*a*; Argos *et al.*, 1984; Goldbach, 1986, 1987).

Purified replication complexes from CPMV-infected cowpea leaves capable of elongating viral plus-sense RNA, the synthesis of which has already been initiated *in vivo*, contain the B-RNA-encoded 110K polypeptide (Dorssers *et al.*, 1984). This polypeptide is believed to be the active viral RNA replicase and is composed of the 24K protease and 87K core polymerase (Fig. 1). So far it has not been possible to obtain a template-dependent

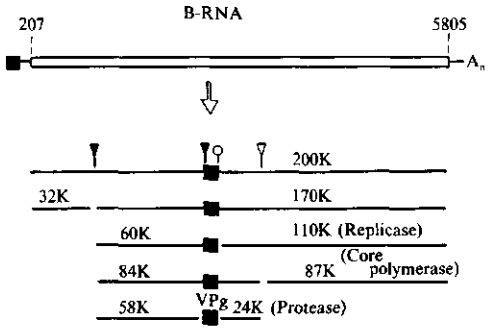


Fig. 1. Diagram of the genetic organization of CPMV B-RNA and its translation products. B-RNA contains a single open reading frame represented by an open bar on which the positions of the start and stop codons are indicated. VPg is indicated by a black square, other proteins by a single line. The initial polyprotein is processed into smaller functional proteins by specific proteolytic cleavages at the indicated sites. ♀, Gln-Met; ▽, Gln-Gly; ▼, Gln-Ser.

CPMV RNA replicase activity from CPMV-infected cowpea plants (Dorssers *et al.*, 1983, 1984) or *Chenopodium amaranticolor* (Eggen *et al.*, 1988). The study of viral RNA replication is further hampered by interfering host plant activities, such as a 130K RNA-dependent RNA polymerase (Dorssers *et al.*, 1983; van der Meer *et al.*, 1984) and a terminal uridylyl transferase (Zabel *et al.*, 1981). As an alternative approach to the study of the role of viral proteins in RNA replication, the B-RNA-encoded 87K and 110K proteins were produced in an *Escherichia coli* expression system (Richards *et al.*, 1989). Neither the 87K nor the 110K protein showed RNA synthesizing activity under conditions where poliovirus polymerase (protein 3D) similarly produced in *E. coli* was highly active (Richards *et al.*, 1989). Reasons for the lack of polymerase activity for the CPMV proteins may be (i) the low level of expression in *E. coli*, (ii) incorrect protein folding or lack of post-translational modifications in a prokaryotic system, (iii) the low stability of the proteins in *E. coli*, (iv) requirement for additional proteins such as the viral 60K protein and (v) the need for a polyprotein from which the polymerase is simultaneously cleaved and incorporated into an active replication complex.

In order to investigate whether a eukaryotic system may alleviate the potential shortcomings of a prokaryotic system, and to produce larger amounts of the proteins encoded by CPMV B-RNA, an animal cell expression system was employed. It has been reported that baculovirus expression vectors produce high amounts of biologically active proteins in insect cells which are post-translationally modified in a fashion similar to that of the authentic proteins (for reviews see Luckow & Summers, 1988*a*; Miller, 1988). The high level of expression is based on the exploitation of the strong polyhedrin promoter by the allelic replacement of the baculovirus polyhedrin gene by a heterologous sequence. So far the gene of phaseolin has been the only plant gene expressed with this system (Bustos *et al.*, 1987); the expression of plant virus genes has not yet been reported. In this paper we report the expression of the 60K, 87K, 110K and 170K coding regions of CPMV B-RNA in insect cells by *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) recombinants.

Methods

Viruses, plasmids and cells. *Ac*NPV, strain E2 (Summers & Smith, 1987) and recombinant viruses were grown in *Spodoptera frugiperda* IPLB-SF-21 cells (Vaughn *et al.*, 1977) in TNM-FH medium (Hink, 1970) supplemented with 10% foetal bovine serum as described by Summers & Smith (1987). Baculovirus transfer vectors and other plasmids were propagated in the *E. coli* strains DH5α(F') and JM109. Single-stranded DNA containing UMP residues was isolated from the *E. coli* strain RZ1032.

Plasmid pTB1G (Eggen *et al.*, 1989) is a full-length cDNA clone of the CPMV B-RNA sequence (Lomonossoff & Shanks, 1983) downstream of the bacteriophage T7 promoter from which infectious RNA transcripts can be generated. The baculovirus transfer vector pAcRP23 (Possee & Howard, 1987) was constructed by cloning the *Eco*RI I fragment of *Ac*NPV, containing the polyhedrin gene, into pUC8.

DNA manipulations. Standard recombinant DNA techniques were used for the isolation and ligation of DNA fragments and transformation of DNA in competent *E. coli* cells (Maniatis *et al.*, 1982). Enzymes were purchased from Gibco-BRL or from Boehringer-Mannheim and used as described by the manufacturer. Oligodeoxynucleotides were synthesized with a Cyclone DNA synthesizer (Biosearch). Site-directed mutagenesis with these oligodeoxynucleotides was performed as described by Kunkel (1985).

Construction of the baculovirus transfer vector pAcHB60. An *Xba*I–*Sst*I fragment [nucleotides (nt) 899 to 2301] from pTB1G was inserted in M13mp18 and a start codon was introduced at positions 1185 to 1187 using the oligonucleotide 5'-GGACAATGCACATAT-GAGTCCTGTTATCCT-3', which, at the same time, created an *Nde*I site (CATATG). The mutagenized *Xba*I–*Sst*I fragment was then reinserted into pTB1G resulting in the plasmid pTBHM60 (HM refers to the histidine–methionine residues encoded by the *Nde*I recognition site). A TAA stop codon was added at the end of the 60K coding region by inserting a *Sst*I–*Sph*I fragment (nt 2301 to 3156) of pTB1G in M13mp19 followed by mutagenesis with the oligonucleotide 5'-GGGCAGACGCACAATAATCTTTGGAT-3'. The newly created stop codon was subsequently inserted in pTBHM60 by exchanging the homologous *Sst*I–*Kpn*I fragments (nt 2301–3134), resulting in plasmid pTBHM60STOP. This plasmid was partially digested with *Nde*I, blunted with Klenow polymerase, partially digested with *Kpn*I and the 1970 bp *Nde*I–*Kpn*I fragment was isolated. The baculovirus transfer vector pAcRP23 was digested with *Bam*HI, the protruding ends were filled in with Klenow polymerase and digested with *Kpn*I. Ligation with the 1970 bp *Nde*I–*Kpn*I fragment from pTBHM60STOP resulted in the 11250 bp expression vector pAcHB60 containing the 60K coding region.

Construction of transfer vector pAcHB87. An *Sst*I–*Bam*HI fragment (nt 2301 to 3857 in the B-cDNA) from pTB1G was inserted in M13mp18 and subjected to mutagenesis using the oligonucleotide

5′-ATTCCTCAGCCATATGCGCTTG-3′. This resulted in an *Nde*I recognition site and an ATG start codon at the beginning of the 87K coding region. The mutation was transferred to pTB1G, resulting in plasmid pTBHM87, which was subsequently cleaved with *Nde*I (partially) and with *Cla*I (nt 3671 to 5935). The baculovirus transfer vector pAcRP23 was digested with *Bam*HI and *Kpn*I and the 9·3 kb fragment was isolated, blunted with Klenow polymerase and dephosphorylated with calf intestinal phosphatase. Linkage of this fragment and the blunted *NdeI–ClaI* fragment, containing the 87K coding region, produced vector pAcHB87. Confirmation of the proper orientation of the CPMV insert downstream of the polyhedrin promoter was established by restriction enzyme analysis and dideoxynucleotide sequencing (Sanger *et al.*, 1977; Korneluk *et al.*, 1985).

Construction of transfer vector pAcHB110. The 1556 bp *Sst*I–*Bam*HI fragment of pTB1G was inserted in M13mp19 and an *Nde*I site was introduced at positions 3045 to 3050 using the oligonucleotide 5′-GCAGACGCACATATGTCTTTGGAT-3′. This mutation was transferred to pTB1G resulting in pTBHM110, which was subsequently digested with *Nde*I (partially) and *Cla*I (nt 3046 to 5935). The isolated *NdeI–ClaI* fragment, containing the 110K coding region, was blunted with Klenow polymerase and ligated to the 9·3 kb linearized and blunted vector described for the construction of pAcHB87. The proper orientation of the 110K coding region downstream of the polyhedrin promoter was confirmed by restriction enzyme analysis and sequencing.

Construction of transfer vector pAcHB170. pAcHB60 was digested with *Kpn*I, blunted with Klenow polymerase and digested with *Sst*I; the 10·4 kbp fragment was isolated. pTB1G was digested with *Bam*HI, filled in with Klenow polymerase and digested with *Sst*I. The 1556 bp fragment (nt 2301 to 3857 of the B-cDNA) was isolated and ligated to the 10·4 kb pAcHB60 fragment so that the *Bam*HI site was repaired. The resulting plasmid pAcHBam was digested with *Bam*HI and *Bst*EII and a 5·7 kb fragment was isolated. pAcHB110 was also digested with *Bam*HI and *Bst*EII and the isolated 8·2 kb fragment was ligated with the 5·7 kb fragment to create pAcHB170.

Selection of recombinant viruses. Monolayers of *S. frugiperda* cells (1 × 10^6 cells in 35 mm tissue culture dishes) were cotransfected with wild-type (wt) *Ac*NPV DNA and transfer vector DNA using the calcium phosphate precipitation procedure of Summers & Smith (1987). After incubation of cells for 5 days at 27 °C the supernatant was collected and putative recombinants, recognized by their polyhedron-negative phenotype, were picked and plaque-purified three times to reach genetic homogenity.

Analysis of proteins synthesized in infected cells. S. frugiperda cells (1 × 10^6 cells per 35 mm Petri dish) were infected with *Ac*NPV recombinants containing CPMV B-cDNA inserts at a m.o.i. of 10 and incubated at 27 °C for 48 h. The cells were pelleted by low-speed centrifugation (300 *g* for 2 min), washed twice in cold phosphate-buffered saline (10 mM-Na_2HPO_4, 100 mM-NaCl pH 7·5) and disrupted in sample buffer (10 mM-Tris–HCl pH 8·0, 1 mM-EDTA, 10% v/v glycerol, 2% w/v SDS, 5% v/v 2-mercaptoethanol and 0·001% w/v bromophenol blue). An equal amount of *S. frugiperda* cells infected with wt *Ac*NPV and mock-infected cells served as controls. Samples were boiled for 3 min and aliquots corresponding to 10^3 to 50×10^3 cells were electrophoresed on 10% SDS–polyacrylamide gels (Laemmli, 1970). These gels were either stained with Coomassie brilliant blue (CBB) or analysed by immunoblotting using either rabbit anti-24K (Wellink *et al.*, 1987), anti-170K (Franssen *et al.*, 1984*b*) or anti-VPg serum (Eggen *et al.*, 1988) with anti-rabbit IgG–alkaline phosphatase conjugate (Promega Biotec) as a second antibody (Blake *et al.*, 1984).

Northern blot analysis. Total RNA was isolated from uninfected and infected *S. frugiperda* cells at 24 h post-infection (p.i.) essentially as described by de Vries *et al.* (1988). Briefly 1 × 10^6 infected cells were mixed at 53 °C with a 1:1 mixture of RNA extraction buffer (100 mM-Tris–NaOH pH 9·0, 100 mM-LiCl, 10 mM-EDTA and 1% w/v SDS) and phenol and vortexed for 5 min. After the addition of 0·5 vol. of chloroform the cells were vortexed for another 10 min followed by 30 min centrifugation at 10000 *g*. The aqueous layer was removed and again extracted with chloroform. The RNA was precipitated in 2 M-LiCl at 0 °C for 16 h and subsequently electrophoresed on a 1% denaturing agarose gel according to McMaster & Carmichael (1977). Blotting was on GeneScreen (NEN Research Products) as recommended by the manufacturer. Nick translations of the 4·6 kb *Acc*I fragment from pTB1G (nt 735 to 5319) and the 0·5 kb *Eco*RV–*Kpn*I (nt −93 to 634 of the polyhedrin gene) fragment from pAcRP23 were used as radioactive probes.

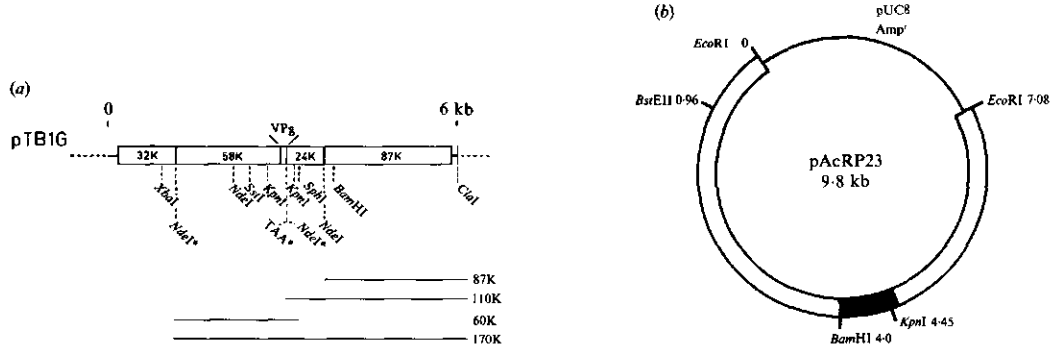
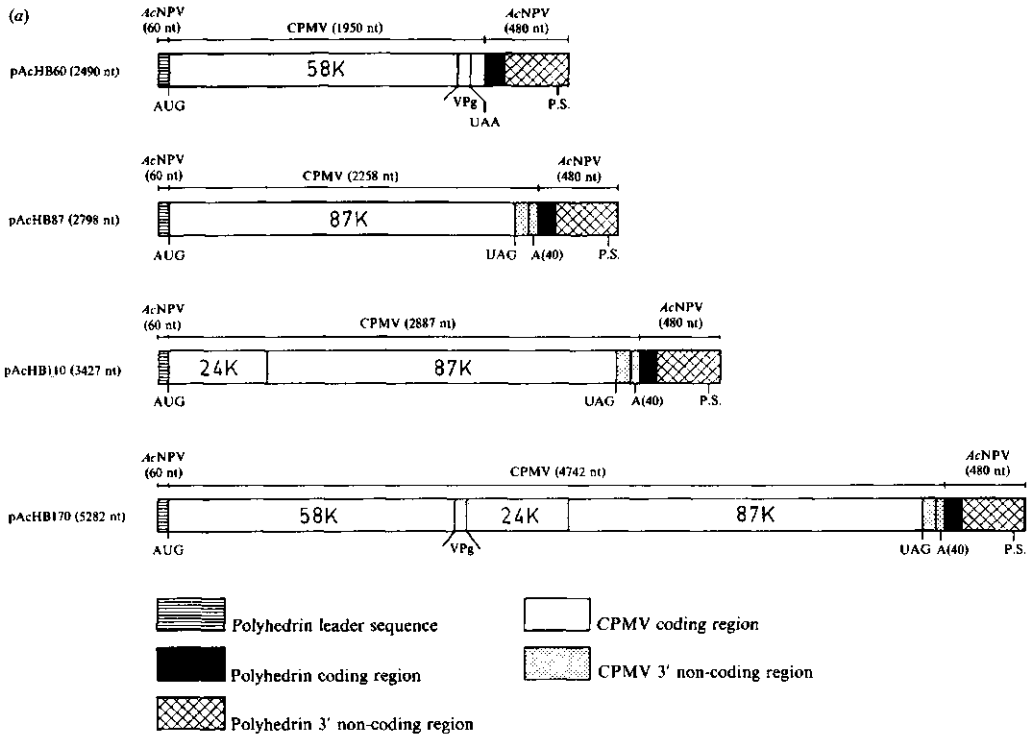


Fig. 2. Schematic diagram of the construction of the transfer vectors. Vector construction is described in Methods. (*a*) The full-length cDNA clone of CPMV B-RNA is represented by an open bar on which the restriction sites that were used in the different cloning steps are indicated. The newly created *Nde*I sites and TAA stop codon are indicated with an asterisk. The regions that were cloned downstream of the polyhedrin promoter of transfer vector pAcRP23 are represented by single lines. (*b*) The *Bam*HI–*Kpn*I fragment of the polyhedrin coding region (black bar) of pAcRP23 was exchanged with the CPMV coding sequences, resulting in the pAcHB transfer vectors.



(*b*)

*Ac*NPV	AAAAAACCTATAAATAATGC
pAcHB60/170	CTATAAATCCGGATCTATGA
pAcHB87	CTATAAATCCGGATCTATGG
pAcHB110	CTATAAATCCGGATCTATGT
	-3 +4

Fig. 3. Transcripts expected to be generated from the polyhedrin promoter for the different recombinant baculoviruses. (*a*) The mRNAs are represented by bars on which the positions of the start and stop codons and the polyadenylation signal (P.S.) are shown. The lines indicate the length of segments of the transcripts as the number of nucleotides (nt) and the virus from which these segments originated. (*b*) Comparison of the leader sequences of the CPMV genes inserted in the transfer vector to that of the *Ac*NPV polyhedrin gene (Hooft van Iddekinge *et al.*, 1983). The sequences were verified by sequence analysis.

Results

Construction of recombinant baculoviruses

For the cloning and expression of the CPMV B-RNA sequences encoding the 87K (core polymerase), 110K (protease and polymerase), 60K (58K and VPg) and 170K (60K and 110K) polypeptides, start and stop codons and restriction enzyme cleavage sites were introduced in the B-cDNA. This was achieved by site-directed mutagenesis (Kunkel, 1985) in such a way that the first codon of a sequence to be expressed was changed into an ATG start codon which was also part of an *Nde*I recognition site (CATATG). Using the newly created restriction sites the modified B-cDNA sequences were then inserted in the baculovirus transfer vector pAcRP23 (Possee & Howard, 1987) behind the intact polyhedrin promoter, resulting in the transfer vectors pAcHB87, pAcHB110, pAcHB60 and pAcHB170 (Fig. 2). In these constructs almost the entire polyhedrin coding region (nt +1 to +633) has been deleted and replaced by a CPMV coding sequence in such a way that the open reading frame is under the control of the polyhedrin promoter and that translation initiation will be at the ATG start codon provided by the CPMV sequence. This start codon was either naturally present (pAcHB110) or introduced by site-directed mutagenesis. In the latter case the

methionine codon substitutes a serine codon (pAcHB60 and pAcHB170) or a glycine codon (pAcHB87) of the authentic CPMV proteins. For the expression of the 60K coding region a TAA stop codon was introduced immediately behind the 60K coding region, at the position of the first amino acid of the 24K protease (Fig. 1; Wellink *et al.*, 1986). In all other constructions the natural stop codon (TAG) of the CPMV B-cDNA is present.

Each of the transfer vectors were used with wt *Ac*NPV DNA for cotransfection of *S. frugiperda* cells. In approximately 0·5% of the infections recombination occurred between the sequences flanking the CPMV inserts and the homologous sequences of the wt *Ac*NPV DNA. The recombinant *Ac*NPV DNAs, denoted *Ac*HB87, *Ac*HB110, *Ac*HB60 and *Ac*HB170, were characterized by their occlusion body-negative phenotype, as they lack the polyhedrin gene. The CPMV-specific mRNAs expected to be generated for each construct are depicted in Fig. 3*a*. Sequencing of the transfer vectors revealed that, as expected, the 5′ untranslated regions were identical for all constructs (Fig. 3*b*).

Identification of CPMV polypeptides in insect cells

Plaque-purified recombinant viruses were used at a m.o.i. of 10 to infect *S. frugiperda* cells and, after incubation for 48 h at 27 °C, proteins from these cells were analysed on SDS–polyacrylamide gels. Following electrophoresis the gels were either stained with CBB (Fig. 4) or used for immunoblotting (Fig. 5, 6). The CBB-stained gel revealed that a 30K protein representing polyhedrin was present in wt *Ac*NPV-infected cells (Fig. 4, lane 2) but not in cells infected with each of the recombinants. Instead, with the recombinants *Ac*HB87 and *Ac*HB110 extra bands of proteins of 87K and 110K were clearly visible which made up about 20% of the total stainable protein content (Fig. 4, lanes 4 and 5). Furthermore, the 110K protein seemed to be partially processed (approximately 10%) into the 87K protein. No additional proteins could be detected by CBB staining with recombinants *Ac*HB60 and *Ac*HB170 as compared to wt *Ac*NPV-infected cells.

The expression products from the recombinants were further analysed on immunoblots by treatment with three different CPMV-specific antisera. The analysis showed that the 87K and 110K proteins, which were detected with CBB, migrated with the same electrophoretic mobility as the 87K and 110K proteins present in plant fractions of CPMV-infected cowpea plants and were immunoreactive with anti-170K serum (Fig. 5, lanes 3, 4, 9 and 10). With anti-24K serum a 24K protein could also be detected in cells infected with *Ac*HB110

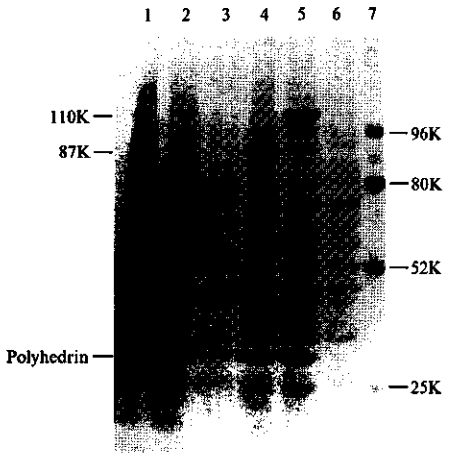


Fig. 4. Expression of CPMV sequences by recombinant baculoviruses. *S. frugiperda* cells were infected with wt *Ac*NPV or recombinant *Ac*NPVs derived from transfer vectors pAcHB60, pAcHB87, pAcHB110 and pAcHB170. At 48 h p.i. cells were collected and protein was fractionated in a 10% polyacrylamide gel. The proteins were visualized by CBB staining. Lane 1, mock-infected Sf21 cells (control); lane 2, wt *Ac*NPV; lane 3, *Ac*HB60; lane 4, *Ac*HB87; lane 5, *Ac*HB110; lane 6, *Ac*HB170; lane 7, M_r markers. The *Ac*NPV polyhedrin protein and the 87K and 110K proteins of CPMV are indicated by pointers.

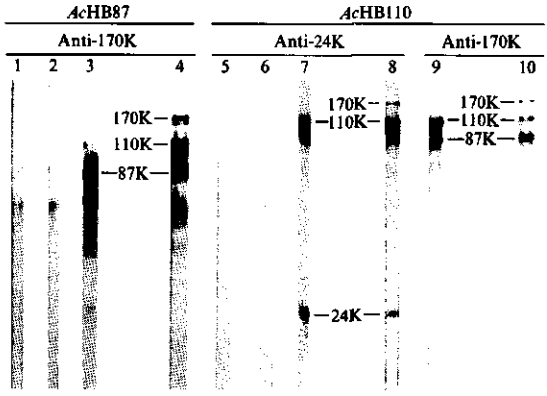


Fig. 5. Immunoblot analysis of proteins synthesized in *S. frugiperda* cells infected with wt *Ac*NPV or recombinant viruses *Ac*HB87 and *Ac*HB110. Detection was with anti-170K or anti-24K serum as a primary antibody and anti-rabbit alkaline phosphatase conjugate as the detection antibody. Cells were collected and fractionated by PAGE as in Fig. 4. Lanes 1 and 5, uninfected Sf21 cells (control); lanes 2 and 6, *Ac*NPV; lane 3, *Ac*HB87; lanes 4, 8 and 10, cytoplasmic fraction of CPMV-infected cowpea leaves (F3); lanes 7 and 9, *Ac*HB110.

(Fig. 5, lane 7), indicating, that the 110K protein was processed into 24K and 87K proteins. In *S. frugiperda* cells infected with recombinant *Ac*HB60, a 60K protein reactive with anti-VPg serum could be shown (Fig. 6, lane 3). This protein was also present in cells infected with *Ac*HB170 (Fig. 6, lane 5) as were proteins of sizes of 170K, 110K, 84K, 87K and 24K, which were immunoreactive with anti-24K serum (Fig. 6, lane 7) or anti-170K serum (Fig. 6, lane 9). All of the immunoreactive species correspond to authentic viral proteins present in fractions of CPMV-infected cowpea plants and were absent in uninfected or *Ac*NPV-infected *S. frugiperda* cells. It is thus concluded that the proteins detected for the *Ac*NPV recombinants could be attributed to expression of CPMV sequences. Furthermore, the results demonstrate that whenever the 24K protein was synthesized, processing of approximately 10% of the 110K protein and almost complete processing of the 170K protein into products of the proper sizes occurred (see also Fig. 1). In addition to the specific products of proteolytic processing, additional cleavage products could also be detected with anti-24K and anti-170K serum.

Differences in expression levels for the various recombinants

Significant differences in the expression levels of the various recombinant baculoviruses was observed (Fig. 4). For the detection of CPMV-specific proteins on immunoblots, a 20-fold greater number of cells was used in the case of recombinants *Ac*HB60 and *Ac*HB170 as compared to *Ac*HB87 and *Ac*HB110. The *Ac*NPV DNA recombinants with low expression levels both have the 60K coding region in common, whereas recombinants with high expression levels do not harbour this sequence. *S. frugiperda* cells producing the 60K protein exhibited abnormal cytopathic effects and showed lysis within 48 h p.i. Furthermore, using electron microscopy, it appeared that these cells contained large numbers of vacuoles in the cytoplasm (results not shown). Therefore, the proteins produced by recombinants *Ac*HB60 and *Ac*HB170, notably the 60K protein, may have a deleterious effect on cells. *S. frugiperda* cells were infected with a mixture of two recombinants at an m.o.i. of 15 for each recombinant in the following combinations: *Ac*HB60+110, *Ac*HB87+110 and *Ac*HB170+110. At 48 p.i. the cells were harvested and assayed for the presence of the 110K protein (Fig. 7). Similar amounts of the 110K protein were found for each set of recombinants (Fig. 7, lanes 1 to 3), demonstrating that the proteins produced in *Ac*HB60- or *Ac*HB170-infected cells were not detrimental to the expression of other genes. To determine whether the low expression of the 60K and 170K proteins by recombinants *Ac*HB60

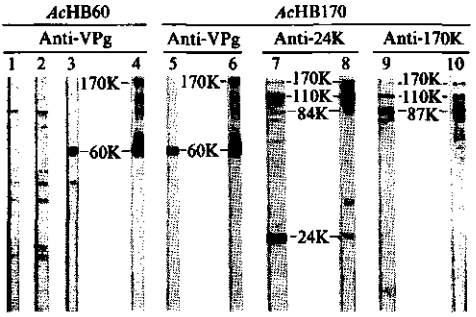


Fig. 6. Immunoblot analysis of proteins synthesized in *S. frugiperda* cells infected with wt *Ac*NPV or the baculovirus recombinants *Ac*HB60 and *Ac*HB170. Detection was with anti-VPg, anti-24K or anti-170K serum as a primary antibody and anti-rabbit alkaline phosphatase conjugate as the detection antibody. Cells were collected and fractionated by PAGE as in Fig. 4. Lane 1, uninfected Sf21 cells (control); lane 2, wt *Ac*NPV; lane 3, *Ac*HB60; lanes 4 and 6, membrane fraction of CPMV-infected cowpea leaves (marker); lanes 5, 7 and 9, *Ac*HB170; lanes 8 and 10, cytoplasmic fraction of CPMV-infected cowpea leaves (marker).

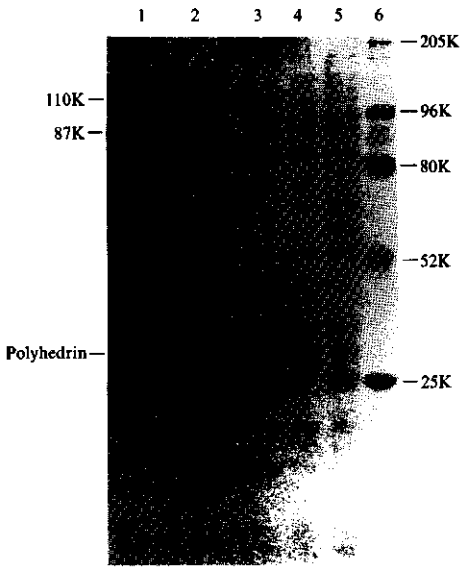


Fig. 7. Analysis of the amount of 110K protein synthesized upon mixed infection of *S. frugiperda* cells with baculovirus recombinants *Ac*HB87+110 (lane 1), *Ac*HB170+110 (lane 2) and *Ac*HB60+110 (lane 3). Cells were infected with an m.o.i. of 15 for each recombinant virus in combination, grown for 48 h and collected and fractionated by PAGE as in Fig. 4. The *Ac*NPV polyhedrin protein and the 110K and 87K proteins of CPMV are indicated. Lane 4, wt *Ac*NPV; lane 5, uninfected Sf21 cells (control); lane 6, *M*, markers (sizes indicated to right).

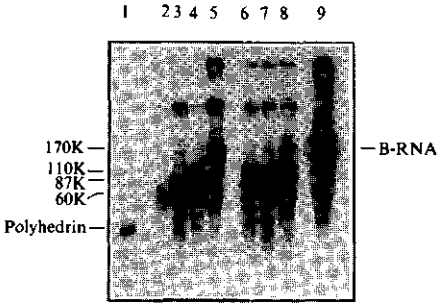


Fig. 8. Northern blot analysis of the transcripts generated from the polyhedrin promoter. Total cellular RNA was extracted from *S. frugiperda* cells infected with *Ac*NPV (lane 1), the recombinant baculoviruses *Ac*HB60 (lane 2), *Ac*HB87 (lane 3), *Ac*HB110 (lane 4) and *Ac*HB170 (lane 5), or with the mixtures of recombinants shown in Fig. 7 (lane 6, *Ac*HB60+110; lane 7, *Ac*HB87+110; lane 8, *Ac*HB170+110; lane 9, CPMV RNA as control). RNA samples were separated on a 1% agarose gel under denaturing conditions. After blotting to GeneScreen the RNA was hybridized to nick-translated probes specific for CPMV B-RNA and the polyhedrin coding region (see Methods). CPMV RNA was included as a control and B-RNA is indicated. The polyhedrin protein messenger RNA and the mRNAs for the 60K, 87K, 110K and 170K proteins of CPMV are also indicated.

and *Ac*HB170 was due to a reduced transcription level, RNA was isolated at 24 h p.i. from cells infected with *Ac*NPV recombinants. The amount and quality of the isolated RNA was analysed on an ethidium bromide-stained agarose gel. Similar quantities of RNA were used for electrophoresis under denaturing conditions and for Northern blotting. It was verified that comparable amounts of viral RNA were present by treating the blot with a probe specific for the gene of the abundant envelope surface glycoprotein (gp67; Whitford *et al.*, 1989) of *Ac*NPV as an internal standard (results not shown). Using probes specific for CPMV B-RNA and polyhedrin coding sequences, no significant differences in mRNA levels transcribed from the polyhedrin promoter were detected for the recombinant with low expression level (*Ac*HB170) and the recombinants with high expression levels (*Ac*HB87 and *Ac*HB110; Fig. 8). For the other recombinant with the low expression level (*Ac*HB60) there seemed to be less mRNA present but this was probably due to masking of the signal by rRNA present at the same position on the gel. Cells infected with a mixture of two recombinants contained comparable amounts of mRNA detectable on Northern blots. Hence, it may be deduced that the low expression of the 60K and 170K sequences was not the result of an impeded transcription level in these cells. The origin of the high M_r bands in Fig. 8 (lanes 2 to 8) is unknown. The same bands appeared with the same intensity with the gp67 probe and are thus not specific for CPMV sequences. The sizes of the mRNAs detected on the Northern blots also demonstrate that transcription does not stop at the poly(A) sequence provided by the CPMV cDNA but proceeds to the polyhedrin transcription termination site.

Discussion

The results described in this paper demonstrate that the baculovirus expression vector system is very useful for the high-level expression of plant virus genes. The CPMV-specific 87K and 110K proteins produced by *Ac*HB87 and *Ac*HB110 amounted to up to 20% of the total CBB-stainable protein content of infected insect cells (Fig. 4). On the other hand, the CPMV-specific 60K and 170K proteins produced by *Ac*HB60 and *Ac*HB170 were detectable only by immunoblotting. Similar differences in the amounts of CPMV-specific proteins were found upon expression in *E. coli* cells (Richards *et al.*, 1989; S. Peters, unpublished results) but overall the amounts of protein produced in the baculovirus expression vector system were larger than those in *E. coli*. Moreover, the polypeptides synthesized in *S. frugiperda* cells were more stable than those produced in *E. coli*, as very little non-specific proteolytic cleavage occurred. Specific proteolytic processing of the CPMV polyproteins was observed in cells infected with *Ac*HB110 or *Ac*HB170, which both produced the 24K protease. About 10% of the 110K protein was processed into 87K and 24K proteins (Fig. 5) whereas the 170K protein was almost completely processed to give 110K, 87K, 84K, 60K and 24K polypeptides (Fig. 6). The 110K protein could be precipitated by low-speed centrifugation from cell extracts and therefore appears to be aggregated into large insoluble complexes. The low degree of processing of the 110K protein as compared to the 170K protein may be attributed to this aggregation of the 110K protein. The polyproteins and products of processing synthesized in insect cells corresponded to the CPMV proteins produced in infected cowpea plants as judged by their electrophoretic mobility and their reaction with CPMV-specific antisera. The occurrence of the specific cleavage products in insect cells indicates that the 24K protease is biologically active and acts at the proper glutamine–methionine cleavage site between the 60K and 110K proteins and the glutamine–glycine cleavage site between the 87K and 84K proteins.

We wondered whether the low amounts of 60K and 170K polypeptides produced in cells infected with *Ac*HB60 and *Ac*HB170 might be due to a deleterious effect of these proteins on the *S. frugiperda* cells. It is

rather striking that these cells showed a c.p.e. characterized by large arrays of vacuoles and lysis of the cells within 48 h p.i. whereas such effects were not found in cells infected with *Ac*HB87 and *Ac*HB110. Production of vacuoles in cells infected with recombinant viruses has been reported before (Matsuura *et al.*, 1987; Luckow & Summers, 1988*b*) but in these cases there was high expression of the foreign gene. Moreover, insect cells infected both by *Ac*HB110 and *Ac*HB60 showed the characteristic c.p.e. whereas the amounts of 110K and 60K proteins produced were not affected. It is therefore not very likely that the c.p.e. is the major cause of the low production of the 60K and 170K proteins. Neither it seems are the low amounts of 60K and 170K polypeptide found in insect cells the result of more rapid turnover of these proteins as compared to the 87K and 110K proteins. In cells infected with *Ac*HB170 low quantities of 170K protein are found, as are similarly low amounts of its cleavage products, 110K, 87K, 84K, 60K and 24K proteins (Fig. 6). If the different CPMV-specific proteins varied in stability, accumulation of the 110K and 87K polypeptides would be expected, as occurs in cells infected with *Ac*HB110 and *Ac*HB87. Northern blot analysis revealed no significant difference in the amounts of mRNA produced from the polyhedrin promoter of the recombinant with the low expression level (*Ac*HB170) as compared to the recombinants with high expression levels (*Ac*HB87 and *Ac*HB110; Fig. 8) but apparently less 60K mRNA was present in *Ac*HB60-infected cells. However, it is not very plausible that this is caused by a transcriptional defect because the promoter region of the transfer vector pAcHB170 originated from pAcHB60. Furthermore, it is unlikely that transcription signals were affected during cloning or recombination because, for each recombinant *Ac*NPV DNA, at least five independent clones were isolated. It is concluded that recombinants *Ac*HB170 and *Ac*HB60 produce similar amounts of mRNA from the polyhedrin promoter and that the hybridization signal for *Ac*HB60 is weakened by the presence of rRNA. Therefore, the relatively low expression level of the 60K and 170K coding sequences as compared to the 87K and 110K coding sequences does not appear to be the result of a transcriptional defect.

The only possibility left is that the low production of 60K and 170K proteins in *Ac*HB60- and *Ac*HB170-infected cells is caused by inefficient translation of the corresponding mRNAs. On the other hand it is difficult to understand what the structural basis of the difference in efficiency of translation of the mRNAs is, since the 5′ non-coding regions were identical in all constructs. Also, the sequence around the AUG start codon in the different mRNAs does not give a plausible explanation for the different translation efficiencies. The mRNAs for the 87K, 110K, 170K and 60K proteins all have T at the −3 position. It has been reported that when a pyrimidine at position −3 is replaced by a purine, translation becomes more sensitive to changes at position +4 (Kozak, 1986). The mRNAs generated from the polyhedrin promoter of *Ac*HB87, *Ac*HB110 and *Ac*HB60/170 have a G, a T and an A residue, respectively, at the +4 position (Fig. 3*b*). Consequently one would expect that these mRNAs would be equally translated (Rohrmann, 1986; Luckow & Summers, 1988*b*). This, however, is not the case. The low translation level seems to be an intrinsic property of the mRNA of the 60K coding region that also occurs at the 5′ end of the mRNA of the 170K coding region. A similar suggestion has been made for the expression of the human tissue type plasminogen activator in insect cells (Luckow & Summers, 1988*b*). Deletion of various domains of the 60K coding sequence may answer the question of which region is responsible for the poor translation.

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Chapter 3

Evidence for dissimilar properties of comoviral and picornaviral RNA polymerases

Evidence for dissimilar properties of comoviral and picornaviral RNA polymerases

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The poliovirus RNA polymerase has been synthesized in *Spodoptera frugiperda* cells by using the baculovirus expression system. Crude sonicates of these cells exhibited an RNA-elongating activity of a synthetic oligo(U) primer with poly(A) or cowpea mosaic virus (CPMV) RNA as a template. A similar polymerase activity was found in extracts of insect cells in which foot-and-mouth disease virus (FMDV) proteins, including the putative polymerase, were produced. The analogous CPMV 87K protein and several of its precursors, synthesized in *S. frugiperda* cells, did not show any detectable polymerase activity in the same assay under a variety of conditions. The results indicate that, in contrast to the picornaviral polymerases, the CPMV polymerase is unable to function in an oligo(U)-primed polymerase assay.

Introduction

The organization and expression of the genome of cowpea mosaic virus (CPMV), type member of the plant comoviruses, is strikingly similar to that of the animal picornaviruses (Franssen *et al.*, 1984; Argos *et al.*, 1984; Goldbach, 1987). The major difference is that the genome of CPMV consists of two separately encapsidated RNA molecules and that of picornaviruses only one (Fig. 1). The single-stranded positive-sense RNA molecules of both the comovirus group and the picornaviruses contain a small protein, VPg, covalently linked to their 5′ ends and a poly(A) tail at their 3′ termini. Translation of the genomic RNAs results in the synthesis of large polyproteins, which are processed into functional proteins by virus-encoded proteases. Significant sequence identity exists between the 60K, 24K and 87K non-structural proteins encoded on CPMV B-RNA and the picornavirus non-structural proteins 2C, 3C and 3D, respectively, which are also similarly arranged on the genome (Fig. 1; Franssen *et al.*, 1984). Protein 3D has been assigned as the primer- and template-dependent RNA polymerase in poliovirus RNA replication (Lundquist *et al.*, 1974; Flanegan & Baltimore, 1977; Van Dyke & Flanegan, 1980) and a similar function has been proposed for protein 3D of foot-and-mouth disease virus (FMDV) (Lowe & Brown, 1981). The 87K protein of CPMV shares 20·9% amino acid identity with the poliovirus 3D protein and contains conserved sequence motifs found in all viral RNA-dependent RNA polymerases characterized so far (Kamer & Argos, 1984). There is evidence that the precursor of the 87K protein, which is 110K (24K and 87K), is the active viral polymerase (Dorssers *et al.*, 1984). This is in contrast with poliovirus where the corresponding protein 3CD has no polymerase activity (Van Dyke & Flanegan, 1980). Both the 24K protein of CPMV and protein 3C of picornaviruses have been identified as proteases (Vos *et al.*, 1988*a*; Hanecak *et al.*, 1984). For poliovirus it has been suggested that protein 3C may regulate the viral replication process by releasing functional proteins from non-functional precursors (Kuhn & Wimmer, 1987; Andino *et al.*, 1990).

RNA polymerase activity, as measured in a poly(A)–oligo(U) assay system, has been demonstrated for the polioviral 3D protein synthesized in *Escherichia coli* (Rothstein *et al.*, 1988; Morrow *et al.*, 1987). In contrast, neither the 87K nor the 110K proteins of CPMV exhibited such an activity when synthesized in *E. coli* and assayed under the same conditions (Richards *et al.*, 1989). Previously we have described the expression of CPMV B-RNA coding sequences in insect cells using the baculovirus vector expression system (van Bokhoven *et al.*, 1990). Here we report assays for RNA polymerase activity of the CPMV proteins as well as the poliovirus and FMDV polymerase, which were also produced in these cells.

Methods

Viruses, plasmids and cells. Autographa californica nuclear polyhedrosis virus (*Ac*NPV, strain E2; Smith & Summers, 1978) and recombinant *Ac*NPVs were grown in *Spodoptera frugiperda* IPLB-SF-21 cells (Vaughn *et al.*, 1977) in TNM-FH medium (Hink, 1970) supplemented

with 10% foetal bovine serum. Baculovirus transfer vectors and other plasmids were propagated in *E. coli* strain DH5α. Expression vector pEXC-3D contains the poliovirus 3CD coding sequence under the control of the TrpE promoter and produced an active 3D polymerase in *E. coli* (Richards *et al.*, 1987). Baculovirus transfer vector pAcRP23 has been constructed by cloning the *Eco*RI I fragment of *Ac*NPV containing the polyhedrin promoter in pUC8 (Possee & Howard, 1987). The construction and expression of recombinant baculoviruses containing CPMV sequences (*Ac*HB60, *Ac*HB87, *Ac*HB110 and *Ac*HB170) or FMDV sequences (*Ac*JR4/MR41) have been described by van Bokhoven *et al.* (1990) and Roosien *et al.* (1990), respectively.

Construction of transfer vectors pAcHB3CD and pAcHB3CDΔ. Plasmid pEXC-3D was partially digested with *Hin*dIII and with *Sal*I to completion and the poliovirus fragment encoding 3CD (about 2 kb) was isolated from an agarose gel. This fragment was inserted into the multiple cloning site of plasmid Bluescript KS$^+$ (Stratagene), to isolate the 3CD coding sequence as a *Bam*HI–*Kpn*I fragment. Baculovirus transfer vector pAcRP23 (Possee & Howard, 1987) was also digested with *Bam*HI and *Kpn*I and ligated with the 3CD fragment to create pAcHB3CD. The number of nucleotides between the polyhedrin promoter and the start codon provided by the 3CD sequence was reduced by deleting a *Sma*I–*Eco*RV fragment originating from the Bluescript plasmid, resulting in pAcHB3CDΔ. Sequence analysis confirmed that the transfer vectors pAcHB3CD and pAcHB3CDΔ contain 38 and 20 additional nucleotides, respectively, upstream of the ATG start codon compared to the polyhedrin gene.

Selection of recombinant baculoviruses. Monolayers of *S. frugiperda* cells (1×10^6 cells per 35 mm Petri dish) were cotransfected with wild-type (wt) *Ac*NPV DNA and transfer vector DNA using the calcium phosphate precipitation procedure of Summers & Smith (1987). After 5 days incubation at 27 °C the supernatant was collected and used for infection of *S. frugiperda* cells under agarose. Putative recombinants recognized by their polyhedron-negative phenotype were picked and plaque-purified three times to reach genetic homogeneity.

Analysis of proteins synthesized in S. frugiperda *cells. S. frugiperda* cells (1×10^6 cells per 35 mm Petri dish) were infected with *Ac*NPV recombinants at an m.o.i. of 10 and incubated for 3 days at 27 °C. The cells were pelleted by low-speed centrifugation (300 *g* for 2 min), washed twice in cold phosphate-buffered saline (PBS; 10 mM-sodium phosphate pH 7·5, 100 mM-NaCl) and lysed by boiling for 3 min in sample buffer (10 mM-Tris–HCl pH 8·0, 1 mM-EDTA, 10% v/v glycerol, 2% w/v SDS, 5% v/v 2-mercaptoethanol and 0·001% w/v bromophenol blue). Aliquots corresponding to 5×10^4 cells were electrophoresed in 10% SDS–polyacrylamide gels (Laemmli, 1970), which were either stained with Coomassie brilliant blue or analysed by immunoblotting using antiserum raised against a TrpE/poliovirus polymerase fusion protein (gift of Dr O. C. Richards) and with anti-rabbit IgG–alkaline phosphatase conjugate (Promega). Equal amounts of *S. frugiperda* cells infected with wt *Ac*NPV and mock-infected cells served as controls.

Polymerase assays. S. frugiperda cells were infected with the *Ac*NPV recombinants at an m.o.i. of 10. At 3 days post-infection the cells were harvested and sedimented by low-speed centrifugation (300 *g* for 2 min). The cells were washed twice in cold PBS and finally resuspended in TGKEDP buffer (50 mM-Tris–acetate pH 8·0, 25% v/v glycerol, 50 mM-potassium acetate, 1 mM-EDTA, 5 mM-DTT and 0·5 mM-PMSF) at 4×10^6 cells per ml. Cell suspensions on ice were sonicated with a Vibra Cell sonicator in two cycles of 10 pulses separated by a 2 min interval for cooling.

Poly(A)–oligo(U) polymerase assays were performed essentially as described by Flanegan & Baltimore (1977). Reaction mixtures contained 50 mM-HEPES pH 8·0, 0·5 mM-ATP, -GTP and -CTP, 10 μM-UTP, 3 mM-magnesium acetate, 60 μM-$ZnCl_2$, 3 μg/ml actinomycin D, 1 μg poly(A) or 2·5 μg of CPMV RNA, 0·34 μg oligo(U) and 2·5 μCi [^{3}H]UTP (Amersham) and 5 to 10 μl of crude sonicate in a total volume of 50 μl. Samples (20 μl) taken at time zero and after 30 min incubation at 30 °C were precipitated in 5% Na_2HPO_4 on Whatman DE81 filter discs, and counted in Insta-Fluor (Packard) scintillation fluid. Each extract was also assayed in the absence of primer and/or template.

For an analysis of the products of the polymerase assays the reactions were performed with 0·5 μCi of [^{32}P]UTP (Amersham) instead of [^{3}H]UTP. A sample of assay mixture (5 μl) was spotted onto a filter and used for scintillation counting. The remaining 45 μl of the reaction mixture was, after a phenol extraction and ethanol precipitation, used for electrophoresis on a denaturing 1% agarose gel (McMaster & Carmichael, 1977).

Results and Discussion

Expression of picornavirus and CPMV sequences in insect cells

The construction and expression of four recombinant *Ac*NPVs containing CPMV B-RNA coding sequences under the control of the polyhedrin promoter has been described (van Bokhoven *et al.*, 1990). These recombinants, *Ac*HB60, *Ac*HB87, *Ac*HB110 and *Ac*HB170, produce the proteins they denote (60 K etc.) and also the proteolytic cleavage products derived from them (Fig. 1; van Bokhoven *et al.*, 1990). In *Ac*HB110-infected cells about 10% of the 110K protein was processed into the 87K and 24K proteins and approximately 80% of the 170K protein produced by *Ac*HB170 was processed into the 110K, 87K, 84K, 60K and 24K polypeptides. Recombinant *Ac*JR4/MR41 has also been described and produces, amongst others, protein 3D of FMDV (Fig. 1; Roosien *et al.*, 1990).

For the synthesis of the poliovirus polymerase in insect cells two transfer vectors, pAcHB3CD and pAcHB3CDΔ, were constructed which contain the poliovirus 3CD coding region under the control of the polyhedrin promoter separated by 38 and 20 additional nucleotides, respectively, directly upstream of the ATG start codon as compared to the polyhedrin gene. Cotransfection of *S. frugiperda* cells with these two transfer vectors together with wt *Ac*NPV DNA generated the recombinant baculoviruses *Ac*HB3CD and *Ac*HB3CDΔ. These recombinants were plaque-purified and used at an m.o.i. of 10 to infect *S. frugiperda* cells. After incubation for 3 days at 27 °C proteins from these cells were analysed by electrophoresis on 10% SDS–polyacrylamide gels. Polioviral proteins could not be detected by staining with Coomassie brilliant blue (results not shown), but on an immunoblot treated with antiserum raised against a TrpE/poliovirus polymerase fusion protein, two bands were clearly detectable at positions corresponding to the bands of poliovirus proteins 3CD and 3D found in infected HeLa cells (Fig.

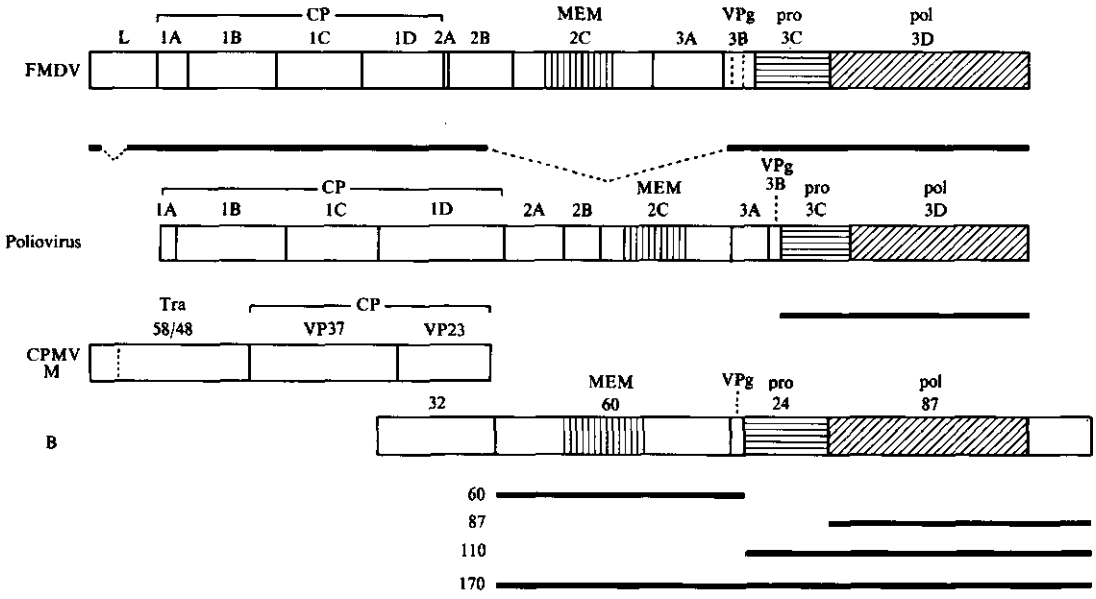

Fig. 1. Diagram showing the genetic organization of the monopartite picornavirus RNAs of FMDV and poliovirus and the bipartite comoviral RNA of CPMV. Each RNA molecule contains a single open reading frame represented by a bar on which the positions of the final proteolytic cleavage products are indicated. Regions of amino acid sequence similarity are shaded similarly. Also indicated are: Tra, putative transport protein(s); CP, coat proteins; MEM, membrane protein; pro, protease; pol, core RNA-dependent RNA polymerase. The dashed lines in protein 3B of FMDV represent three tandemly arranged VPgs. The coding regions that were cloned downstream of the polyhedrin promoter of a baculovirus transfer vector are indicated by single solid lines drawn underneath the genomic RNA.

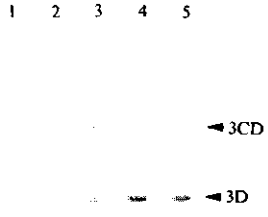

Fig 2. Immunoblot analysis of proteins synthesized in *S. frugiperda* cells (lane 1) infected with wt *Ac*NPV (lane 2) or recombinants *Ac*HB3CD (lane 4) and *Ac*HB3CDΔ (lane 5) visualized with antiserum raised against a TrpE/poliovirus polymerase fusion protein and anti-rabbit IgG–alkaline phosphatase conjugate as the detection antibody. An extract of poliovirus-infected HeLa cells (lane 3) was used as a source of 3CD and 3D marker proteins.

2, lanes 3 to 5). These bands were not present in uninfected or *Ac*NPV-infected *S. frugiperda* cells (Fig. 2, lanes 1 and 2). The amounts of 3CD and 3D proteins produced in the *Ac*HB3CD- and *Ac*HB3CDΔ-infected cells were similar. Furthermore, the immunoblot demonstrates that partial processing of the 3CD protein has occurred resulting in the release of approximately 75% of the 3D protein from its precursor 3CD.

The amount of 3CD and 3D protein produced in *Ac*HB3CD- and *Ac*HB3CDΔ-infected *S. frugiperda* cells is lower than is usually found with the baculovirus expression system. In comparison with the polyhedrin gene, *Ac*HB3CD has 38 and *Ac*HB3CDΔ has 20 additional nucleotides upstream of the ATG start codon and it is possible that these extra nucleotides prevent the high expression level that is possible with this system. On the other hand, it is clear that the amount of protein produced in the baculovirus expression system is also influenced by the nature of the protein or the efficiency with which the corresponding messenger RNAs are translated. For example, the CPMV 87K and 110K proteins synthesized by *Ac*HB87 and *Ac*HB110 in *S. frugiperda* cells amounted to 20% of the total cellular

Table 1. *Oligo(U)-dependent polymerase activity of crude sonicates of wt and recombinant* Ac*NPV-infected* S. frugiperda *cells*

Extract	[^{3}H]UTP incorporated (c.p.m.)	
	Poly(A) template	CPMV RNA template
*Ac*NPV	70*	70
*Ac*HB3CD	109000	31000
*Ac*JR4/MR41	1100	ND†
*Ac*HB60	10	10
*Ac*HB87	60	40
*Ac*HB110	60	50
*Ac*HB170	70	50

* The amount of [^{3}H]UTP label incorporated in the absence of primer and/or template and at time zero varied between 10 and 100 c.p.m.

† ND, Not determined.

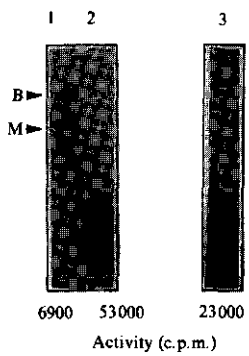

Fig. 3. Products synthesized by crude *Ac*HB3CD-infected *S. frugiperda* extracts in oligo(U)-dependent polymerase assays with either CPMV RNA (lanes 1 and 2) or poly(A) RNA (lane 3) as a template. Except for CTP (0·5 mM) that was present in all reactions, the assays were performed with (lanes 1 and 3) or without (lane 2) additional unlabelled nucleotides. The positions of CPMV B-RNA (5900 nucleotides) and M-RNA (3500 nucleotides) in the gel, as determined from an ethidium bromide-stained marker, are indicated. The amount of incorporated label was determined by scintillation counting.

protein content, whereas the CPMV 60K and 170K coding sequences in constructs with the same 5′ non-coding region produced much lower amounts of protein (van Bokhoven *et al.*, 1990), which were comparable to the amounts of 3CD and 3D in *Ac*HB3CD(Δ)-infected cells. It has previously been shown that the FMDV-specific proteins 3CD, 3C and 3D synthesized in *S. frugiperda* cells infected with *Ac*JR4/MR41 together with ΔL-1AB, 1AB, 1CD, 1C, 1D and P1 are produced in amounts that are detectable only by immunoprecipitation (Roosien *et al.*, 1990). Therefore it was suggested that the low level of production may be due to the toxicity of the 3C protease to the baculovirus system. If so, the low amounts of poliovirus proteins might likewise be explained by a toxic effect of the poliovirus 3C protease on the insect cells.

Polymerase activity in crude sonicates of infected S. frugiperda *cells*

S. frugiperda cells were infected at an m.o.i. of 10 with each of the *Ac*NPV recombinants described and, after 3 days incubation at 27 °C, crude extracts of these cells were tested for polymerase activity using poly(A) as a template and oligo(U) as a primer (Table 1). A substantial RNA synthesizing activity was detected in cells infected with *Ac*HB3CD which was not present in the absence of primer and/or template (results not shown), indicating that poliovirus protein 3D synthesized in *S. frugiperda* cells exhibits a primer- and template-dependent RNA polymerase activity. Polymerase activity was also detected in cells producing FMDV-specific proteins, but this activity was much lower than the activity present in *Ac*HB3CD-infected cells (Table 1). This is not surprising as the FMDV-specific proteins are produced in much smaller amounts than the poliovirus proteins (Roosien *et al.*, 1990). This result shows that one of the FMDV proteins synthesized in insect cells, most likely 3D (Lowe & Brown, 1981), exhibits an oligo(U)-primed RNA-synthesizing activity similar to that of 3D polymerase of poliovirus. We were unable to retrieve recombinants expressing only the FMDV 3CD sequence, which might be due to the toxic effect of protease 3C for the host cell (Falk *et al.*, 1990).

In striking contrast to the extracts with picornavirus polymerase proteins, the extracts containing CPMV proteins did not show [^{3}H]UTP-incorporating activity above background levels in these assays either with poly(A) or with CPMV RNA as a template (Table 1). Remarkably, when crude extracts containing the poliovirus proteins were assayed with 2·5 μg of CPMV RNA as a template, the amount of incorporated label was only three- to fourfold lower than that with 1 μg poly(A) as a template.

The polymerase assays with *Ac*HB3CD extracts were repeated with [^{32}P]UTP to perform a product analysis. A range of products, migrating around 0·2 kb, was found when poly(A) was used as a template (Fig. 3). On the other hand, when CPMV RNA was used as a template the products were almost exclusively present in two distinct bands at the B- and M-RNA positions (Fig. 3). The M-RNA band was reproducibly found to be more intense than the B-RNA band. This can be explained by the longer average length of the poly(A) tail of M-RNA (20 to 400 nucleotides) as compared to B-RNA (10 to 170 nucleotides; Ahlquist & Kaesberg, 1979), but it may also

be caused by a higher affinity of poliovirus 3D for the M-RNA template. It is surprising that CPMV RNA seems to be such an efficient template and that even full-length copies were synthesized with crude insect cell extracts as the source of the polymerase.

The polymerase assay with CPMV RNA as a template was repeated in the absence of unlabelled nucleotides, except that CTP (0·5 mM) was maintained in the reaction mixture as it was found that the presence of at least one of the nucleotides gave a more than 10-fold stimulation of the RNA-synthesizing activity of poliovirus 3D polymerase in a poly(A)–oligo(U) polymerase assay (results not shown). A high incorporation of label was found, due to the absence of competing unlabelled nucleotides, but only short products were formed (Fig. 3), indicating that oligo(U) was elongated to a limited extent. This shows that the B- and M-RNA bands were the result of genuine polymerase activity and not of a terminal transferase.

Differences between the comoviral and picornaviral RNA polymerases

It is remarkable that the poliovirus 3D polymerase and also the presumed FMDV 3D polymerase display such an easily detectable RNA-elongating activity whereas the analogous CPMV proteins do not. Mixing of extracts from cells infected with *Ac*HB3CD with any of the extracts in which CPMV proteins were present revealed that there was no activity inhibiting RNA elongation in extracts containing CPMV proteins (results not shown). The assays for CPMV polymerase activity were performed under a large number of different conditions (pH, temperature, monovalent and divalent ions) including conditions optimal for RNA elongation in crude replicase extracts from CPMV-infected cowpea plants (Dorssers *et al.*, 1984). Since no stimulation of incorporation of [^{3}H]UTP was observed, it seems unlikely that the conditions in the assay system are responsible for the lack of polymerase activity of CPMV proteins. The observed proteolytic processing activities of CPMV proteins which contained the protease domain suggest that precursor proteins are folded properly in insect cells. Thus, the absence of RNA polymerase activity is probably not due to incorrect protein folding. It remains possible that some form of post-translational modification, that does not occur in *S. frugiperda* cells, is required for CPMV polymerase activity but not for the proteolytic activity or functioning of picornaviral polymerases. However, it is known that the 87K and 110K proteins found in CPMV-infected plants are not glycosylated (H. van Bokhoven & J. Wellink, unpublished results).

It is also conceivable that the CPMV polymerase is not able to function in an oligo(U)-primed assay. In the case of poliovirus at least two host factors have been described that can replace oligo(U) as a primer to allow 3D polymerase to initiate RNA synthesis (Dasgupta *et al.*, 1980; Morrow *et al.*, 1985; Andrews & Baltimore, 1986), although the relevance of these proteins in the replication of poliovirus RNA has been questioned (Hey *et al.*, 1987). In studies with crude replication complexes from poliovirus-infected HeLa cells the presence of another activity has been demonstrated that catalyses the formation of VPg–pUpU (Takegami *et al.*, 1983), which may serve as a primer for RNA synthesis (Takeda *et al.*, 1986). Similar activities have not been demonstrated in CPMV-infected cowpea leaves, but it is known that CPMV RNA replication is inhibited by actinomycin D during early stages of infection, suggesting the requirement for a host factor (Rottier *et al.*, 1979; de Varennes *et al.*, 1985). Mixing of insect cell extracts containing CPMV proteins with plant extracts from CPMV-infected or non-infected cowpea plants did not result in any detectable RNA-synthesizing activity (results not shown). However, it is possible that during the formation of a viral replication complex a host factor is needed to obtain functional proteins. Obviously, expression of the CPMV polymerase genes in a homologous system is required to demonstrate such a mechanism. Finally, another viral protein may be required for polymerase activity. It is known that the B-RNA of CPMV encodes all viral functions necessary for viral RNA replication (Goldbach *et al.*, 1980). The only B-RNA-encoded proteins which were not tested in our polymerase assays were the 32K protein and the entire 200K polyprotein. Vos *et al.* (1988*b*) have shown that a mutation of the 32K protein renders the B-RNA non-infectious. Recent experiments suggest a function for the 32K protein in the regulation of polyprotein processing (J. Wellink, J. Wery & R. Milek, unpublished results). Assuming that processing and the formation of an active replication complex are tightly linked, the 32K protein may have a crucial role in the establishment of such a complex.

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Chapter 4

Synthesis of the complete 200K polyprotein encoded by cowpea mosaic virus B-RNA in insect cells

Synthesis of the complete 200K polyprotein encoded by cowpea mosaic virus B-RNA in insect cells

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The coding sequence for the entire 200K polyprotein of cowpea mosaic virus (CPMV) B-RNA was expressed in insect cells by using baculovirus expression vectors. The 200K polyprotein, which harbours all virus functions required for RNA replication, is completely cleaved into 170K and 32K products by the 24K protease activity contained within the polyprotein. Further processing of the 170K protein into CPMV-specific products of 60K, 84K, 87K, 110K and 112K occurred to a limited extent, similar to that observed in cowpea cells. Electron microscopy of insect cells in which the 200K protein was produced revealed the presence of membranous vesicles and electron-dense structures which were not seen in cells infected with wild-type baculovirus. Similar cytopathic structures develop in the cytoplasm of CPMV-infected cowpea cells and are thought to be the site of membrane-bound viral RNA replication. The electron-dense structures in insect cells could be preferentially labelled with several CPMV-specific antisera and Protein A–gold. Since electron-dense structures were not observed in cells in which the 170K protein only was produced, it seems that the 32K protein has a role in keeping the B-RNA-encoded proteins in these structures together. Membranous vesicles were also observed in insect cells in which the 60K protein only was produced. Use of specific antibodies and Protein A–gold showed that the 60K protein is associated with these vesicles, indicating that the 60K protein may induce the formation of vesicles. Although proteolytic processing of the 200K polyprotein and the induction of cytopathic structures indicate that the CPMV proteins produced in insect cells are functional, it has not been possible to demonstrate RNA polymerase activity in extracts of these cells using an oligo(U)-primed assay. The results indicate that in the assay an additional component is lacking and/or that the CPMV polymerase is not able to start RNA synthesis on an exogenous template.

Introduction

The two genomic RNA molecules of cowpea mosaic virus (CPMV) are both required for successful infection of cowpea plants. Individual steps in the infection process involve proteins encoded by either one of the viral RNAs and presumably also proteins encoded by the host. For instance, all virus functions necessary for viral RNA replication are confined to the larger B-RNA molecule as it can replicate independently of the smaller M-RNA in cowpea protoplasts (Goldbach *et al.*, 1980). On the other hand, cell-to-cell movement of the virus in the plant is dependent on the coat proteins and the transport protein(s), which are encoded by M-RNA (Wellink & van Kammen, 1989).

Translation of the open reading frames (ORFs) of each RNA yields large polyproteins which are processed into smaller proteins by a B-RNA-encoded protease (Wellink *et al.*, 1986; Vos *et al.*, 1988*a*; see also Fig. 1). Our understanding of the exact activities of the separate B-RNA-encoded proteins, i.e. final cleavage products and precursors thereof, is still incomplete, but several functional domains within the B polyprotein have been identified (Fig. 1).

The 32K protein is involved in regulation of B polyprotein processing (Peters *et al.*, 1992) and is required as a cofactor in the cleavage of the M polyprotein (Vos *et al.*, 1988*a*). The 24K protein has been identified as the viral protease (Verver *et al.*, 1987) and, based on sequence homologies with other viral polymerases, the 87K protein is thought to be the core polymerase (Franssen *et al.*, 1984*a*; Poch *et al.*, 1989). The 110K protein, which encompasses the 24K and 87K proteins, is the sole viral protein that can be detected in highly purified replication complexes (Dorssers *et al.*, 1984) and therefore has been identified as the viral replicase. The 60K protein (58K + VPg) contains an amino acid sequence motif that is found in proteins with

NTPase activity, such as helicases (Gorbalenya *et al.*, 1988, 1989). Furthermore, it has been suggested that the 60K protein has a role in anchoring the viral RNA replication complex to vesicular membrane structures, which are induced in infected cells by the virus (de Zoeten *et al.*, 1974; Eggen & van Kammen, 1988).

So far it has not been possible to isolate template-dependent CPMV polymerase from infected plant material (Dorssers *et al.*, 1983, 1984; Eggen *et al.*, 1988). In attempts to obtain an active CPMV polymerase, individual B-RNA-encoded proteins have been produced in *Escherichia coli* (Richards *et al.*, 1989) and in insect cells (van Bokhoven *et al.*, 1990). The viral proteins obtained with these expression systems are structurally identical to the authentic virus proteins as judged by electrophoretic mobility, immunoreactivity and the faithful proteolytic processing patterns of proteins containing the 24K protease domain. Nevertheless, none of the CPMV proteins show RNA-synthesizing activity in oligo(U)-primed polymerase assays (Richards *et al.*, 1989; van Bokhoven *et al.*, 1991). This observation was rather unexpected as the poliovirus polymerase, which shows considerable amino acid sequence homology with the B-RNA-encoded 87K protein, and which was synthesized and tested under identical conditions, is active in such assays. The polymerases of poliovirus and CPMV appear to have different requirements for RNA-synthesizing activity.

One of the explanations for the lack of activity of the CPMV-encoded polymerase is that other viral proteins or host proteins from cowpea plants are required to yield or maintain an active enzyme (van Bokhoven *et al.*, 1991). For example, the regulation of proteolytic processing of the 200K polyprotein by the 32K protein (Peters *et al.*, 1992) might be essential for the formation of a functional replicase complex. To study this we have expressed the entire 200K coding sequence of CPMV B-RNA in insect cells using a baculovirus vector and tested fractions of insect cells for RNA-dependent RNA polymerase activity. In CPMV-infected cowpea cells, RNA replication complexes are associated with vesicular membranes of cytopathic structures, the formation of which is induced by a B-RNA-encoded function (de Zoeten *et al.*, 1974; Rezelman *et al.*, 1982). Here we report an electron microscopy study to investigate whether the 200K polyprotein or one of its proteolytic cleavage products is capable of inducing similar cytopathic structures in insect cells.

Methods

Viruses, plasmids and cells. Plasmid pTB1G contains a full-length cDNA of CPMV B-RNA (Lomonossoff & Shanks, 1983) from which infectious RNA transcripts can be generated using T7 polymerase (Eggen *et al.*, 1989). Plasmid pTB32* contains a TGA stop codon immediately downstream of the coding sequence for the 32K protein of B-RNA (Vos *et al.*, 1988*b*). Transcripts of this plasmid produce 32K protein only upon *in vitro* translation. Baculovirus transfer vector pAcRP23 has the polyhedrin promoter-containing *Eco*RI I fragment of *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) in pUC8 (Possee & Howard, 1987). Transfer vectors pAcHB170, pAcHB110, pAcHB87 and pAcHB60 contain the coding sequences for the respective B-RNA-specific proteins in pAcRP23 (van Bokhoven *et al.*, 1990). All plasmids were propagated in *E. coli* DH5α(F'). UMP-containing ssDNA was obtained from *E. coli* strain RZ1032.

*Ac*NPV strain E2 (Summers & Smith, 1987) and recombinants derived from it were grown in *Spodoptera frugiperda* IPLB-SF-21 cells (Vaughn *et al.*, 1977) in TNM-FH medium (Hink, 1970) supplemented with 10% foetal bovine serum.

DNA manipulations. Standard recombinant DNA techniques were used for construction of baculovirus transfer vectors (Sambrook *et al.*, 1989). Site-directed mutagenesis was performed as described by Kunkel (1985) using oligodeoxynucleotides synthesized on a Cyclone DNA synthesizer (Biosearch).

Construction of baculovirus transfer vectors. A *Sal*I–*Xba*I fragment [nucleotides (nt) −44 to 899 of B-RNA] from pTB1G was inserted into M13mp18 and subjected to mutagenesis using the oligonucleotide 5′ GTCTTGACCCCATATGGGTCTCCC 3′. This resulted in the creation of a *Nde*I recognition site (underlined) at positions 204 to 209 while leaving the natural start codon at position 207 intact. A *Sal*I–*Pst*I fragment containing the mutation was exchanged with the homologous fragment from pTB1G to produce plasmid pTB200N.

The baculovirus transfer vector pAcHB200 was constructed in a three-point ligation reaction. Plasmid pTB200N was partially digested with *Nde*I, filled in with the Klenow fragment of DNA polymerase I and cleaved with *Sst*I, and the *Nde*I–*Sst*I fragment (nt 206 to 2301) was isolated. Transfer vector pAcHB170 was cleaved with *Sst*I and *Bst*EII, and the 10·4 kb fragment containing vector sequences and B-cDNA sequences spanning the *Sst*I site and the 3′ terminus was isolated. Plasmid pAcRP23 was digested with *Bam*HI, blunt-ended with the Klenow fragment of DNA polymerase I and then cleaved with *Bst*EII. A 3·0 kb fragment containing the remainder of the vector and the polyhedrin promoter was isolated. The proper ligation of the three fragments into pAcHB200 was confirmed by restriction enzyme analysis and dideoxynucleotide sequencing (Korneluk *et al.*, 1985).

Three plasmids were also used for the construction of transfer vector pAcHB32. Transfer vector pAcHB200 was digested with *Bst*EII and *Pst*I (3·18 kb), pAcHB60 with *Bst*EII and *Sst*I (7·09 kb) and pTB32* with *Pst*I and *Sst*I (0·84 kb). Ligation of the indicated fragments resulted in pAcHB32 in which the 32K coding region of CPMV B-RNA followed by a stop codon was present under control of the polyhedrin promoter.

Selection of recombinant baculoviruses. Monolayers of *S. frugiperda* cells (1×10^6 cells/35 mm Petri dish) were cotransfected with wild-type (wt) *Ac*NPV DNA and transfer vector DNA using either the calcium phosphate precipitation procedure (Summers & Smith, 1987) or lipofectin reagent (Gibco BRL) as described by Groebe *et al.* (1990). After 5 days incubation at 27 °C, the supernatant containing extracellular virus particles was collected and used for a plaque assay. Five days post-infection (p.i.) putative recombinant viruses characterized by a polyhedron-negative phenotype were picked and plaque-purified three times to achieve genetic homogenity.

Analysis of proteins synthesized by recombinant baculoviruses. *S. frugiperda* cells (1×10^6 cells/35 mm dish) were infected with *Ac*NPV recombinants at an m.o.i. of 10 and incubated for 2 days at 27 °C. The cells were collected by centrifugation at 300 *g* for 2 min, washed twice in cold PBS (10 mM-sodium phosphate pH 7·5, 100 mM-

NaCl) and disrupted by boiling for 3 min in sample buffer (10 mM-Tris–HCl pH 8·0, 1 mM-EDTA, 10% v/v glycerol, 2% w/v SDS, 5% v/v 2-mercapoethanol, 0·001% w/v bromophenol blue). Equal numbers of cells infected with wt *Ac*NPV and mock-infected cells served as controls. Aliquots obtained from 5×10^4 cells were electrophoresed in 10% SDS-polyacrylamide gels (Laemmli, 1970). These gels were either stained with Coomassie blue or analysed by immunoblotting using antisera raised in rabbits against synthetic peptides (anti-24K, Wellink *et al.*, 1987; anti-VPg, Eggen *et al.*, 1988), purified viral protein (anti-32K, Franssen *et al.*, 1984*b*) or purified 110K protein from baculovirus expression vector pAcHB110 (H. van Bokhoven, unpublished data). To stain the blot, anti-rabbit IgG–alkaline phosphatase conjugate (Promega) was used as a second antibody (Blake *et al.*, 1984).

Preparation of extracts for polymerase assays. S. frugiperda cells were infected with *Ac*NPV recombinants at an m.o.i. of 10. At 2 days p.i. the cells were harvested and collected by low-speed centrifugation (300 *g* for 2 min). The cells were washed twice, resuspended in cold 50 mM-Tris–acetate pH 8·0, 25% glycerol, 50 mM-potassium acetate, 1 mM-EDTA, 5 mM-DTT and 0·5 mM-PMSF at 4×10^6 cells/ml, and sonicated with a Vibra Cell sonicator in two cycles of 10 pulses each. The resulting crude extract was subjected to sequential centrifugation steps to obtain pellet and supernatant fractions. The crude insect cell extract was first centrifuged for 30 min at 10000 *g*, then the resulting supernatant was centrifuged for 30 min at 30000 *g* and the supernatant fraction thereof was finally centrifuged for 60 min at 100000 *g*. All fractions were tested for polymerase activity as described previously (Flanegan & Baltimore, 1977; van Bokhoven *et al.*, 1991).

Electron microscopy. S. frugiperda cells were infected with wt and recombinant *Ac*NPVs at an m.o.i. of 10 $TCID_{50}$ units/cell. At 48 h p.i., cells were dehydrated and embedded in LR White or LR gold, and sections were immunogold-labelled with CPMV-specific antibodies and Protein A–gold complexes with 7 nm diameter gold particles, followed by silver enhancement as described previously (van Lent *et al.*, 1990).

Results

Cloning and expression of the B-RNA coding sequence in insect cells

To clone the entire 200K coding sequence of CPMV B-RNA in a baculovirus expression vector a *Nde*I recognition site was created in pTB1G at position 204 (Fig. 1). *In vitro* T7 polymerase transcripts obtained from a full-length cDNA clone containing this mutation (pTB200N) were as infectious in cowpea protoplasts as were transcripts of the original wt clone (results not shown). This demonstrates that neither the cloning and mutagenesis steps nor the changing of bases at positions 204 (A to C) and 206 (C to T) had any adverse effect on virus viability.

The newly created *Nde*I site was used to construct baculovirus transfer vectors pAcHB32 and pAcHB200, which contained the 32K and 200K ORFs of CPMV B cDNA, respectively, under control of the strong polyhedrin promoter (Fig. 1). *S. frugiperda* cells were cotransfected with either of the two transfer vectors together with wt *Ac*NPV DNA resulting in the recombinant baculoviruses *Ac*HB32 and *Ac*HB200. Following plaque purification, these recombinants were used at an m.o.i. of 10 to infect *S. frugiperda* cells. After incubation for 48 h at 27 °C, the total protein content of these cells was analysed on SDS–polyacrylamide gels (Fig. 2*a*).

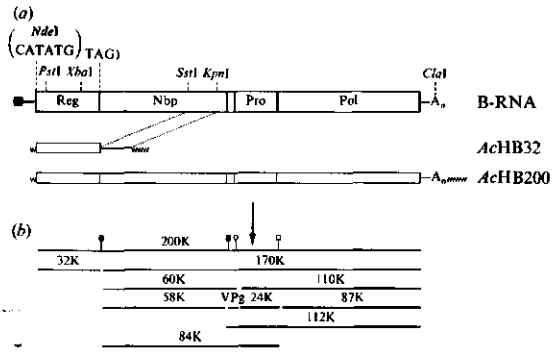


Fig. 1. Schematic diagram of the baculovirus expression vectors and their expected protein products. (*a*) The genetic organization of CPMV B-RNA. Non-coding regions are represented by a single line and VPg is drawn as a black square at the 5′ terminus. Polypeptide domains with a known or presumed function are indicated: Reg, regulation of polyprotein processing; Nbp, nucleotide binding; Pro, protease; Pol, polymerase. Also shown are the restriction sites and mutagenized sequences (in parentheses) in the cDNA that were used in the cloning procedures. The transcripts expected from the polyhedrin promoter for expression vectors *Ac*HB200 and *Ac*HB32 with baculovirus sequences represented as wavy lines are shown. (*b*) The B polyprotein and proteolytic cleavage products are shown with approximate sizes indicated. Proteolytic cleavage sites: ⚲, Gln/Met; ⚳, Gln/Gly; ⚳, Gln/Ser.

Coomassie blue staining of these gels revealed that cells infected with *Ac*HB200 contained proteins with M_rs of approximately 170K and 32K which were not found in *Ac*NPV-infected (not shown) or uninfected insect cells. The proteins produced by *Ac*HB200 were further analysed by immunoblotting. The 170K protein found in *Ac*HB200-infected cells was indistinguishable from the 170K protein present in CPMV-infected plants as it reacted with anti-VPg, anti-24K, anti-110K and anti-170K antisera (Fig. 3; data not shown). Using anti-32K antiserum, it was confirmed that the 32K protein synthesized in *Ac*HB200-infected insect cells was indeed CPMV-specific and of the proper size as it migrated with the same electrophoretic mobility as the 32K protein found in CPMV-infected plants (Fig. 2*b*). The 200K polyprotein was not detected, indicating that upon synthesis in insect cells it is completely cleaved into 170K and 32K proteins. Moreover, use of the CPMV-specific antisera revealed that in addition to this primary cleavage further proteolytic processing of the 170K protein had occurred in insect cells. The presence of

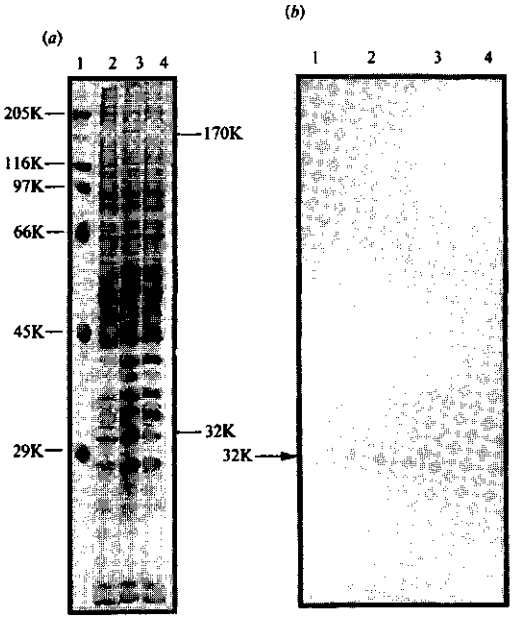


Fig. 2. Expression of CPMV B-RNA sequences by baculovirus recombinants. (*a*) Coomassie blue staining of proteins present in total insect cell extracts separated in a 10% polyacrylamide gel. Lane 1, *M*, markers; lane 2, mock-infected Sf21 cells; lane 3, *Ac*HB200- and lane 4, *Ac*HB32-infected cells. (*b*) Immunoblot analysis of proteins synthesized in insect cells infected with wt *Ac*NPV (lane 4), *Ac*HB200 (lane 2) or *Ac*HB32 (lane 3). A cytoplasmic fraction of CPMV-infected cowpea leaves was used as a control (lane 1). Proteins were separated in a 7·5% polyacrylamide gel, transferred to nitrocellulose and visualized with anti-32K antiserum and anti-rabbit IgG–alkaline phosphatase conjugate staining.

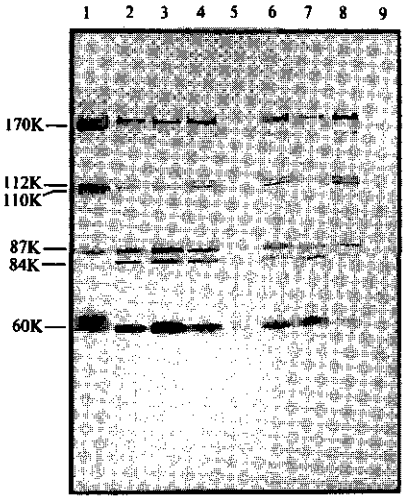


Fig. 3. Determination of the cellular distribution of CPMV proteins. Insect cells infected with *Ac*HB200 were sonicated and the resulting crude lysates (lane 2) were subjected to sequential centrifugation steps to give pellet (lanes 3, 5 and 7) and supernatant (lanes 4, 6 and 8) fractions. Centrifugal forces applied were 10000 *g* for 30 min (lanes 3 and 4), 30000 *g* for 30 min (lanes 5 and 6) and 100000 *g* for 60 min (lanes 7 and 8). The proteins were separated in a 10% polyacrylamide gel and blotted onto nitrocellulose. Visualization was with anti-VPg and anti-110K antisera followed by anti-rabbit IgG–alkaline phosphatase conjugate. A crude extract of *Ac*NPV-infected cells (lane 9) and a membrane fraction of CPMV-infected cowpea leaves (lane 1) served as controls.

proteins of sizes of 112K, 110K, 87K, 84K and 60K, comigrating with viral proteins from CPMV-infected cowpea plants, suggested that processing of precursor proteins had taken place at the authentic cleavage sites (Fig. 3). It is noteworthy that proteolytic processing of the 170K protein originating from the 200K polyprotein was less extensive than processing of the 170K protein that had been produced in insect cells in the absence of the 32K protein (van Bokhoven *et al.*, 1990). In addition to the specific proteolytic processing products, other protein species immunoreactive with CPMV-specific antisera were detected. These polypeptides become more apparent upon repeated freezing and thawing of the harvested insect cells, and therefore are probably due to non-specific degradation.

The amount of CPMV-specific proteins produced in *Ac*HB200-infected cells was significantly higher than that obtained upon expression of the 170K protein coding sequence from the polyhedrin promoter (van Bokhoven *et al.*, 1990). Apparently the presence of the 32K protein coding sequence upstream of the 170K protein coding region has a beneficial effect on the level of expression. Furthermore, *Ac*HB200 and *Ac*HB32 showed equally high levels of expression as judged by the amount of 32K protein detected on immunoblots and after Coomassie blue staining (Fig. 2).

Distribution of CPMV proteins in S. frugiperda *cells*

The cellular distribution of CPMV proteins produced by the baculovirus vector that contained the 200K coding sequence of B-RNA was tested by subjecting crude sonicated fractions of insect cells to sequential centrifugation steps (Fig. 3). Crude lysates of cells in which the 200K protein was produced were centrifuged at 10000 (lanes 3 and 4), 30000 (lanes 5 and 6) and 100000 *g* (lanes

7 and 8) successively, and pellet and supernatant fractions were analysed by electrophoresis and immunoblotting using anti-VPg and anti-110K anti-sera. It appeared that after low-speed centrifugation most CPMV-specific proteins were distributed equally between the pellet and supernatant fractions (lanes 3 and 4). Almost none of the protein from the 10000 *g* supernatant was sedimented at 30000 *g*, but, upon application of high centrifugal forces (100000 *g*), CPMV-specific polypeptides were found in the pellet (lanes 5 to 8). The 60K protein was present in the different pellet fractions in slightly higher amounts than the other CPMV-specific proteins, which occurred in each fraction in roughly equal amounts.

To obtain more detailed information on the cellular localization of the CPMV proteins, *S. frugiperda* cells infected with *Ac*HB200 were prepared for electron microscopy and immunogold labelling at 48 h p.i. (Fig. 4). Except for the absence of occlusion bodies, *Ac*HB200-infected *S. frugiperda* cells exhibited the cytopathic typical features of an *Ac*NPV infection, such as the presence of an enlarged nucleus containing fibrillar structures, electron-dense 'spacers' and virogenic stroma (van der Wilk *et al.*, 1987). However, in the cytoplasm of *Ac*HB200-infected cells, electron-dense structures as well as membranous vesicles were present, reminiscent of the characteristic cytopathic structures found in CPMV-infected cowpea cells (Fig. 4*a*). The appearance of electron-dense structures and membranous vesicles was not observed in cells infected with wt *Ac*NPV (not shown).

The unusual cytopathic effects, characterized by large arrays of vacuoles and rapid cell lysis, manifested by recombinant *Ac*NPVs expressing the 60K and 170K coding regions of CPMV B-RNA have been noticed before (Fig. 4*c*; van Bokhoven *et al.*, 1990). Electron microscopic evaluation of cells in which either the 60K or 170K protein is synthesized showed that membranous vesicles were also present in the cytoplasm of most of these cells, whereas no electron-dense structures were detected (Fig. 4*d*). The induction of membrane structures was very obvious in these cells, despite the relatively low expression level of the 60K/170K coding sequences. In contrast, neither membranous vesicles nor electron-dense structures were detected in insect cells in which very high amounts of the 87K or 110K proteins of CPMV were synthesized (Fig. 4*e*).

Ultrathin sections of insect cells infected with baculovirus recombinants were further analysed by immunocytochemistry using antisera reactive against CPMV proteins and Protein A–gold. Cells in which the 87K and 110K proteins were synthesized independently of other B-RNA-encoded proteins and which were incubated with anti-24K or anti-110K antiserum showed gold label distributed randomly over the cell (Fig. 4*f*). Sections of cells in which the 60K protein was produced separately were preferentially labelled on the membranous vesicles upon incubation with anti-VPg antiserum (Fig. 4*d*). In sharp contrast, strong labelling of the electron-dense structures in the cytoplasm of *Ac*HB200-infected cells was observed with anti-32K antiserum (Fig. 4*b*), and the anti-24K, anti-110K and anti-VPg antisera (not shown). These results indicate that the majority of CPMV proteins produced in insect cells are present in these electron-dense structures.

Table 1. *Poly(A)/oligo(U)-dependent polymerase activity in extracts of infected insect cells*

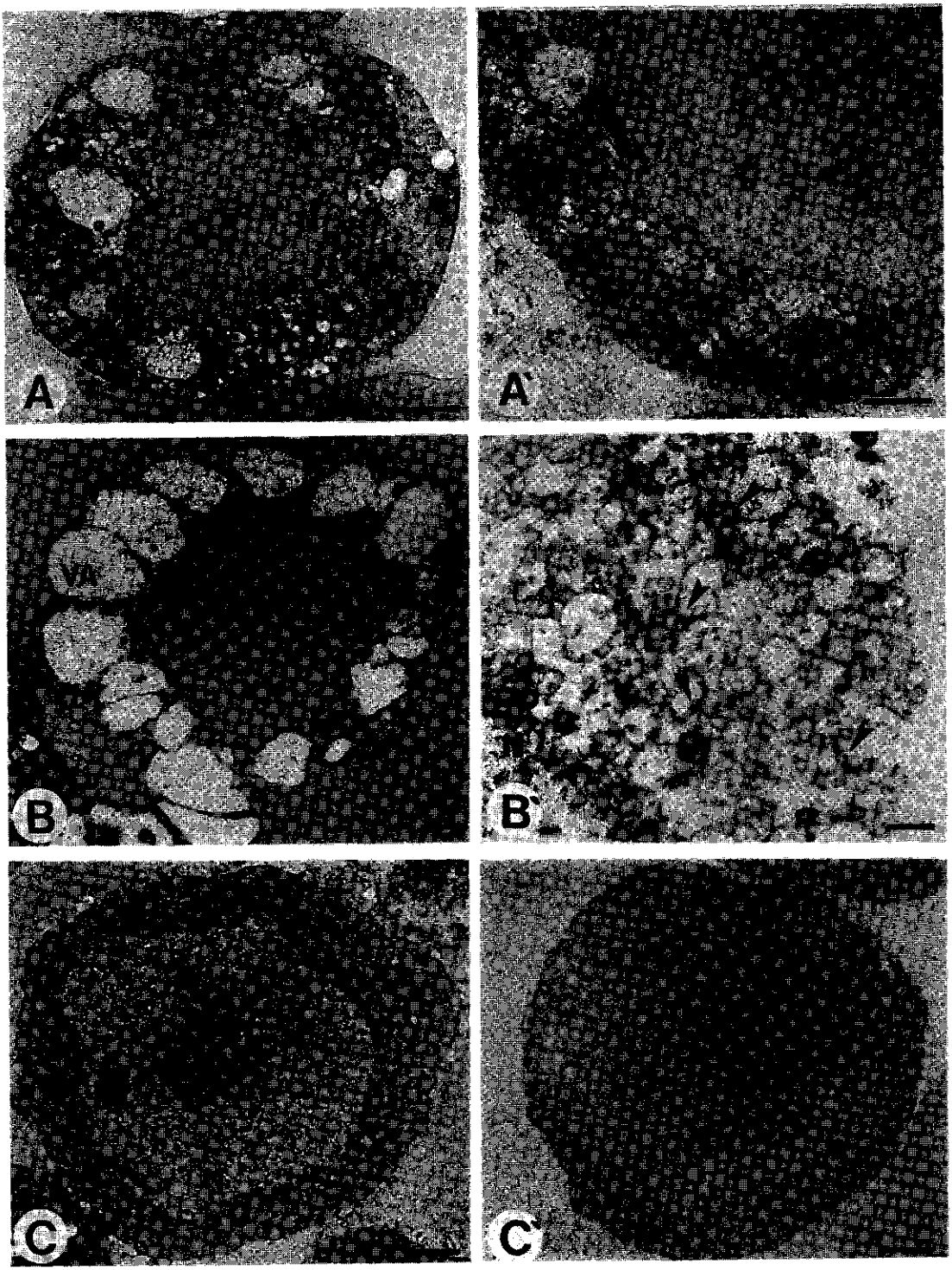
Extract*	Poly(A)/oligo(U)† (c.p.m.)	No template/primer (c.p.m.)
*Ac*NPV	350	280
*Ac*HB3CD	168000	350
*Ac*HB200C	180	120
*Ac*HB200P3	110	100
*Ac*HB200S3	210	160

* Extracts were prepared as indicated in Methods. *Ac*HB200C, crude; *Ac*HB200P3, 100000 *g* pellet; *Ac*HB200S3, 100000 *g* supernatant.

† The assays were performed as described in Methods. Polymerase activity was determined as the amount of [^{3}H]UTP incorporated after 30 min incubation. The incorporation of [^{3}H]UTP at time 0 varied between 100 and 400 c.p.m.

Polymerase assays

S. frugiperda cells, infected at an m.o.i of 10 with *Ac*HB200, were used at 48 h p.i. for the preparation of crude extracts, and pellet and supernatant fractions as described for the experiment in Fig. 3. All extracts were tested for the presence of RNA-dependent RNA polymerase activity in an assay containing poly(A) as a template, oligo(U) as a primer and [^{3}H]UTP. To ensure that no false positive activity was registered the assay was also performed without any primer and template, and with extracts of insect cells that had been infected with wt *Ac*NPV. Crude insect cell extracts in which poliovirus polymerase was produced by baculovirus recombinant *Ac*HB3CD (van Bokhoven *et al.*, 1991) served as a positive control for the assays. Indeed, poliovirus polymerase produced in insect cells exhibited, as before (van Bokhoven *et al.*, 1991), a clearly detectable RNA-synthesizing activity but, in sharp contrast, none of the insect cell fractions containing CPMV proteins showed any detectable polymerase activity (Table 1). Several modifications of the standard polymerase assay were used to determine whether the lack of polymerase activity in extracts of *Ac*HB200-infected insect cells was caused by suboptimal conditions in the assay mixture. However, none of the variations in monovalent (Na, K,


A
A'
VR
B
B'
C
C'

NH_4) or divalent (Mg, Mn, Zn, Ca) cations, pH, rNTP concentration, or DTT, spermidine, polyethylene glycol, actinomycin D and several combinations thereof, had any stimulatory effect on polymerase activity. Also, when CPMV RNA was added as template instead of poly(A), there was no polymerase activity. Furthermore, the addition of crude membrane fractions of CPMV-infected cowpea plants, which have the capacity to elongate nascent RNA chains and might contain essential host proteins, did not cause polymerase activity to act upon exogenous poly(A)/oligo(U).

Discussion

Elucidation of the mechanism of RNA replication for CPMV requires detailed knowledge of the biochemical properties of the B-RNA-encoded proteins and would be especially helped by the definitive identification of the viral RNA polymerase. In this paper we report the high-level synthesis of the entire 200K polyprotein of CPMV B-RNA in insect cells to provide all the viral factors required for replication of this RNA in plants. In insect cells, the 200K polyprotein was faithfully processed into smaller size proteins which were present in cytopathic structures resembling those found in CPMV-infected cowpea cells. Despite these results, which indicate the native character of CPMV proteins in insect cells, viral RNA polymerase activity was not demonstrated in extracts of these cells.

The 200K polyprotein produced by recombinant baculovirus *Ac*HB200 is completely cleaved into proteins of 170K and 32K by the action of the 24K protease moiety of the protein. Presumably, nascent 200K chains undergo this cleavage (Franssen *et* . Further processing of the 170K protein occurred to a limited extent to give CPMV-specific proteins of 112K, 110K, 87K, 84K and 60K in ratios similar to those found in CPMV-infected cowpea leaves. In contrast, previous observations have shown that the 170K protein synthesized by *Ac*HB170 is almost completely processed into products of 110K, 87K, 84K, 60K and 24K (van Bokhoven *et al.*, 1990). These 170K proteins differ only in their N-terminal amino acid. The 170K protein produced by processing of the 200K polyprotein from *Ac*HB200 has a N-terminal serine, whereas the N-terminal amino acid of the 170K protein produced by *Ac*HB170 is methionine. It is known that the N-terminal amino acid residue may influence the stability of the protein *in vivo* (e.g. Tobias *et al.*, 1991), but it seems unlikely that the Ser to Met change would have a dramatic influence on the further proteolytic processing of the 170K protein. More likely, the striking difference in the level of proteolytic cleavage of the 170K protein produced by *Ac*HB170 and *Ac*HB200 in insect cells reflects the regulatory role of the 32K protein on protein processing. Recently, *in vitro* translation studies have revealed that the 32K protein inhibits the processing of the 170K protein, probably by interaction with the 58K protein domain (Peters *et al.*, 1992).

The level of expression of foreign sequences with the baculovirus expression system is influenced by the nature of the protein to be synthesized (Roosien *et al.*, 1990) and the efficiency of translation of the corresponding mRNAs (Luckow & Summers, 1988). The amount of CPMV-specific protein produced by the recombinant baculoviruses containing B-RNA coding sequences under control of the same polyhedrin promoter is also subject to large variations. Very high yields of the 87K and 110K proteins were obtained with *Ac*HB87 and *Ac*HB110 respectively, whereas recombinants that encoded either the 60K (*Ac*HB60) or the 170K (*Ac*HB170) polypeptide produced low amounts of CPMV-specific protein. Baculovirus recombinants *Ac*HB32 and *Ac*HB200 show intermediate levels of expression. It has been proposed that the production of the 60K and 170K proteins in *Ac*HB60- and *Ac*HB170-infected insect cells is impaired as a result of poor translation caused by an intrinsic property of the mRNA for the 60K coding region (van Bokhoven *et al.*, 1990). The increased expression level of *Ac*HB200 in comparison to that of *Ac*HB170 further supports this suggestion. In *Ac*HB200 the same 170K protein coding region as in *Ac*HB170 is almost 1000 bp further downstream of the start codon. The 1000 bp encoding the 32K protein appear to be sufficient to abolish interference of the 60K coding sequence with translation.

Electron micrographs of *S. frugiperda* cells infected with *Ac*HB200 reveal electron-dense structures and membranous vesicles not found in wt *Ac*NPV-infected cells. Similar cytopathic structures, characterized by large arrays of membranous vesicles surrounded by

Fig. 4. Electron micrographs of *S. frugiperda* cells infected with *Ac*HB200 (*a*, *b*), *Ac*HB60 (*c*, *d*) or *Ac*HB110 (*e*, *f*). Typical CPMV cytopathic structures are apparent in *Ac*HB200-infected cells (*a*), showing electron-dense structures (arrows) and membranous vesicles (arrowheads) in the cytoplasm. Electron-dense structures contain CPMV non-structural proteins as indicated by specific gold/silver labelling using anti-32K antiserum (*b*). Large vacuoles (VA; *c*) and vesiculated areas (*d*) are formed in the cytoplasm of cells infected with *Ac*HB60. The 60K protein is associated with the vesicular membranes (arrowheads) as revealed by gold labelling with an anti-VPg serum (*d*). No CPMV cytopathic structures were observed in *Ac*HB110-infected cells (*e*), but CPMV-specific proteins are distributed in the cytoplasm and nucleus of these cells, as shown by labelling with anti-24K antiserum (*f*). N, Nucleus; C, cytoplasm; F, fibrillar structure; S, electron-dense 'spacer'; V, virogenic stroma. Bar markers represent 2 μm (*a* to *c*, *e* and *f*) and 0·5 μm (*d*).

electron-dense material, develop in the cytoplasm of cowpea cells early in infection with CPMV (de Zoeten *et al.*, 1974). The induction of cytopathic structures has also been observed in cowpea protoplasts inoculated with purified B components of CPMV, indicating that the formation of these structures is induced by a B-RNA-encoded function (Rezelman *et al.*, 1982). It has been demonstrated that viral RNA replication complexes are associated with the vesicular membranes of the cytopathic structures in CPMV-infected cowpea cells (de Zoeten *et al.*, 1974; Assink *et al.*, 1973; Zabel *et al.*, 1974). Moreover, isolation of the crude membranes of CPMV-infected cells yields an RNA-dependent RNA polymerase activity capable of fully elongating viral plus-sense RNA, the synthesis of which had already been initiated *in vivo* (Dorssers *et al.*, 1983). Here, it has been shown that induction of electron-dense structures and membranous vesicles is a property of the B-RNA-encoded proteins *per se*, independent of typical plant factors and the occurrence of viral RNA replication.

Furthermore, membrane proliferation into vesicular structures occurs similarly in *S. frugiperda* cells in which only the 60K protein of CPMV B-RNA was produced. Immunogold labelling with anti-VPg serum revealed an association between the 60K protein and these membrane structures. These observations indicate that the 60K polypeptide appears to act as a vesicle-inducing protein and also reinforces the proposed function of the 60K protein in anchoring the replication complex to membranes (Eggen & van Kammen, 1988). For poliovirus, similar activities have been ascribed to proteins of the P2 genomic region. It has been shown by Bienz *et al.* (1983) that poliovirus protein 2BC is involved in the induction of membrane proliferation, leading to the formation of vesicular membranes. Furthermore, protein 2C, which shows significant sequence identity with the 60K protein of CPMV, is likely to be responsible for attachment of RNA replication complexes to vesicular membranes (Bienz *et al.*, 1987, 1990).

The presence of electron-dense structures in the cytoplasm of *S. frugiperda* cells was only observed upon infection with *Ac*HB200. The electron-dense structures could be labelled specifically with anti-CPMV polypeptide antisera (anti-24K, -VPg, -32K, -110K) and Protein A–gold, suggesting that the bulk of the CPMV proteins is present in these structures in *Ac*HB200-infected insect cells, as has been observed in CPMV-infected cowpea protoplasts (Wellink *et al.*, 1988). On the other hand, the 87K and 110K proteins synthesized in very high amounts by *Ac*HB87 and *Ac*HB110, respectively, are dispersed all over the infected cell. In addition to the proteins synthesized by *Ac*HB170, which do not induce the formation of electron-dense structures, recombinant *Ac*HB200 produces the 32K protein. The 32K polypeptide does not occur as a free protein in CPMV-infected protoplasts but is complexed to other B-RNA-encoded proteins (170K, 84K, 60K and 58K; Franssen *et al.*, 1984*b*). Therefore, it is tempting to assume that the 32K protein keeps the other B-RNA-encoded proteins together in complexes which are seen as electron-dense structures. The function of these complexes in the virus life-cycle, if any, remains to be elucidated.

We have not yet been able to demonstrate the presence of RNA-dependent RNA polymerase activity in any of the insect cell extracts containing B-RNA-specific proteins, even when the entire 200K coding region is expressed. There are several observations which indicate that the CPMV proteins produced in insect cells are able to exercise their characteristic activities. (i) The proteolytic processing pattern of the 200K protein in insect cells is indistinguishable from that in cowpea cells; (ii) the induction of electron-dense structures and membranous vesicles, the site of RNA replication in cowpea cells, occurs just as in cowpea cells and may be induced by an activity of the 32K and 60K proteins, respectively; (iii) the 170K, 112K, 110K and 87K proteins produced by *Ac*HB200 have the capacity to bind ribonucleotides, a property shared with the authentic viral proteins (S. Peters, unpublished observations). These four polypeptides all contain the core polymerase domain that is present within the 87K protein. Thus, it seems that the CPMV proteins produced in insect cells are either not able to accept an exogenous primer and template for polymerase activity and/or need an additional host component to function as a polymerase (for discussion see van Bokhoven *et al.*, 1991). To investigate whether a host factor is indeed required by the CPMV polymerase, we are now expressing the CPMV replicative genes in cowpea protoplasts using a transient expression system based on the 35S promoter of cauliflower mosaic virus.

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Chapter 5

Protoplasts transiently expressing the 200K coding sequence of cowpea mosaic virus B-RNA support replication of M-RNA

Protoplasts transiently expressing the 200K coding sequence of cowpea mosaic virus B-RNA support replication of M-RNA

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In order to identify the viral polymerase involved in cowpea mosaic virus (CPMV) RNA replication the 87K, 110K and 170K proteins as well as the complete 200K polyprotein of CPMV B-RNA have been produced in cowpea protoplasts, using expression vectors based on the 35S promoter of cauliflower mosaic virus. CPMV-specific proteins were obtained that were indiscernable from proteins found in CPMV-infected protoplasts. Proteolytic processing of precursor proteins synthesized from the expression vectors proved that the 24K protease contained within these proteins is active. Moreover, it was established that protoplasts transfected with the expression vector containing the entire 200K coding sequence, but not those transfected with vectors containing the 170K, 110K or 87K coding sequences, were able to support replication of co-inoculated M-RNA. Despite the ability to support replication of M-RNA for protoplasts transiently expressing the 200K coding region, CPMV-specific RNA polymerase activity dependent on exogenous added template RNA could not be detected in extracts of these protoplasts in assays using poly(A)/oligo(U) or other template/primer combinations. In contrast, extracts of protoplasts in which poliovirus polymerase (3D^{pol}) was produced exhibited RNA polymerase activity in such assays. These results indicate that the CPMV polymerase, unlike the poliovirus polymerase, is not able to use oligo(u) as a primer or cannot function on exogenous template and primer RNA.

INTRODUCTION

The genetic information of the comovirus cowpea mosaic virus (CPMV) is located on two positive-sense RNA molecules, designated B- and M-RNA. Upon translation of both RNA molecules large polyproteins are produced from which functional proteins are generated by a number of defined proteolytic cleavages executed by a B-RNA-encoded protease (Wellink *et al.*, 1986; Verver *et al.*, 1987). The viral functions that are required for viral RNA replication are all supplied by B-RNA (Goldbach *et al.*, 1980), whereas the proteins encoded by M-RNA are indispensable for virus cell-to-cell movement (Wellink & van Kammen, 1989).

Characteristic cytopathic structures arise in the cytoplasm of cowpea cells at early stages of infection with CPMV (de Zoeten *et al.*, 1974). These structures consist of numerous vesicular membranes surrounded by electron-dense material in which the bulk of the non-structural proteins encoded by B-RNA are found (Wellink *et al.*,1988). Replication of CPMV RNA is associated with these membranous vesicles (de Zoeten *et al.*, 1974) and, accordingly, viral RNA-dependent RNA polymerase activity capable of elongating *in vivo* initiated (+) sense RNA has been detected in the crude membrane fraction of CPMV-infected leaves (Dorssers *et al.*, 1983). Extensive purification of the RNA polymerase activity from the membrane fraction resulted in a preparation in which the B-RNA-encoded 110K protein was the sole detectable viral protein and this protein was therefore assigned the viral RNA polymerase (Dorssers *et al.*, 1984). In addition to the 110K protein, the active preparation contained two major host proteins of 57K and 68K, but it is not known whether these polypeptides also fulfil a role in viral RNA replication. At the other hand, the inhibition of RNA replication in cowpea protoplasts by actinomycin D early in infection with CPMV suggests that one or more host proteins are needed for CPMV RNA replication (Rottier *et al.*, 1979; de Varennes *et al.*, 1985).

The 87K protein of CPMV, which together with the 24K protease constitutes the 110K protein, has significant sequence homology with poliovirus polymerase 3D^{pol} and also shares conserved sequence motifs with the putative RNA-dependent RNA polymerases of other viruses (Kamer & Argos, 1984; Franssen *et al.*, 1984a; Poch *et al.*, 1989). However, unlike poliovirus (Flanegan & Baltimore, 1977; Van Dyke & Flanegan, 1980, Baron & Baltimore, 1982) and several plant RNA viruses such as brome mosaic virus (Miller & Hall, 1983; Quadt *et al.*, 1988), alfalfa mosaic virus (Houwing & Jaspars, 1986) and cucumber mosaic virus (Hayes & Buck, 1990), RNA polymerase activity dependent on exogenous template has never been obtained from CPMV-infected cells (Dorssers *et*

al., 1983, 1984; Eggen *et al.*, 1988). Furthermore, poliovirus protein $3D^{pol}$ synthesized in *Escherichia coli* (Morrow *et al.*, 1987; Rothstein *et al.*, 1988) and insect cells (van Bokhoven *et al.*, 1991) exhibited RNA polymerase activity in a poly(A)-oligo(U) assay. We have previously employed the same heterologous expression systems to produce the putative 110K polymerase as well as the other proteins encoded by B-RNA (Richards *et al.*, 1989; van Bokhoven *et al.*, 1990, 1991, 1992). Although the proteins thus produced were very similar to the corresponding viral proteins found in plant cells, it was not possible to demonstrate oligo(U)-primed RNA polymerase activity in cell extracts containing CPMV-specific proteins. Even insect cells in which the complete 200K polyprotein encoded by B-RNA was synthesized did not contain any virus-specific RNA-dependent RNA polymerase activity (van Bokhoven *et al.*, 1992). It was concluded that CPMV polymerase, in contrast to poliovirus $3D^{pol}$, is not able to make use of exogenous added template and primer RNA or requires an additional plant component to function as a polymerase.

In this paper we have exploited a plant expression vector containing the 35S promoter of cauliflower mosaic virus to produce the 87K, 110K, 170K and 200K proteins encoded by CPMV B-RNA in cowpea protoplasts. Transfected protoplasts were subsequently assayed for CPMV-specific RNA polymerase activity.

METHODS

Construction of plasmids

Escherichia coli strain DH5α was used for growth and maintance of all plasmids. Plant expression vector pMON999 was kindly provided by Dr. C. Hemenway of Monsanto Company. Plasmid pBI121, containing the gene for β-glucuronidase (GUS) has been described by Jefferson *et al.* (1987). Plasmids pTB1G and pTM1G contain full-length cDNA copies of CPMV B- and M-RNA respectively from which infectious RNA transcripts can be generated using T7 RNA polymerase (Eggen *et al.*, 1989). Plasmids pTBHM60, pTBHM87, pTBHM110 and pTB200N are derivatives of pTB1G with an engineered *Nde*I recognition site just at the 5' end of the coding sequence for the protein they denote (van Bokhoven *et al.*, 1990, 1992). Baculovirus transfer vector pAcHB3CDΔ contains the poliovirus 3CD coding region (Van Bokhoven *et al.*, 1991). All DNA manipulations were carried out by standard recombinant DNA techniques (Sambrook *et al.*, 1989).

Plasmid pMGUS was constructed by ligating the *Xba*I-*Sst*I fragment from pBI121 containing the GUS gene into the corresponding sites of pMON999.

To create pMB87, plasmid pTBHMB87 was digested with *Nde*I (position 3672; Lomonossoff & Shanks, 1983), filled in with Klenow polymerase and digested with *Cla*I just downstream of the poly (A) sequence at position 5935. The 2.3 kb *Nde*I-*Cla*I fragment was ligated into pMON999 cleaved with *Bgl*II (filled in) and *Cla*I, thus restoring the *Bgl*II site. Plasmid pMB110 was constructed by digestion of pTBHM110 with *Nde*I (position 3048; filled in) and *Xho*I (position 3978) and insertion of the isolated 0.9 kb fragment into pMB87 digested with *Bgl*II (filled in) and *Xho*I.

For construction of pMB170, a *Nde*I (filled in)-*Bam*HI fragment (position 1185 to 3857) from pTBHM60 was first subcloned in pUC19 digested with *Asp*718 (filled in) and *Bam*HI. The 60K coding region was retrieved from this plasmid by digestion with *Sst*I and *Bam*HI and subsequently ligated into the corresponding sites of pMON999 to give pMB60. Expression vector pMB170 was then created in a three-fragment ligation using DNA fragments from pTB1G (*Nde*I-*Xho*I; position 2080 to 3978), pMB60 (*Pst*I-*Nde*I) and pMB87 (*Pst*I-*Xho*I).

To create expression vector pMB200, subclone pMBΔ32 was constructed by insertion of a 0.7 kb *Nde*I (position 207, filled in)-*Xba*I (position 899) fragment from pTB200N into pMON999 digested with *Bgl*II (filled in) and *Xba*I. DNA fragments from pTB1G (*Xba*I-*Xho*I; position 899 to 3978), pMBΔ32 (*Xba*I-*Bam*HI) and pMB170 (*Xho*I-*Bam*HI) were used in a three-fragment ligation to give expression vector pMB200.

Plasmid pMP3CD was constructed by insertion of the poliovirus 3CD coding region, contained in a *Bam*HI-*Kpn*I fragment from pAcHB3CDΔ, in the *Bgl*II-*Kpn*I sites of pMON999.

For the generation of (-)-sense transcripts of CPMV B-RNA a *Sna*BI site was introduced at position -4 to +2 of the B-cDNA sequence. For this, the *Sal*I-*Sst*I (position -40 to 2301) fragment of pTB1G was inserted in M13mp19 and subjected to mutagenesis (Kunkel, 1985) using the oligonucleotide 5'-GATTTTAA<u>TACGTA</u>TAGTGAGTC-3', resulting in an insertion of a C nucleotide in the complementary sequence (represented by the bold G) and thereby the generation of a *Sna*BI recognition site (underlined). This clone was digested with *Sal*I and *Nde*I (position 2080) and the 2.1 kb fragment carrying the mutation as well as the T7 promoter was isolated and exchanged with the homologous sequence of pTB1G to give pTBSna. The T7 promoter of plasmid Bluescript (SK^-) was removed by deletion of the *Pvu*II-*Sal*I fragment. Plasmid pSnaB was then created by insertion of the *Sal*I-*Cla*I fragment of pTBSna, comprising the T7 promoter and the entire B-cDNA sequence, into this modified Bluescript clone. Upon linearization of pSnaB with *Sna*BI and *in vitro* transcription with T3 polymerase, B-RNA transcripts of (-)-sense polarity were obtained which carried only one additional C at the 3' end as compared to B(-)-RNA found in CPMV-infected plants.

Transfection of cowpea protoplasts

Cowpea (*Vigna unguiculata* L.) mesophyll protoplasts were prepared essentially as described previously (Rezelman *et al.*, 1989), except that 10 mM $CaCl_2$ was included in the enzyme solution. Cesium chloride-purified vector DNA (10 µg in 25 ml ice-cold water) was added to 1 x 10^6 protoplasts in 100 ml ice-cold 0.6 M mannitol containing 10 mM $CaCl_2$, immediately followed by addition of a 0.5 ml solution of 40% polyethylene glycol (M_r 6000), 0.5 M mannitol and 0.1 M $Ca(NO_3)_2$ (Negrutiu *et al.*, 1987). The protoplast suspension was gently mixed for 10-15 seconds, diluted with 4.5 ml 0.5 M mannitol, 15 mM $MgCl_2$ and 0.1% MES (pH 5.6) and kept at room temperature for 20 min. The protoplasts were then washed with 0.6 M mannitol and 10 mM $CaCl_2$ and incubated for 16-66 h as described previously (Rottier *et al.*, 1979).

Determination of GUS activity

GUS activity in extracts of protoplasts was determined essentially as described previously (Jefferson, 1987). One unit of GUS activity is defined as the amount of enzyme required to produce 1 pmole of methylumbelliferone in 1 min at 37 °C using 1mM 4-methylumbelliferyl glucuronide as a substrate.

GUS activity in individual protoplasts was assayed at 42 h post transfection (p.t.). For this, the protoplast culture medium was replaced with a solution of 100 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 2 mM X-Gluc substrate. Reactions were carried out at 37 °C until an indigo precipitate was visible (usually after a few hours). After staining, protoplasts were rinsed in 70% ethanol and examined by light microscopy.

Analysis of proteins synthesized in cowpea protoplasts

At 16-42 h p.t. protoplasts were harvested to determine expression of sequences under control of the CaMV 35S promoter. Transfected protoplasts were stained by the indirect fluorescent antibody technique (Hibi *et al.*, 1975; Maule *et al.*, 1980), using one of the following antisera: anti-24K (Wellink *et al.*, 1987), anti-VPg (Eggen *et al.*, 1988), anti-32K (Franssen *et al.*, 1984b), anti-110K (van Bokhoven *et al.*, 1992) or anti-poliovirus 3D (kindly provided by Dr O. C. Richards, University of Utah Medical Center). Upon treatment with goat anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC; Nordic) the protoplasts were examined by fluorescence microscopy.

For immunoblot analysis of the proteins synthesized by the expression vectors, protoplasts were collected by centrifugation at 100 x g for 2 min, washed and resuspended in 50 mM Tris-acetate pH 8.0, 25% glycerol, 50 mM potassium acetate, 1 mM EDTA, 5 mM DTT and 0.5 mM PMSF at 1 x 10^7 protoplasts per ml. Cells were disrupted by centrifugation at 30,000 x g for 30 min. Aliquots, corresponding to 2 x 10^5 protoplasts, of the resulting pellet (P30) and supernatant (S30) fraction were electrophoresed in 10% SDS-polyacrylamide gels as described by Laemmli (1970). Immunoblots of these gels were prepared as described previously (van Bokhoven *et al.*, 1990) using the antisera mentioned above.

Detection of RNA polymerase activity

At 60 h p.t. protoplasts, co-transfected with plasmid DNA and M-RNA transcript derived from pTM1G, were collected by centrifugation and pellets were frozen at -80 °C. Total RNA was extracted from the protoplasts as described by de Vries *et al.* (1988) and electrophoresed in a 1% denaturing agarose gel (McMaster & Carmichael, 1977). The separated RNAs were transferred to GeneScreen (NEN Research Products) and hybridized with ^{32}P-labelled probe prepared by random-primer labelling (Feinberg & Vogelstein, 1984) of a *Hind*III fragment (position 482 to 2231) of pTM1G for detection of M-RNA.

RNA polymerase assays were performed on crude extracts and S30 supernatant fractions, prepared as described above. Determination of RNA polymerase activity in these extracts was as described previously (Flanegan & Baltimore, 1977; van Bokhoven *et al.*, 1991).

RESULTS

Optimal conditions for transfection of cowpea protoplasts

An expression system based on the CaMV 35S promoter was developed for use in cowpea protoplasts. All expression vectors used in this study are derived from plasmid pMON999 by insertion of sequences of interest in the multiple cloning site in this vector that is located between an enhanced CaMV 35S promoter and a nopaline synthase terminator (Fig. 1). Plasmid pMGUS, containing the bacterial reporter gene coding for β-glucuronidase (GUS; Jefferson, 1987), was used to determine the optimal conditions for transfection of cowpea protoplasts. Cowpea protoplasts were transfected with plasmid DNA using electroporation (Hibi *et al.*, 1986), liposomes (Malone *et al.*, 1989) or polyethylene glycol (PEG) (Negrutiu *et al.*, 1987). The highest GUS activity, as determined by fluorometry, was found if protoplasts were transfected in a medium with 40% PEG, 0.5 M mannitol and 0.1 M $Ca(NO_3)_2$. Under such conditions, using 10 µg DNA per 1 x 10^6 protoplasts, approximately 70% of the protoplasts survived and expressed the GUS gene as seen with X-gluc staining. In a typical experiment the GUS activity was 1500 units/ 100,000 protoplasts at 16 h p.t. and 2000 units/ 100,000 protoplasts at 42 and 66 h p.t..

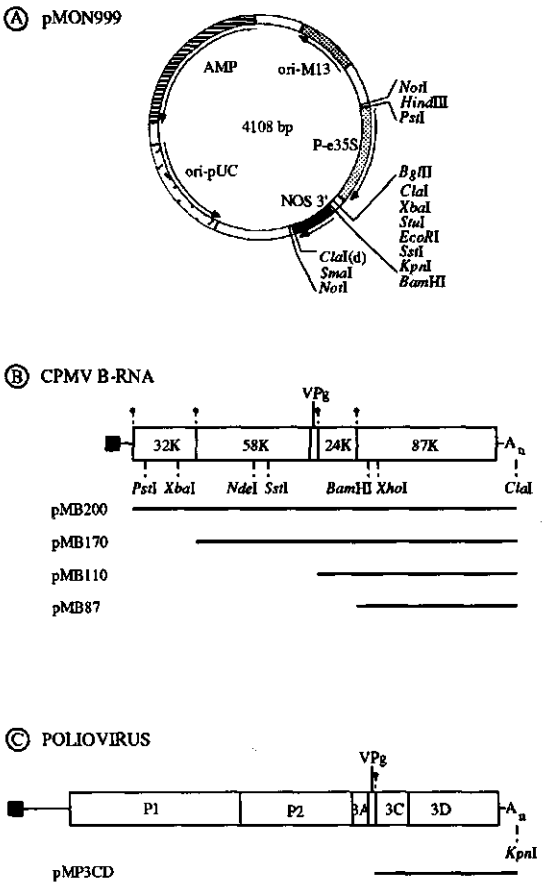

FIGURE 1. Schematic diagram of the expression vectors used in this study. A) Expression vector pMON999. Indicated are the restriction sites and the relevant sequences contained within the plasmid: P-e35S, enhanced CaMV 35S promoter; NOS 3', nopaline synthase terminator; ClaI(d), dam-methylated *Cla*I site. B) Top, genetic organization of CPMV B-RNA. VPg at the 5' end of the RNA is drawn as a black square. The single open reading frame codes for a 200K polyprotein and is represented by an open bar in which final cleavage products (the 32K, 58K, 24K and 87K proteins and VPg) are indicated. The other protein species mentioned in the text are composed of these final cleavage products as follows: 170K = 58K-VPg-24K-87K; 60K = 58K-VPg; 84K = 58K-VPg-24K; 112K = VPg-24K-87K and 110K = 24K-87K. The position of the restriction sites in the B-cDNA sequence used for the construction of the expression vectors are indicated. The open circles represent the engineered *Nde*I sites. Bottom, expression vectors containing CPMV B-cDNA sequences. The sequences represented by solid lines were cloned in the polylinker of pMON999. C) Genetic organization of poliovirus RNA and relevant position of the cDNA sequence inserted in pMON999 to create expression vector pMP3CD. For details of vector constructions see Methods.

The transfection conditions for introducing DNA into protoplasts were also very efficient for inoculation of cowpea protoplasts with viral RNA. As little as 0.1 µg CPMV RNA sufficed to infect 80% of 1 x 10^6 protoplasts, while inoculation of 1 x 10^6 protoplasts with 1 µg B-RNA transcript resulted in B-RNA replicating in 40% of these protoplasts as determined by immunofluorescence staining with anti-110K serum.

Expression of CPMV and picornaviral sequences in protoplasts

The multiple cloning site of pMON999 was used to insert the coding sequences for the 87K, 110K, 170K and the complete 200K polyprotein of CPMV B-RNA, resulting in expression vectors pMB87, pMB110, pMB170 and pMB200 respectively (Fig. 1). Cowpea protoplasts were transfected with these plasmids or inoculated with CPMV RNA under the conditions mentioned above. At 16 h p.t. the protoplasts were harvested, fractionated into 30,000 x g pellet (P30) and supernatant (S30) fractions and proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. Use of CPMV-specific antibodies revealed that approximately 90% of the proteins produced by each of the expression vectors resided in the supernatant fraction (data not shown).

Protoplasts transfected with plasmids pMB200, pMB110 and pMB87 contained amounts of CPMV-specific proteins, that could be detected on polyacrylamide gels stained with Coomassie brilliant blue. The expression from pMB170 was less but still sufficient to detect proteolytic processing products of the 170K protein on immunoblots (Fig. 2).

The 87K protein produced by pMB87 showed an electrophoretic mobility and immunoreactivity with anti-110K serum identical to the authentic 87K protein found in CPMV-infected protoplasts (Fig. 2, lanes 5 and 7). Expression of the 110K coding sequence by pMB110 resulted in the synthesis of a proteins, co-migrating with the authentic 110K protein from CPMV-infected protoplasts. Proteolytic cleavage of the 110K protein occurred very inefficiently as hardly any 87K protein was visible on immunoblots of proteins extracted from protoplasts at 16 h p.t.. Also at later time points (> 42 h p.t.) only about 5% of 110K protein encoded by pMB110 was processed into proteins of 24K and 87K (data not shown). These results are in agreement with *in vitro* translation studies that have revealed that efficient cleavage at the 24K and 87K junction requires sequences upstream of the 24K protease (Dessens & Lomonossoff, 1992).

The processing patterns of the 200K polyproteins synthesized in protoplasts transfected with either pMB200 or CPMV B-RNA were very similar (Fig. 2, lanes 2 and 7; see legend of Fig. 1 for the composition

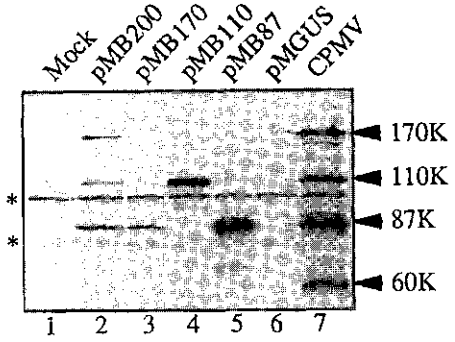

FIGURE 2. Immunoblot analysis of CPMV proteins produced in cowpea protoplasts. Protoplasts were collected at 16 h p.t., disrupted by centrifugation and proteins from the supernatant (S30) fraction were fractionated in 10% polyacrylamide gels. Detection of CPMV-specific proteins was with anti-110K and anti-VPg serum. Cowpea proteins that cross-react with these antisera are indicated by asteriks. Lane 1, mock-inoculated protoplasts; lanes 2-6, protoplasts transfected with indicated expression vectors; lane 7, protoplasts inoculated with CPMV RNA.

of the different protein species). In both cases proteolytic processing of the 200K polyprotein into proteins 170K and 32K appeared very efficient as the 170K protein was the only CPMV-specific protein that could be detected on Coomassie brilliant blue-stained polyacrylamide gels (data not shown). Further processing of the 170K protein into 112K, 110K, 87K, 84K and 60K proteins occurred only to a limited extent (Fig. 2, lanes 2 and 7 and data not shown). The 60K protein seems to be underrepresented in pMB200 transfected protoplasts. We have no explanation for this discrepancy. In protoplasts transfected with pMB170 the B-RNA-specific proteins that could be identified were the 112K, 110K and 87K C-terminal cleavage products (Fig. 2, lane 3 and data not shown), indicating that the 170K protein was processed very efficiently. Remarkably, the 60K and 84K proteins were not detected in protoplasts transfected with pMB170.

Expression vector pMP3CD was created for the synthesis of poliovirus protein 3CD in cowpea protoplasts. On an immunoblot of protoplasts transfected with pMP3CD a protein of approximately 53K was visible that was immunoreactive with anti-3D serum and co-migrated with poliovirus protein 3D found in infected HeLa cells (data not shown). This protein band was not present in extracts of protoplasts transfected with pMGUS (data not shown), indicating that indeed poliovirus protein 3D had been produced by pMP3CD. The presence of 3D protein in protoplasts strongly suggests that proteolytic processing of precursor protein 3CD had occurred.

Localization of CPMV proteins by immunofluorescence

By immunofluorescence staining with anti-110K serum and goat-anti-rabbit IgG conjugated with FITC it was possible to quantitate the number of protoplasts in which considerable amounts of CPMV-specific protein were produced (Table 1). For each of the expression vectors and RNAs used for transfection, the percentage of protoplasts showing fluorescence was in good agreement with the amount of protein detected on immunoblots (Table 1).

Immunofluorescent staining of transfected protoplasts was also used to roughly locate B-RNA-specific proteins within the protoplasts. A remarkable difference in the localization of the fluorescent label was observed between, on the one hand, protoplasts transfected with CPMV-RNA or pMB200 and, on the other hand, protoplasts transfected with pMB87, pMB110 or pMB170. Typical examples of these localization studies are shown in Fig. 3. In protoplasts transfected with CPMV-RNA (Fig. 3A) or pMB200 (Fig. 3B) the fluorescent signal after treatment with anti-VPg serum was concentrated in one or a few restricted areas in the cytoplasm of the protoplast. Use of anti-32K, anti-24K and anti-110K antisera resulted in a similar localized immunofluorescence, except that the anti-110K serum always gave also fluorescence in other parts of the protoplast (Fig. 3C). These results indicate that majority of the different viral proteins had accumulated at restricted areas in these protoplasts. In contrast, such accumulation of CPMV-specific proteins was not observed in protoplasts transfected with pMB87, pMB110 or pMB170 (Fig. 3 and data not shown). In these cases the fluorescence after treatment with anti-110K serum occurred dispersed over the entire cell (Fig. 3D).

Expression of cDNA from the 200K coding region supports replication of co-inoculated M-RNA

Having established that the proteins synthesized by the expression vectors are similar to the authentic viral proteins in immunogenic properties, electrophoretic mobility and, at least in one case activity (24K protease), it was of interest whether the B-RNA-specific proteins produced by the expression vectors were capable of replicating viral RNA. For that purpose protoplasts were transfected with the different expression vectors together with M-RNA. As a control, protoplasts were transfected with B- and M-RNA transcripts.

Immunofluorescent staining (Table 1) with anti-CPMV serum at 16 h p.t. detected the presence of the capsid proteins encoded by M-RNA in 20% of the protoplasts transfected with B- and M-RNA transcripts, indicative of the occurrence of replication of M-RNA in

Table 1: *Immunofluorescent staining of cowpea protoplasts*

protoplasts*	immunofluorescence†	
	anti-110K	anti-CPMV
B-RNA	40	20
pMB200	20	10
pMB170	20	0
pMB110	50	0
pMB87	50	0
pMGUS	0	0

*) Cowpea protoplasts were transfected with B-RNA transcripts or expression vector DNA in the presence of M-RNA transcripts.
†) Immunofluorescent staining was with the indicated antisera. The numbers in the table represent the percentage of fluorescing protoplasts as obtained in a typical experiment.

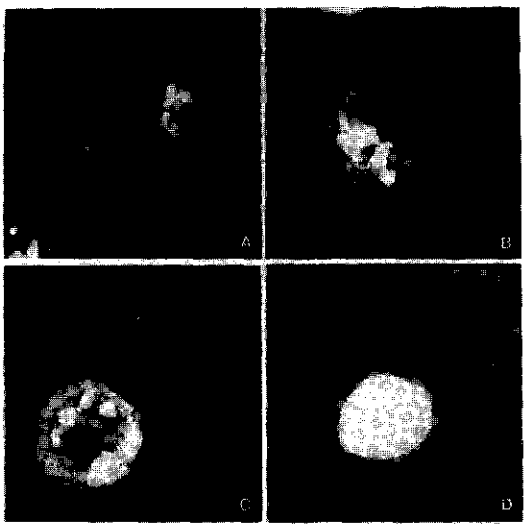

FIGURE 3. Immunofluorescent staining of cowpea protoplasts inoculated with CPMV RNA (A) or transfected with pMB200 (B, C) or pMB170 (D). Detection of CPMV-specific proteins was with anti-VPg (A, B) or anti-110K serum (C, D) followed by treatment with goat-anti-rabbit IgG conjugated with FITC. Note that the immunofluorescent staining in protoplasts transfected with CPMV RNA or pMB200 is concentrated in a few restricted areas.

these protoplasts. Replication of M-RNA was also observed in approximately 10% of the protoplasts transfected with pMB200 together with M-RNA, as determined by immunofluorescent staining. No fluorescent staining with anti-CPMV serum was observed in protoplasts transfected with pMB170, pMB110 or pMB87 in the presence of M-RNA, while immunofluorescent staining with anti-110K serum verified that indeed expression of the B-RNA coding sequences had occurred in these protoplasts. Thus it appeared that the proteins expressed from pMB200 but not those from pMB170, pMB110 and pMB87 are able to support replication of M-RNA. This was confirmed by analysis of the RNA content of the protoplasts. At 42 h p.t. total RNA was extracted from protoplasts and equal amounts of RNA were analyzed on a Northern blot hybridized with an M-RNA-specific probe. Figure 4 shows that RNA from protoplasts transfected with pMB200 gave a hybridization signal on the blot at the same migrational position as M-RNA produced in protoplasts inoculated with B- and M-RNA. In none of the other lanes was a hybridization signal detected even after prolonged autoradiography.

Polymerase assays

Cowpea protoplasts isolated 16 h after transfection with the different expression vectors were used to prepare fractions to test for RNA-dependent RNA polymerase activity using poly(A) as a template and oligo(U) as a primer (Table 2). Partially purified poliovirus 3D polymerase obtained from poliovirus-infected HeLa cells and a crude extract of insect cells in which $3D^{pol}$ was produced by a baculovirus expression vector (van Bokhoven *et al.*, 1991) were used as a control for the assays and indeed exhibited a template and primer dependent polymerase activity. An S30 fraction of pMP3CD-transfected protoplasts contained a similar RNA-synthesizing activity dependent on addition of both template and primer, indicating that biochemically active poliovirus polymerase ($3D^{pol}$) was synthesized in cowpea cells. In striking contrast, no polymerase activity was detected in any of the extracts (S30, P30 or crude extracts containing CPMV proteins (Table 2). The assays were repeated with other template/primer combinations to investigate whether the absence of polymerase activity might be due to specificity of CPMV polymerase for its homologous RNA. But none of the other substrates used, i.e. CPMV RNA with or without oligo(U) and (-) sense B-transcripts with or without (+) sense B-primer, resulted in detectable CPMV polymerase activity, whereas poliovirus polymerase was active with these templates (data not shown). No stimulatory effect on CPMV polymerase activity was obtained by varying the assay conditions, including conditions which have been used to demonstrate RNA-elongating activity in crude

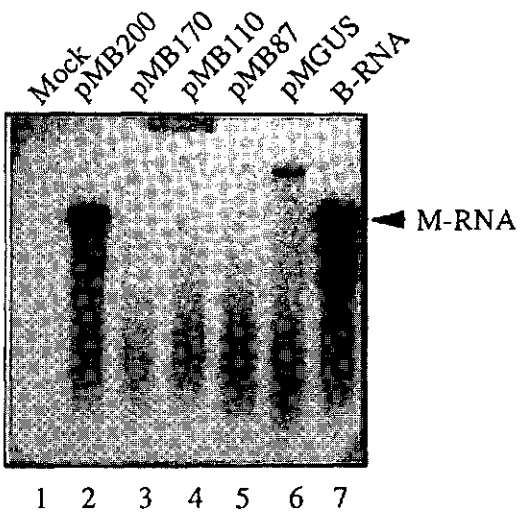

FIGURE 4. Determination of M-RNA replication in cowpea protoplasts. Total RNA extracted from protoplasts at 16 h p.t. was separated in a 1% denaturing agarose gel followed by transfer of the RNAs onto GeneScreen. The blot was hybridized with a probe specific for M-RNA and exposure was for 16 h (with a screen) to a Kodak XAR film. The position of M-RNA is indicated by an arrow head. Lane 1 contains RNA from protoplasts inoculated with M-RNA only (mock). Lanes 2 to 6 contain RNA from protoplasts transfected with the indicated expression vectors together with M-RNA. Lane 7, RNA from protoplasts inoculated with B- and M-RNA.

Table 2: *Poly(A)/oligo(U) polymerase activity in cell extracts*

extract*	poly(A)/oligo(U)	no template/primer
AcHB3CD	126,000†	160
HeLa	7,200	150
pMP3CD	1,500	140
pMB200	160	170
pMB170	160	150
pMB110	130	160
pMB87	100	130
pMGUS	150	150

*) Extracts of protoplasts were prepared as described in the text. AcHB3CD is a crude extract of insect cells containing poliovirus $3D^{pol}$ and HeLa is a partially purified preparation of $3D^{pol}$ from poliovirus-infected HeLa cells.
†) Polymerase activity is expressed as the amount of [^{3}H]-UTP incorporated label after 30 min incubation.

membrane fractions of CPMV-infected cowpea plants (Dorssers *et al.*, 1984).

DISCUSSION

We have shown that expression vector pMON999 lends itself very well for synthesis of CPMV-specific proteins in cowpea protoplasts. The expression levels obtained with this system were remarkably high, especially for the 87K, 110K and 200K coding regions of CPMV B-RNA, even though the amount of these proteins was lower than obtained for these proteins with the baculovirus expression system (van Bokhoven *et al.*, 1990; 1992). Expression in cowpea protoplasts has an advantage over the baculovirus expression system as it provides a tool for examining the characteristics and behavior of individual CPMV proteins and mutants thereoff in plant cells. Hitherto, such experiments have not been possible since the production of viral proteins in cowpea cells was dependent on the occurrence of viral RNA replication. Furthermore, the system proves to be useful for the production of proteins of animal virus origin, as is illustrated by the synthesis of functional $3D^{pol}$ of poliovirus.

The proteins produced by pMB200 were active in supporting replication of M-RNA transcripts in cowpea protoplasts. No replication of M-RNA was observed in protoplasts transfected with either pMB170, pMB110 or pMB87, suggesting that expression of the entire 200K coding region is required for replication. Since the 32K protein is the only additional viral protein produced by pMB200 in comparison to pMB170, the 32K protein seems to serve a crucial role in achieving viral RNA replication. Recently, it was demonstrated that the 32K protein has a regulatory role in the proteolytic processing of the 170K protein and it was proposed that this regulation may be important in building an active replication complex (Peters *et al.*, 1992a). The present results show that in cowpea protoplasts the rate of proteolytic processing is also strongly affected by the presence of the 32K protein. In extracts of protoplasts transfected with pMB200 or with CPMV RNA, the 170K protein was the most abundant viral protein and processing of this primary cleavage product occurred to a limited extent only. In contrast, no 170K protein could be detected in pMB170-transfected protoplasts in which the 32K protein is not produced. Rather strikingly only the 112K, 110K and 87K cleavage products from the C-terminal part of the 170K protein and not the 60K and 84K proteins from the N-terminal end were found in cells transfected with pMB170 (see legend of Fig. 1 for the composition of these proteins). It could be argued that VPg was removed from the 60K and 84K proteins, preventing their detection with anti-VPg serum. However, in *in vitro* proteolytic processing studies the 60K and 84K

proteins are clearly detectable (Peters *et al.*, 1992b). Therefore, it seems likely that the 60K and 84K proteins are not stable in protoplasts transfected with pMB170. This may point to a role of the 32K protein in keeping the proteins with a function in viral RNA replication together and/or in guiding them in forming an active replication complex. As long as we do not understand the instability of the 60K and 84K proteins in pMB170 transfected cells we should be cautious in concluding that pMB170 will not be capable of replicating co-inoculated M-RNA.

Upon infection of cowpea cells with CPMV characteristic cytopathic structures appear in the cytoplasm, consisting of numerous membranous vesicles and amorphous electron-dense material (Assink *et al.*, 1973; De Zoeten *et al.*, 1974). It has been shown previously that in protoplasts inoculated with CPMV RNA most of the B-RNA-encoded proteins accumulate in these electron-dense structures (Wellink *et al.*, 1988). Immunofluorescent staining of protoplasts transfected with pMB200 or inoculated with CPMV RNA revealed that the non-structural proteins encoded by B-RNA were aggregated in one or a few areas in the cytoplasm of the protoplast. In protoplasts transfected with either pMB87, pMB110 or pMB170 viral proteins were found dispersed over the entire cell. Thus it seems that the 32K protein, which is the only unique domain in the 200K polyprotein in comparison with the other B-proteins that have been synthesized, is somehow involved in keeping together B-RNA-encoded proteins in defined areas of the cell. In this context it is remarkable that the 32K protein is known to interact with the 170K, 84K, 60K and 58K proteins encoded by B-RNA (Franssen *et al.*, 1984b; Peters *et al.*, 1992a). The immunofluorescent localization studies are in agreement with the results on the expression of B-RNA-encoded proteins in insect cells by means of the baculovirus expression system (van Bokhoven *et al.*, 1992). Insect cells that produce the 200K polyprotein of B-RNA developed electron-dense structures, whereas the independent synthesis of the 170K, 110K, 87K and 60K proteins did not result in the induction of electron-dense structures in such cells (van Bokhoven *et al.*, 1992). Therefore, the localized areas of fluorescent staining likely represents the occurrence of electron-dense structures. The biological significance of the electron-dense structures in CPMV-infected cells remains obscure. Perhaps, the electron-dense structures are the storage-sites where B-RNA-encoded proteins are maintained in an active conformation for RNA replication or other processes of the viral life-cycle. Alternatively, the electron-dense material may be waste-baskets for abundant viral proteins.

Although pMB200-transfected protoplasts supported replication of co-inoculated M-RNA, it was not possible to demonstrate *in vitro* RNA-dependent RNA polymerase activity in extracts of these protoplasts, nor in extracts of protoplasts transfected with pMB170, pMB110 or pMB87. In contrast, poliovirus polymerase $3D^{pol}$ produced in cowpea protoplasts by pMP3CD exhibited RNA polymerase activity *in vitro*, providing evidence that the polymerase assay functions in extracts of protoplasts. Why then is the CPMV polymerase not able to function under these conditions? Although we can not exclude that an essential host factor could have been dissociated from the CPMV proteins prior to the *in vitro* polymerase assays we consider it more likely that the CPMV polymerase can not use oligo(U) as a primer or can not function on any exogenous template and/or primer. Recently, we have obtained evidence that replication of both B- and M-RNA is tightly linked to translation of the RNAs (van Bokhoven et al., in press). Therefore, it may be anticipated that *in vitro* CPMV replicase activity using exogenous template RNA will only be measurable under conditions that also facilitate translation of the added template RNA molecules. Poliovirus $3D^{pol}$, at the other hand, is capable of *in vitro* RNA synthesis in a template- and primer-dependent fashion (Flanegan & Baltimore, 1977). Perhaps, the ability of the poliovirus polymerase to use poly(A)/oligo(U) (or any other template/primer combination) may be a fortuitous property not shared by the CPMV polymerase, which may be more fastidious in accepting other than the natural primer. Indeed, the oligo(U)-primed polymerase activity of $3D^{pol}$ is not at all specific for the poliovirus RNA template and furthermore does not reflect the *in vivo* initiation of replication events in which VPg is likely to be involved (Kuhn & Wimmer, 1987). Thus far, true replication *in vitro* of poliovirus RNA, i.e. synthesis on genomic RNA template of a (-) strand RNA which is subsequently used as a template for the synthesis of the complementary (+) strand, has been accomplished only in extracts of uninfected HeLa cells that support translation of the exogenous RNA molecules (Molla *et al.*, 1991).

Notwithstanding the difficulties in the study of CPMV RNA replication *in vitro*, the expression system described in this paper offers new opportunities for the examination of the viral RNA replication process *in vivo*. One interesting question to be answered is whether mRNAs generated by expression vector pMB200 can be used as templates for the synthesis of complementary (-)-strand RNA. Although the mRNAs produced by pMB200 have additional nucleotides at their 3' termini as compared to viral B-RNA, this should not interfere with (-)-strand RNA synthesis as addition of about 400 non-viral nucleotides at the 3' end of B-RNA did not have any effect on virus infectivity (Eggen *et al.*, 1989). The pMB200 mRNAs further differ from B-RNA in that they lack the viral 5' non coding region. It will be of interest to create expression vectors which contain the 5' end of the B-cDNA and to examine if the mRNAs that will be generated are replicated by the proteins they encode. Another question now amenable to further

investigations is whether a functional replication complex can only be obtained with viral proteins obtained by proteolytic processing of a 200K polyprotein or whether an active replication complex can also be constructed from 32K and 170K proteins (or other combinations) produced by separate expression in one cell.

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Chapter 6

Cis- and trans-acting elements in cowpea mosaic virus RNA replication

Cis- and *Trans*-acting Elements in Cowpea Mosaic Virus RNA Replication

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Cowpea mosaic virus (CPMV) B-RNA encodes the viral proteins required for viral RNA replication while M-RNA does so for the capsid proteins and functions required in cell-to-cell movement of the virus. Accordingly, B-RNA can replicate by itself, whereas M-RNA can only replicate in the presence of B-RNA. We have made heterologous sequence insertions at different positions in the open reading frame of B-RNA, leaving the 5′ and 3′ non-coding ends intact. None of these mutant B-RNAs were able to replicate. Furthermore, it was not possible to support replication of these mutant B-RNAs by co-inoculating wild-type B-RNA as a helper, indicating that B-RNA can not be replicated *in trans*. In contrast, replication of M-RNA must occur *in trans*, as the viral replicative proteins are encoded by B-RNA. Mutant M-RNA transcripts containing 5′ and 3′ non-coding regions of B-RNA are still efficiently replicated in protoplasts if co-inoculated with B-RNA, indicating that *in cis* or *in trans* replication of the CPMV RNAs is not primarily determined by the non-coding regions. Remarkably, for replication of M-RNA, the N-terminal domain of the 58K protein encoded by M-RNA was found to be required.

INTRODUCTION

Cowpea mosaic virus (CPMV) is the type member of the plant comoviruses, which are characterized by a bipartite RNA genome of positive polarity (for reviews see Goldbach and Van Kammen, 1985; Eggen and Van Kammen, 1988). Upon translation of both RNA molecules, denoted B- and M-RNA, large polyproteins are produced from which functional proteins are generated by a cascade of defined proteolytic cleavages. These cleavages are controlled and executed by proteins derived from the 200K B-polyprotein. B-RNA can replicate independently from M-RNA in cowpea protoplasts (Goldbach *et al.*, 1980) and, therefore, B-RNA encodes all the viral functions required for replication of B-RNA at least. Initiation of translation on M-RNA occurs on two alternative start codons to give carboxy-coterminal polyproteins of 95K and 105K, which are processed into the 48K/58K transport protein(s) and the 37K and 23K capsid proteins. The M-RNA-encoded proteins are required for cell-to-cell movement of virus particles in plants (Wellink and Van Kammen, 1989; Van Lent *et al.*, 1990). In contrast to B-RNA, CPMV M-RNA does not support its own replication, but requires the viral replicative functions encoded by B-RNA.

The genomic organization and expression strategy of CPMV is similar to that of the animal picornaviruses, except for the genetic information in picornaviruses being present on one RNA molecule (Argos *et al.*, 1984; Goldbach, 1987). Furthermore, extensive sequence homology exists between the proteins encoded on CPMV B-RNA with these proteins involved in viral RNA replication of picornaviruses (Franssen *et al.*, 1984). For poliovirus, defective interfering (DI) particles have been identified, which contain RNAs with in-frame deletions in the region coding for the capsid proteins (Cole *et al.*, 1971; Nomoto *et al.*, 1979). The purified DI particles are able to initiate a normal poliovirus replication cycle but fail to synthesize capsid proteins and therefore cannot produce progeny virions. Studies with transcripts of *in vitro* mutagenized cDNAs have shown that RNAs that are unable to produce viral non-structural proteins are not co-replicated by wild-type (wt) poliovirus as DI particles (Hagino-Yamagishi and Nomoto, 1989). These and other results are taken for evidence that translation and replication of poliovirus RNA are tightly linked and/or that at least one of the non-structural proteins is *cis*-acting in replication (Bernstein *et al.*, 1986). Considering the similarities in genome organization and expression strategy between CPMV and poliovirus it is possible also for CPMV B-RNA that translation and replication of the RNA are linked. If there is indeed a tight linkage between translation and replication for CPMV B-RNA, the

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question arises how replication of M-RNA is achieved, because all viral replicative functions are encoded by the B-RNA.

In this paper we address the question of whether CPMV B-RNA can be replicated *in trans* and which sequences of the M-RNA are essential for its replication. For this purpose, defined mutations were made in the cDNA sequences of B- and M-RNA and T7 RNA transcripts from these mutated cDNAs were used for transfection of cowpea protoplasts. The ability of the mutated RNAs to be replicated by the proteins provided by wild-type helper B-RNA transcripts was determined by immunofluorescent staining and analysis of the RNAs from transfected protoplasts by Northern blotting.

MATERIALS AND METHODS

Construction of mutant clones

For growth and maintanance of plasmids *Escherichia coli* strain DH5αF' was used. Single-stranded DNA containing dUMP residues was isolated from *E. coli* strain RZ1032. Plasmids pTM1G and pTB1G are full-length cDNA clones of M- and B-RNA, respectively, from which infectious RNA transcripts can be generated using T7 RNA polymerase (Eggen *et al.*, 1989a) and served as starting material for introduction of mutations. The construction of mutant B-cDNA clones pTB58I (Peters *et al.*, 1992) and pTBlac (Richards *et al.*, 1989) and mutant M-cDNA clones pTMΔNco, pTMΔBgl, pTMΔP (Wellink and Van Kammen, 1989) and pTM58ΔH, pTMΔAUG1, pTMbL, pTMγBgl, and pTMγHind (Verver *et al.*, 1991) has been described. Plasmid constructions described in this paper were carried out using standard recombinant DNA techniques (Sambrook *et al.*, 1989). The fidelity of all new constructs was checked by sequencing of the junctions at the ligation sites, restriction enzyme mapping, and *in vitro* translation of the RNA transcripts.

Plasmid pTM58GS was created by digestion of pTM1G with *Bgl*II (nucleotide position 189 of M-cDNA; Van Wezenbeek *et al.*, 1983), filling in of the protruding ends with Klenow polymerase and religation, while inserting an 8-mer *Sma*I linker (5' CCCCGGGG 3').

To construct plasmid pTM58S a 2-kb *Bam*HI–*Cla*I fragment from pTM1G was inserted in the multiple cloning site of plasmid Bluescript SK$^+$ (Stratagene). The resulting plasmid was used for the preparation of single-stranded dUMP-containing DNA by growing in the presence of M13 helper phage M13K07. Site-directed mutagenesis (Kunkel, 1985) with oligonucleotide 5' GGCAAACAAGTTTTAGGCCTATTGTGGAAAAGC 3' created, in the complementary sequence, a double stop codon (bold) just downstream of the 58/48K coding sequence, and a *Stu*I recognition site (underlined). Insertion of the mutagenized *Bam*HI–*Cla*I fragment into pTM1G resulted in plasmid pTM58S, which was further used for making deletions of increasing length in the region coding for the coat protein precursor. For this, fragments were removed spanning from the newly created *Stu*I site to either of the recognition sites for *Sca*I (position 1838; pTM58SΔ1), *Eco*RV (position 2242; pTM58SΔ2 and position 3026; pTM58SΔ3), *Nco*I (position 3068, filled in with Klenow polymerase; pTM58SΔ4), or *Nae*I (position 3172; pTM58SΔ5).

In plasmid pTMbT the 3' terminus of the M-cDNA has been exchanged for the 3' end of B-cDNA. To construct this plasmid, a *Sma*I restriction site was created in the B-cDNA at position 5395 (Lomonossoff and Shanks, 1983) by digestion with *Bst*XI, where upon the protruding four nucleotides were removed with the exonuclease activity of Klenow polymerase followed by insertion of a 10-mer *Sma*I linker. This plasmid (pTB87BS) was digested with *Sal*I and *Sma*I and the 2.9-kb fragment, containing the 3' end of B-cDNA and the vector sequences, was ligated with the *Sal*I–*Nae*I fragment (position −40 to 3172 of M-cDNA) from pTM1G, to give plasmid pTMbT.

Plasmids pTBγB and pTBγ contain part of the gene I sequence of cauliflower mosaic virus (CaMV) consisting of nucleotides from the 5' non-coding region of gene I, the first 457 nucleotides of the gene I open reading frame and linker sequences. To construct pTBγB and pTBγ we used plasmid pTBΔA4, a derivative of pTB1G that yielded transcripts that were not infectious due to an insertion of three nucleotides at an *Ava*II site at position 3941. Plasmid pTBγB was obtained by an in-frame substitution of the PstI (protruding ends removed)–*Sst*I fragment (position 345–2301) of pTBΔA4 for a 0.5-kb *Pst*I (protruding ends removed)–*Sst*I fragment from pBSγI (Verver *et al.*, 1991), containing the CaMV gene I sequence. In pTBγ the B-RNA coding sequence downstream of the gene I insert was shifted out of frame by removing the protruding ends of the *Sst*I site in pTBγB.

In plasmids pTBlacS and pTB58IS the B-RNA sequences downstream of the insertions were also rendered non-coding. Plasmid pTBlacS was created by filling in the unique *Bam*HI site of pTBlac that was used for insertion of lacZ sequences in pTB1G. In plasmid pTB58I the *Kpn*I site at the 3' junction of the M-cDNA insertion was removed with Klenow polymerase to give pTB58IS.

In vitro transcription and inoculation of protoplasts

In vitro RNA synthesis with T7 RNA polymerase was performed as described previously (Vos *et al.*, 1988a). The yield and integrity of the RNA was checked on by

agarose gel electrophoresis and *in vitro* translation of the RNA transcripts was carried out as an additional control on the proper construction of the mutant clones. Cowpea protoplasts were prepared and inoculated with RNA transcripts as described previously (Eggen *et al.*, 1989a).

Detection of RNA replication in cowpea protoplasts

At 42-hr post-inoculation (p.i.) protoplasts were harvested for immunofluorescent staining (Vos *et al.*, 1988b), using anti-48K and/or anti-CPMV virion serum for detection of protoplasts in which M-RNA has been replicated and anti-24K serum for detection of protoplasts in which B-RNA has been replicated (Wellink *et al.*, 1987). Only when an RNA has replicated to a certain (unknown) level in a protoplast enough viral proteins will be synthesized from the progeny RNAs to allow their detection in this assay. Therefore, the number of protoplasts showing immunofluorescence is a measure for the efficiency by which an RNA is replicated. For Northern blot analysis the protoplasts were collected by centrifugation at 68 hr p.i. and the pellets were frozen at −80°. Total RNA was isolated from the protoplasts as described by de Vries *et al.* (1988) and electrophoresed in a 1% denaturing agarose gel (McMaster and Carmichael, 1977). The separated RNAs were transferred to GeneScreen (NEN Research Products) and hybridized with a ^{32}P-labeled probe, which was obtained upon random-primer labeling (Feinberg and Vogelstein, 1984) of the appropriate DNA fragments.

RESULTS

Evidence that B-RNA cannot be replicated *in trans*

To determine whether B-RNAs defective in replication can be replicated when wild-type (wt) B-RNA is co-inoculated as a helper, B-RNA mutants were created carrying in-frame insertions at different positions in the open reading frame (Fig. 1; BγB, B58I, and BIac). Heterologous sequences were used in the construction of these B-RNA mutants to allow their detection on Northern blots among the excess of co-transfected wt B-RNA. To exclude the possibility that the recognition of these B-RNA mutants as templates for replication would be obstructed by shielding of recognition sites on the RNA by defective replicative proteins, mutants were constructed in which B-RNA coding sequences were disrupted (Fig. 1; Bγ, BIacS, and B58IS). In these mutants the B-RNA coding region downstream of the foreign sequences was rendered non-coding. Upon *in vitro* translation all of the mutants produced proteins predicted by their sequence as indicated in Fig. 1 (data not shown).

None of the mutant B-RNAs was able to replicate, as judged by immunofluorescent staining of the protoplasts with anti-24K serum (data not shown). Upon inoculation of BγB-RNA in the absence of helper B-RNA, a smear of hybridizing material up to the position of the input RNA was found on Northern blots (Fig. 2, lane 2), indicating that even at 68 hr p.i. residual inoculum RNA was still present in cowpea protoplasts. Therefore, an inoculation of a mutant RNA without helper B-RNA was always included as a control in order to discriminate between detection of input RNA and RNA that had been synthesized *de novo* by replication. Inoculation of cowpea protoplasts with the B-RNA mutants together with wt B-RNA resulted in similar levels of accumulation of mutant RNAs as in inoculations without helper B-RNA (Fig. 2, lanes 2 to 4 and data not shown). It was verified that the co-inoculations had succeeded as immunofluorescent staining with anti-24K serum revealed that co-transfected wt B-RNA was expressed in approximately 40% of the total protoplasts; a similar percentage of infected protoplasts as upon inoculation by wt B-RNA alone. Furthermore, Northern blots hybridized with a B-RNA-specific probe showed that in all experiments replication of helper B-RNA had occurred at similar levels (data not shown). These results indicate that B-RNA mutants that are defective in replication are not replicated effectively by the replicative functions provided by co-inoculated wt B-RNA molecules.

Evidence that the N-terminus of the 58K protein of M-RNA is involved in replication of M-RNA

The CaMV gene I sequence was also inserted as a marker sequence at two positions in the M-RNA (Fig. 1); at nucleotide 482 (MγHind) and at position 189 (MγBgl). *In vitro* translation of both MγBgl- and MγHind-RNA resulted in the synthesis of an intact 95K polyprotein and an aberrant 105K polyprotein (Verver *et al.*, 1991). Northern blot analysis revealed that MγHind-RNA was still amenable to replication by helper B-RNA (Fig. 2, lane 6), albeit at a 10-fold lower level than wt M-RNA (not shown). Strikingly, the same CaMV insertion in MγBgl-RNA decreased almost to zero the ability of the RNA to accumulate (Fig. 2, lane 5). Co-inoculations of protoplasts with MγBgl- or MγHind-RNA together with CPMV RNA were performed to test whether a protein activity provided by M-RNA could enhance accumulation of the mutated RNAs. It appeared that the presence of wt M-RNA gave rise to even lower levels of MγBgl- and MγHind-RNA (data not shown), suggesting that M-RNA is successfully competing with and not complementing the accumulation of mutant RNAs.

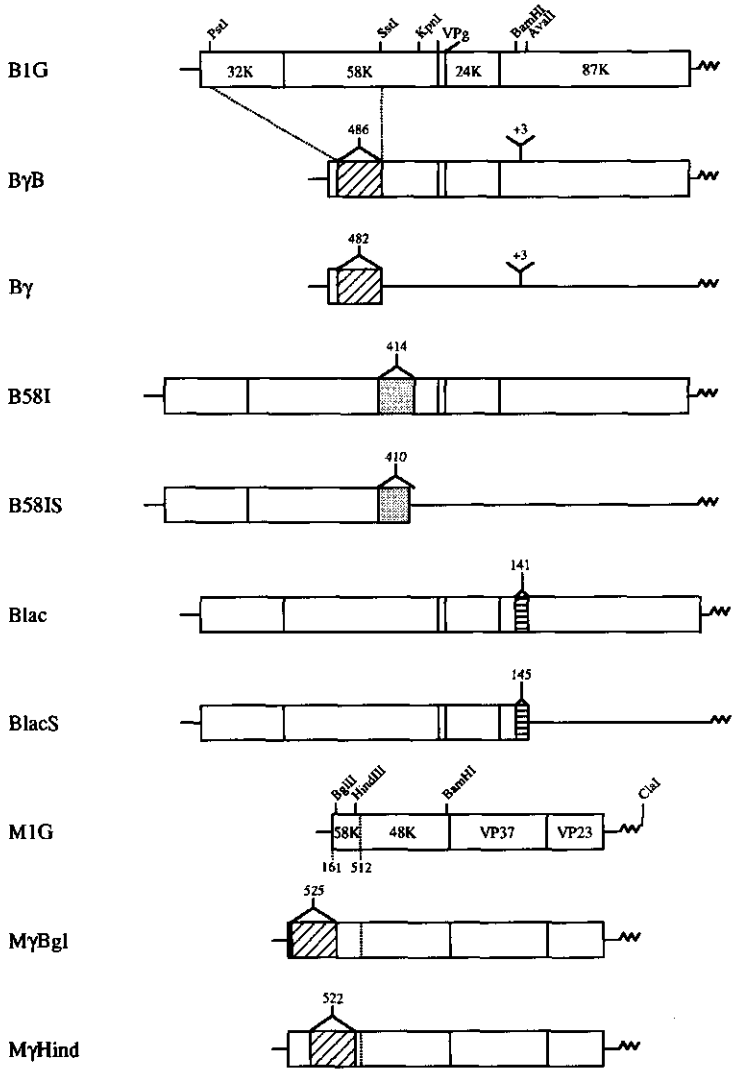

FIG. 1. Schematic representation of the CPMV B- and M-RNA insertion mutants used in this study. Open bars denote open reading frames present in the transcripts. The 5' and 3' nontranslated regions are designated by a single line and the poly(A) tail is designated by a waved line. Translation of B-RNA results in a 200K polyprotein which is proteolytically processed at peptide bonds located by vertical lines, to give proteins of indicated sizes. Translation of M-RNA from AUG 161 results in a 105K protein and from AUG 512 in a 95K protein. Processing of these polyproteins yields the 58K/48K proteins and the 60K precursor of the capsid proteins. The positions of restriction enzyme sites used for the insertion of foreign sequences are shown above the wt transcripts (B1G and M1G). Insertions originate from parts of the coding sequences of CaMV gene I (hatched), lacZ (horizontal lines) and CPMV M-RNA (stippled) with sizes indicated above the mutant transcripts. Further details of these constructions are given under *Materials and Methods*.

The decreased accumulation of both MγBgI- and MγHind-RNA as compared to wt M-RNA suggests that the N-terminal domain of the 105K polyprotein that is specific for the 58K protein may be important for replication of M-RNA (Holness *et al.*, 1989). Alternatively, RNA sequences that are involved in M-RNA replication or encapsidation might have been disturbed by the CaMV insertion in these mutants (Holness *et al.*, 1989). To discriminate between these possibilities several other M-RNAs with mutations in the 58K coding region were constructed (Fig. 3A). M58ΔH-RNA contains a large in-frame deletion in the 58K-specific region and

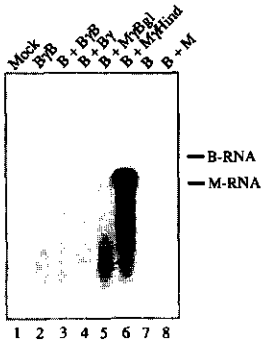

FIG. 2. Northern blot analysis of B- and M-RNA mutants containing CaMV gene I insertions. Total RNA was extracted from protoplasts at 68 hr p.i. and each lane was loaded with RNA from 3×10^5 protoplasts. Protoplasts were inoculated with the indicated mutant RNAs together with wt B-RNA, except for lane 2 where B-RNA was omitted and lane 8 where CPMV RNA was used instead of B-RNA. Lane 1 contains RNA from mock-inoculated protoplasts. The blot was hybridized with a probe specific for CaMV gene I sequences and exposure was for 16 hr (with a screen) to a Kodak XAR film. The positions of CPMV B- and M-RNA were determined after stripping the gene I probe from the blot and re-hybridization with B- and M-RNA-specific probes.

synthesizes a truncated 105K polyprotein and a normal 95K protein (Verver *et al.*, 1991). Northern blot analysis and immunofluorescent staining of protoplasts inoculated with this mutant M-RNA and wt B-RNA revealed that the ability of M58ΔH-RNA to accumulate in protoplasts was completely lost (Figs. 3A and 3B, lane 4), emphasizing the importance of the 58K-specific region for M-RNA replication. The M-RNA mutants MΔBgI and M58GS contain small insertions of 4 and 12 nucleotides, respectively, at position 189 (Fig. 3A). *In vitro* translation of M58GS-RNA results in synthesis of both the 95K and 105K polyproteins, the latter presumably containing an insertion of amino acids Pro-Arg-Gly-Ile (data not shown). In MΔBgI-RNA the reading frame for the 105K protein is disrupted and only the 95K polyprotein can be synthesized (Wellink and Van Kammen, 1989). It appeared that for M58GS-RNA, with the in-frame insertion, replication was maintained at wt levels, whereas for MΔBgI-RNA, where the out frame insertion prevented synthesis of the 58K protein, the level of replication was about 50-fold lower than for wt M-RNA as deduced by the combined results of the Northern blot analysis (Fig. 3B, lanes 3, 5, and 6) and immunofluorescent staining (Fig. 3A). The striking difference in replication level between MΔ58GS-RNA and MΔBgI-RNA suggests that it is the protein rather than the nucleotide sequence that is important for replication. Further support for this is provided by the mutant MΔAUG1-RNA, in which the start codon for the 105K polyprotein at nucleotide 161 has been changed to CUG. Replication of MΔAUG1-RNA was about 50 times less efficient than replication of M-RNA (Figs. 3C and 3D, lanes 1 and 4). Finally, MΔP-RNA has an in-frame deletion in the 48K coding region, resulting in the synthesis of polyproteins of approximately 90K and 80K (Wellink and Van Kammen, 1989). Replication of MΔP-RNA was almost as good as wt M-RNA (Figs. 3C and 3D, lanes 1 and 11), suggesting that only the N-terminal part of the 58K protein is required for replication.

Capsid proteins are not essential for M-RNA replication

It is possible that in addition to the N-terminal part of the 58K protein, the capsid proteins or their coding regions are required for *in trans* replication of M-RNA. An involvement of the coat proteins in replication was investigated with M58S-RNA and its derivatives, which all contain a UAG stop codon directly downstream of the 58K/48K coding sequence. In addition, the M58S-derived mutants M58SΔ1 to Δ5 have increasing truncations at the 5′ end of the coding region for the coat protein precursor (Fig. 3A). The amount of viral RNA accumulating in cowpea protoplasts was about 200 times less for M58S-RNA than for wt M-RNA, as estimated by the intensity of the hybridization on the Northern blot, which is even considerably lower than for MΔAUG1-RNA (Figs. 3C and 3D, lanes 1, 4, and 5). Remarkably, the hybridization signal for the M58SΔ1- to Δ5-RNAs increased parallel to the length of the deletion in the coat protein region. The hybridization signal of M58SΔ5-RNA, which has the largest deletion in the coat protein region, was approximately 15% of the signal found for wt M-RNA. Although the hybridization signal on Northern blots was still higher for wt M-RNA as compared to M58SΔ3-, M58SΔ4-, and M58SΔ5-RNA, the intensity of the fluorescent signal as well as the percentage of protoplasts showing fluorescence upon treatment with anti-48K serum are identical for all these RNAs (Fig. 3A and data not shown). This indicates that these RNAs had the same infectivity and supported the synthesis of similar amounts of protein per cell. The apparent discrepancy between the immunofluorescence data and the amount of RNA detected on Northern blots for these mutants might be explained by the absence of coat proteins, preventing accumulation of these RNAs in protoplasts as they are not encapsidated (De Varennes and Maule, 1985). The immunofluorescence for the mutant M58S- and M58SΔ1- and Δ2-RNAs was difficult to quantitate because the fluorescent signal in these cells was rather faint. Apparently, these RNAs are inefficiently replicated.

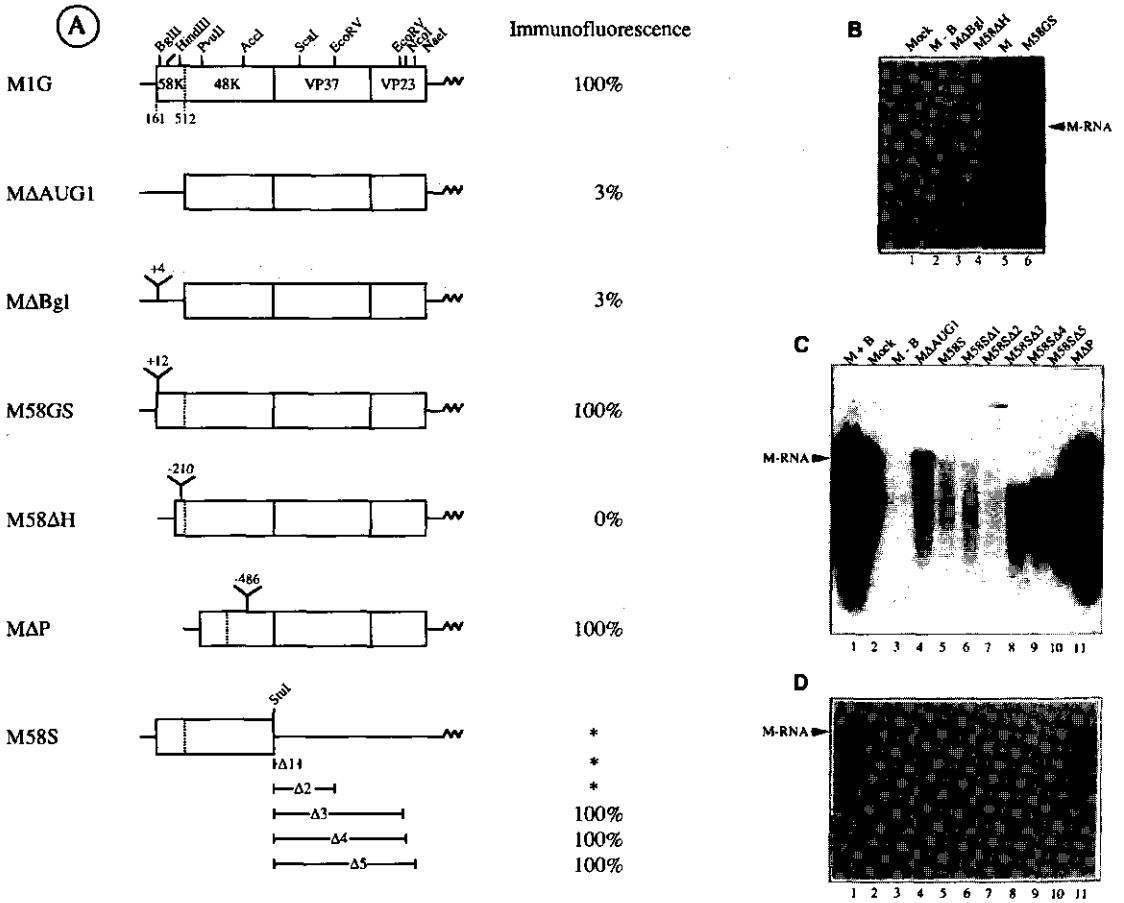

FIG. 3. (A) Schematic representation of constructs containing mutations in the coding region of M-RNA. The relative positions of the restriction enzymes where the insertions and deletions were introduced are indicated above the wt transcript. The newly created *StuI* site in M58S was used to make deletions in the coat protein region (M58SΔ1 to Δ5). The immunofluorescence of protoplasts inoculated with these transcripts was determined at 42 hr p.i. using anti-48K or anti-CPMV virion serum. The immunofluorescence for M1G-RNA was arbitrarily set on 100%, corresponding to 35% of the total protoplasts. The immunofluorescence for M58S and M58SΔ1 and Δ2 could not be quantitated (*) due to the weak fluorescence signal. (B) Northern blot analysis of transcripts containing mutations in the 58K coding region. Total RNA was prepared from protoplasts at 68 hr p.i. and analyzed as described for Fig. 2, using a M-RNA-specific probe. The position of M-RNA on the autoradiogram is indicated. Lane 1, RNA from mock-inoculated protoplasts; lane 2, RNA from protoplasts inoculated with M-RNA only; lanes 3 to 6, RNA from protoplasts inoculated with the indicated transcripts together with wt B-RNA. Lanes 1 to 4 were exposed for 3 days with a screen and lanes 5 and 6 for 3 hr with a screen. (C) Northern blot analysis of transcripts containing mutations in the 48K and coat protein coding regions of M-RNA. Total RNA was extracted from protoplasts inoculated with the indicated RNAs together with wt B-RNA, except for lanes 2 and 3 which contain RNA from mock-inoculated protoplasts and protoplasts inoculated with M-RNA only, respectively. (D) Shorter exposure of (C).

The 5' and 3' non-coding regions of B- and M-RNA are exchangeable

Except for the N-terminal part of the 58K protein none of the other M-RNA coding sequences and proteins derived thereof seem to be necessary for replication. Therefore, only the 5' and 3' non-coding regions of M-RNA may contain sequences that in cooperation with the N-terminus of the 58K protein, allow *in trans* replication of M-RNA. Comparison of the non-coding regions of M-RNA with those of B-RNA reveals a high degree of homology at the 5' terminal 44 nucleotides and 3' terminal 65 nucleotides (Eggen and Van Kammen, 1988; Eggen *et al.*, 1989b). However, proximal to the coding region the nucleotide sequences of B- and M-RNA have widely diverged and especially striking is a stretch of 100 nucleotides in the 3' non-coding region of M-RNA that is lacking at the 3' end of B-RNA. To

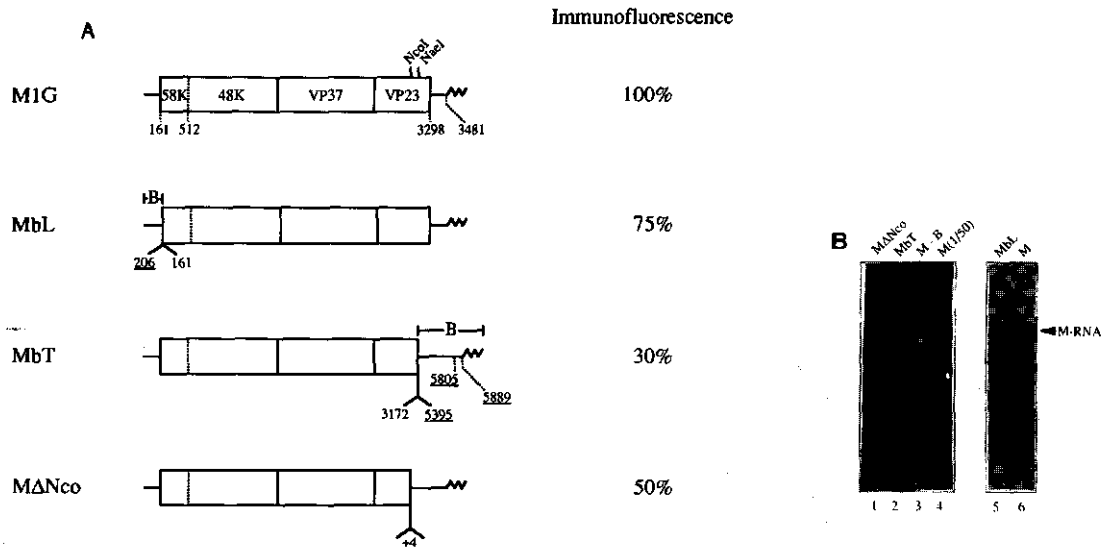

FIG. 4. (A) Schematic representation of M-RNA mutants containing 5′ and 3′ non-coding regions of B-RNA. B-RNA-derived sequences are indicated above the transcripts. Relevant nucleotide positions are shown with respect to numbering of M-cDNA (not underlined) and B-cDNA (underlined). Immunofluorescence of wt transcript (M1G) was arbitrarily set to 100%, and was determined with anti-CPMV virion serum. (B) Northern blot analysis of total RNA from inoculated protoplasts. Blots were prepared and hybridized as described for Fig. 2. Lane 4 (wt M-RNA) contains 50 times less RNA than the other lanes, which contain RNA from 3×10^5 protoplasts. Lanes 1 to 4 and lanes 5 and 6 were exposed to a Kodak XAR and a Konica X-ray film, respectively, and represent two separate experiments. Lane 3 contains RNA extracted from protoplasts inoculated with M-RNA only. Other lanes contain RNA from protoplasts inoculated with the indicated transcripts together with wt B-RNA.

study whether these deviant nucleotides in the non-coding regions of M-RNA are required for *in trans* replication, mutant M-RNAs were made which have the 5′ and 3′ ends individually replaced by the corresponding sequences of B-RNA, which flank an RNA molecule that is replicated only *in cis* (Fig. 2).

In MbL-RNA an exact exchange has been made between the 5′ untranslated regions of M- and B-RNA (Fig. 4A). This mutant RNA was replicated almost as efficiently as wt M-RNA as determined by Northern blotting (Fig. 4B, lanes 5, 6) and immunofluorescent staining (Fig. 4A). In MbT-RNA the 3′ non-coding region of M-RNA and part of the reading frame for the C-terminus of VP23 has been replaced by the 3′ 500 nucleotides of B-RNA (Fig. 4A). Since disruption of the reading frame in the coding sequence for VP23 in MbT-RNA will prevent RNA encapsidation, a low hybridization signal on Northern blots is expected for this mutant. Therefore, as a control MΔNco-RNA was used, which contains a 4 nucleotide insertion that disrupts the coding region of VP23 in a similar position as in MbT-RNA. Mutant MbT- and pTMΔNco-RNA accumulated to similar levels in cowpea protoplasts (Fig. 4B, lanes 1 and 2). Due to the lack of encapsidation the amount of RNA detected on Northern blots for protoplasts inoculated with these mutant RNAs is about 25 times less than for protoplasts inoculated with wt M-RNA (Fig. 4B, lane 4). However, the percentage of protoplasts that show immunofluorescent staining with anti-CPMV virion serum is only 2 to 3 times lower for MbT- and MΔNco-RNA than for wt M-RNA (Fig. 4A). Hence, the substitution of the 5′ and 3′ non-coding regions of B-RNA that is not replicated *in trans* and for those of M-RNA that is replicated *in trans* did not dramatically affect the replication.

DISCUSSION

CPMV B-RNA encodes all the viral proteins that are required for replication of at least the B-RNA molecule. The results described in this paper show that replication in cowpea protoplasts of mutated B-RNA molecules could not be supported by co-inoculated wt B-RNA, indicating that these B-RNA mutants were not replicated *in trans*. This is clearly different for M-RNA, which is replicated *in trans* requiring the viral functions provided by B-RNA. We have now demonstrated that the 5′ and the 3′ non-coding regions of M-RNA can be exchanged for those of B-RNA without dramatic loss in the efficiency in M-RNA replication. These results make it very unlikely that the 5′ and 3′ non coding regions of M-RNA contain signals that are determinative

for the in trans replication of M-RNA. Remarkably, *in trans* replication of M-RNA appeared to be dependent on the N-terminus of the 58K protein, as suggested earlier by Holness *et al.* (1989).

The replication-defective CPMV B-RNA mutants presented in this paper were not co-replicated by the viral proteins provided by wt helper B-RNA, implying that functional replicative proteins could not complement the defect in these mutants. One possible explanation for the lack of co-replication of the mutant B-RNAs is that the heterologous sequence insertions disrupt the structure of the RNA and thereby create *cis*-acting defects. Although this possibility cannot be ruled out as yet, it is not a very likely one because the various B-RNA mutants had the heterologous sequences inserted at different positions in the open reading frame. A more plausible explanation for the inability of the B-RNA mutants to be replicated *in trans* is that they do not produce functional replicative proteins themselves. The possibility that recognition of the mutant B-RNAs as a template for replication was obstructed by shielding of recognition sites on the RNA by defective replicative proteins was excluded. Mutant B-RNAs in which the coding sequences were disrupted were improper templates for *in trans* replication, even when almost the entire B-RNA coding region was disrupted. Our results suggest that replication of a B-RNA molecule is tightly linked to its translation and/or that at least one of the B-RNA-encoded replicative proteins functions *in cis* only. In this respect CPMV B-components have characteristics similar to poliovirus DI particles (Hagino-Yamagishi and Nomoto, 1989).

The results obtained with the various M-RNA-derived mutants strongly indicate that the N-terminus of the 58K protein, but not the RNA sequence coding for this protein, is required for replication of M-RNA. The mutant MΔBgl- and MΔAUG1-RNA, which due to their mutations should not be able to produce any 58K protein, were not completely defective in replication. The low replication levels for these RNAs may be caused by rare events as ribosome shifting during translation (Wellink and Van Kammen, 1989), errors of T7 polymerase during *in vitro* transcription of the mutant M-cDNAs, or initiation of translation at non-AUG codons (Peabody, 1989), which would produce small amounts of 58K or 58K-related proteins.

A possible requirement of the coat proteins in replication of M-RNA (Holness *et al.*, 1989) was excluded with M58S-RNA and deletion mutants derived thereof (M58SΔ1 to Δ5). A complication in the interpretation of the results with these coat protein mutants is that the stability of these mutant RNAs in protoplasts is greatly reduced due to the absence of encapsidation (De Varennes and Maule, 1985). Yet, the redundancy of the coat proteins for viral RNA replication could unequivocally be demonstrated by immunofluorescent staining of the protoplasts using CPMV-specific antisera. It appeared that replication of M58S-RNA was very much debilitated. However, the increasing replication levels for M58SΔ1- to Δ5-RNA with the increasing size of the deletions in the coat protein coding region, showed that the poor replication of M58S-RNA was not caused by an absolute requirement of coat proteins for RNA replication. More likely, next to a lack of encapsidation, the stability of M58S-RNA (and M58SΔ1 and Δ2) is even more reduced by the large 3' non-translated regions in these mutant RNAs. A decreased stability of mRNAs caused by a premature translational stop has also been described for a frameshift mutant of the soybean Kunitz trypsin inhibitor gene (Jofuku *et al.*, 1989) and for certain pseudogenes in transgenic tobacco (Voelker *et al.*, 1990; Vancanneyt *et al.*, 1990). Alternatively, for M-RNA mutants M58S and M58SΔ1 and Δ2 a linkage between translation and replication is hampered due to their large 3' non-translated regions (see below).

Having established that the 58K polypeptide is the sole M-RNA-specific factor that is required for *in trans* replication of M-RNA, the question remains how it works. A screening of protein databases did not reveal any significant amino acid homology with other known proteins. Remarkably, the N-terminus of the 58K protein is not conserved between comoviruses, except for the presence of many hydrophobic and aromatic amino acid residues (Chen and Bruening, 1992). Hydrophobic and aromatic amino acid motifs are often found in members of the highly heterologous "family" of RNA-binding proteins (Kenan *et al.*, 1991). Therefore, it is tempting to speculate that the N-terminal domain of the 58K protein of M-RNA is involved in RNA-binding. The lack of complementation of M-RNAs with mutations in the 58K coding region by wt M-RNA indicates that replication of M-RNA depends on translation of the 58K polypeptide from the very same RNA molecule. A possible mechanism explaining this linkage between M-RNA translation and replication is that the translator ribosomes transport the N-terminal domain of the 58K protein, contained in the 105K polyprotein, to the 3' end of the RNA molecule. Then, a ribonucleoprotein complex may occasionally be formed, consisting of the 105K polyprotein, viral RNA, and possibly a ribosomal factor(s), which is recognized by the B-RNA-encoded replicative machinery to start (−)-strand RNA synthesis. Also for poliovirus it has been proposed that the proper substrate for binding the viral replicase is a ribosome-associated viral RNA and, consequently, that initiation of RNA replication must require concurrent translation of the RNA (Hagino-Yamagishi and Nomoto, 1989). A model for viral RNA replication involving ribosomal transport of viral proteins to the 3' end of the

RNA is consistent with the low replication level of M-mutants that contain large 3' non coding regions, which would thereby interfere with the formation of a ribonucleoprotein complex. For B-RNA, the observed linkage between translation and replication may be effected similarly by transportation of the replicative proteins to the 3' end of the RNA. Each M-RNA molecule must be translated many times in order to produce enough coat proteins to encapsidate the viral RNAs (120 times for one B- and one M-component). Interestingly, the virus prevents (over)production of the 105K (58K) protein by synthesizing the 95K (48K) protein from an internal AUG (Vos *et al.*, 1984; Holness *et al.*, 1989).

If replication of both RNAs is indeed indissolubly linked to their translation then this would explain the difficulties in obtaining an *in vitro* RNA replication complex capable of copying exogenously added CPMV RNA (Eggen, 1989; Van Bokhoven *et al.*, 1992). The search for an *in vitro* replication system for poliovirus RNA has met with similar difficulties as for CPMV and may be explained likewise by the linkage between translation and replication of polioviral RNA. Indeed, complete replication of poliovirus RNA in a cell-free system has thus far only been achieved in extracts of HeLa cells that support translation of poliovirus RNA (Molla *et al.*, 1991).

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Chapter 7

Discussion

I. EXPRESSION OF B-RNA SEQUENCES IN INSECT CELLS

We started our study with producing the separate proteins encoded on CPMV B-RNA in an expression system, with the aim of obtaining functional viral proteins and reconstituting, if possible, a CPMV-specific RNA replicase activity capable of using exogenous template RNA. For this, an eukaryotic expression system was employed, as the previous lack of polymerase activity for CPMV proteins produced in *Escherichia coli* might have been caused by lack of post-translational modifications or improper folding of heterologous proteins in prokaryotic cells (Richards et al., 1989). The baculovirus expression vector system in insect cells was opted for as this system has proven to be very useful for the production of biologically active proteins in large quantities (for reviews: Luckow and Summers, 1988; Miller, 1988).

Sequence motifs characteristic of RNA polymerases are found in the 87K protein of CPMV B-RNA as well as the 110K, 170K and 200K proteins, which are precursors of the 87K protein. The 87K, 110K, 170K and entire 200K proteins of CPMV B-RNA were expressed in insect cells with the dual purpose to investigate which of these proteins is the actual CPMV RNA polymerase and to find out whether proteolytic processing of precursor proteins is prerequisite for generating a functional CPMV RNA polymerase. Besides, each of the 32K and 60K B-RNA-encoded proteins were also produced in insect cells. The 60K protein, which has homology to NTP-binding helicases, and the 32K protein, the function of which was not understood at the beginning of this study, may both be needed for CPMV polymerase activity (chapters 2, 3 and 4).

Upon expression of these sequences, proteins were obtained that under denaturing conditions, could not be distinguished in electrophoretic mobility and immunogenic properties from the viral proteins found in CPMV-infected plants. In addition, close examination of the proteins produced by the recombinant baculoviruses and the cells in which they were produced revealed that the CPMV proteins obtained from insect cells were in several aspects functionally identical to the native proteins from infected cowpea cells.

Precursor proteins that contained the 24K protease domain were faithfully processed in insect cells. For example, insect cells in which the 200K polyprotein had been produced contained also cleavage products of 170K, 112K, 110K, 87K, 84K, 60K and 32K. Hardly any other protein species related to the CPMV proteins was detected in insect cells, indicating that non-specific degradation of CPMV proteins had not occurred in insect cells. Also the proteolytic processing pattern of the 200K polyprotein in insect cells was indistinguishable from that in plants. In CPMV-infected plant cells the 200K polyprotein is rapidly cleaved into the 32K and 170K proteins, followed by relaxed further processing of the 170K polypeptide, which is the most abundant viral protein in these cells (Rezelman et al., 1980). A similar result was found in insect cells in which the 200K polyprotein was synthesized. The 170K primary cleavage product is the predominant viral protein in these cells and secondary cleavage products of the 170K protein were found in limited amounts. Recently, in vitro translation studies have revealed that the 32K protein has a regulatory role in processing of the 170K protein (Peters et al., 1992). When the 170K protein was synthesized in insect cells from a baculovirus vector in the absence of the 32K protein the 170K protein was almost completely cleaved. Apparently, also in insect cells the 32K protein, if present, regulates the processing of the 170K protein.

Electron microscopic examination of insect cells in which the 200K polyprotein was produced revealed the presence of numerous membranous vesicles and electron-dense structures, resembling

the characteristic cytopathic structures found in CPMV-infected cowpea cells. The induction of membranous vesicles and electron-dense structures therefore appears a property of the B-RNA-encoded proteins per se, independent of viral RNA replication taking place or the occurrence of typical plant factors. Interestingly, the appearance of membranous vesicles was also observed in insect cells in which the 60K protein encoded by B-RNA was produced, whereas in cells in which the 87K or 110K were produced no such structures were seen. Immunogold labelling of these cells using anti-VPg serum and protein A-gold revealed that the 60K protein was associated with these vesicles. These observations suggest that the 60K protein is responsible for the induction of membrane proliferation in insect cells, and may also have that role in plant cells. Furthermore, the association of the 60K protein with these vesicles strengthens the suggestion made previously (Eggen and van Kammen, 1988) that the 60K protein has a role in anchoring replication complexes to membranes. For poliovirus, the induction of membranous vesicles and attachment of replication complexes to these vesicles has been attributed to protein 2C, that has extensive sequence identity with the 60K protein of CPMV (Bienz et al., 1987). These results consolidate the notion that the CPMV 60K protein and poliovirus protein 2C fulfil analogous functions in viral RNA replication.

The electron-dense structures were only found in the cytoplasm of insect cells in which the complete 200K B-polyprotein was produced and not in cells that expressed the 170K, 110K, 87K or 60K coding sequences. The electron-dense structures could specifically be labelled with antisera against the B-RNA-encoded proteins and protein A-gold. This suggests that the bulk of CPMV proteins is present in the electron-dense structures, just as is in CPMV-infected cowpea cells (Wellink et al., 1988). Since electron-dense structures were not observed in cells in which alone the 170K protein was produced, it looks like the 32K protein having a role in keeping together the other B-proteins in complexes, clustered into electron-dense structures visible with the electron microscope. Given the abundancy of the 170K protein in CPMV-infected cells it may be anticipated that the electron-dense structures contain mainly 32K and 170K protein.

The 170K, 112K, 110K and 87K proteins, generated by proteolytic processing of the 200K polyprotein produced in insect cells or in CPMV-infected plants, have each the capacity to bind ribonucleotides (Peters et al., submitted). These four polypeptides all contain the conserved polymerase motif that is present in the 87K protein. NTP-binding is a property of RNA polymerases and apparently also the putative CPMV polymerase produced in insect cells contains this quality. Similarly, NTP-binding has been observed for poliovirus polymerase $3D^{pol}$ obtained from the *E. coli* expression system (Richards et al., 1991). In this case it was shown that stable cross-linking of $3D^{pol}$ with GTP coincides with a loss in polymerase activity in a poly(A)/oligo(U) assay, suggesting that reversible binding of NTPs is required for the RNA-elongating activity of $3D^{pol}$.

In spite of these observations, which prove that the CPMV proteins produced in insect cells have native functions, it has not been possible to demonstrate RNA polymerase activity in cell extracts containing these proteins. In sharp contrast, the picornaviral polymerases of poliovirus and foot-and-mouth disease virus, which were similarly produced in insect cells, displayed easily detectable activity in a poly(A)/oligo(U) polymerase assay. In pursuit of achieving RNA polymerase activity for the CPMV proteins a wide variety of conditions was tested to examine if it was necessary to adjust the standard polymerase assay, as initially developed for the poliovirus polymerase. However, none of the choosen conditions, among them those that are optimal for the RNA-elongating activity of the highly purified CPMV polymerase from infected cowpea, resulted in any detectable polymerase activity for the CPMV proteins. Neither was polymerase activity found when

CPMV RNA was used as a template instead of poly(A) RNA, whereas poliovirus polymerase could use CPMV RNA as a template. It was therefore concluded that the lack of activity for the CPMV polymerase must be attributed to either or both of the possibilities: 1) CPMV polymerase requires a (plant) host factor in order to show activity; or 2) The CPMV polymerase can not use oligo(U) as a primer in RNA synthesis or is not able to accept any exogenous RNA, -template and/or primer-, to start RNA synthesis.

II. EXPRESSION OF B-RNA-CODING SEQUENCES IN PROTOPLASTS

For investigating whether the CPMV polymerase needs a plant host factor for activity, the pMON999 expression vector system was used in cowpea protoplasts (chapter 5). The 87K, 110K, 170K and complete 200K coding regions of CPMV B-RNA were each inserted in the vector under control of the 35S promoter of cauliflower mosaic virus. In cowpea protoplasts transfected with the vectors large amounts of the expected CPMV-specific proteins were synthesized. These proteins exhibited the same characteristic activities (proteolytic processing, the induction of cytopathic structures and NTP-binding) manifested by the viral proteins produced in protoplasts transfected with B-RNA.

In comparison with the baculovirus expression system, transient expression of the B-RNA coding sequences in cowpea protoplasts offered the unique opportunity to investigate directly whether the produced proteins indeed possessed the activities required for viral RNA replication. For this, cowpea protoplasts were co-transfected with the expression vectors coding for the B-proteins and M-RNA transcripts. It appeared that under such conditions M-RNA was not replicated in protoplasts in which the 170K, 110K or 87K protein were individually synthesized. However, M-RNA was replicated in cowpea protoplasts if supported by the expression of the entire 200K coding sequence of B-RNA. It was concluded that all the viral replicative proteins, including the viral polymerase, are functionally intact upon their synthesis in cowpea protoplasts. Apparently, expression of the complete 200K coding region of B-RNA is required for CPMV RNA replication.

Although RNA replicase activity of the B-RNA-encoded proteins was found *in vivo*, it was not possible to demonstrate *in vitro* RNA polymerase activity, in a poly(A)/oligo(U) assay, in extracts of protoplasts containing these proteins. Use of natural templates for the CPMV polymerase, such as CPMV RNA and (-)-strand transcripts of B-RNA, whether or not in combination with a oligoribonucleotide primer did not result in any detectable RNA-synthesizing activity either. In contrast, poliovirus polymerase $3D^{pol}$, obtained upon expression in cowpea protoplasts, displayed polymerase activity under the same conditions. Once more, these results demonstrate that the polymerases of CPMV and poliovirus have different requirements for activity *in vitro*. Although it was not possible to demonstrate CPMV polymerase activity *in vitro*, this observation does not preclude the possibility that a plant host factor is essential for CPMV polymerase activity, as such host factor may have dissociated from CPMV proteins prior to the *in vitro* polymerase assay.

So far it has been proven impossible to obtain CPMV polymerase activity capable of using exogenous template RNA. Expression of B-RNA coding sequences in heterologous systems, -either *E. coli* or insect cells-, or in a homologous system, i.e. cowpea protoplasts, were unsuccessful in producing template-dependent CPMV polymerase activity *in vitro*. In earlier experiments, replication complexes isolated from CPMV-infected cowpea or *Chenopodium amaranticolor* did not appear capable of using added template RNA but only capable of elongating nascent RNA chains (Dorssers et al., 1984; Eggen et al., 1988). Hence, all these results indicate that the CPMV polymerase is

unable to accept an exogenous template and/or is unable to initiate RNA synthesis *in vitro*. In the next paragraph the problem of template recognition by the CPMV polymerase will be discussed.

III. REPLICATION OF THE CPMV RNAs IS LINKED TO THEIR TRANSLATION

In the multiplication of the genome of CPMV two different RNA molecules are replicated. Replication of CPMV B-RNA can occur in cowpea protoplasts by itself, indicating that the B-RNA component provides all the viral functions for replication of B-RNA (Goldbach et al., 1980). In addition these B-RNA-encoded functions also have to replicate M-RNA. In order to investigate how the RNAs of CPMV are recognized as templates for viral RNA synthesis, defined mutants of both B- and M-RNA were tested for their ability to be replicated by the proteins provided by wt helper B-RNA (chapter 6).

B-RNA mutants were created with heterologous sequence insertions at different positions in the coding region to allow detection of replication of these mutants by Northern blotting (chapter 6). All such mutants appeared to be replication-defective and, in addition, none of these B-RNA insertion mutants could be replicated by the viral functions provided by wild type helper B-RNA. These results can be interpreted as CPMV B-RNA only functions as a template for RNA replication if replicative proteins are synthesized from the same RNA molecule. Hence, replication of B-RNA seems to occur in cis only.

If there is a tight linkage between translation and replication for CPMV B-RNA, the question arises how replication of M-RNA is achieved. Since all viral replicative proteins are encoded by B-RNA, replication of M-RNA has to occur in trans. Remarkably, also for M-RNA a linkage between replication and translation was observed. It was found that the N-terminal part of the 58K protein of M-RNA, rather than its nucleotide sequence, is required for replication of M-RNA. The N-terminus of the 58K protein probably functions in cis only, since replication of mutants that are not able to produce 58K protein could not be complemented by co-inoculated CPMV RNA. None of the other M-RNA-encoded proteins, i.e. the 48K protein and the two capsid proteins, nor the coding sequence thereof was required for the replication of M-RNA. Strikingly, exchange of the 5' and 3' non coding regions of M-RNA with those of B-RNA maintained the capacity of these RNAs to be replicated in trans. Thus, how important the 5' and 3' non coding regions of M-RNA may be in RNA replication, they are not determinative in allowing the replication to be in trans.

The demonstration that in trans replication of M-RNA requires the N-terminus of the 58K protein in cis, imposes the question how it works. A screening of protein databases did not reveal any significant amino acid homology of the 58K protein with other known proteins. The N-terminus of the 58K protein is not conserved in the comoviruses bean pod mottle virus (BPMV), cowpea severe mosaic mosaic virus (CPSMV), red clover mottle virus (RCMV) and CPMV, except for the presence of many hydrophobic and aromatic amino acid residues (Chen and Breuning, 1992). Hydrophobic and aromatic amino acid motifs are often found in members of the highly heterologous 'family' of RNA-binding proteins (Kenan et al., 1991). Therefore, it is tempting to speculate that the 58K protein encoded by M-RNA is involved in RNA-binding and will specifically bind to M-RNA, thereby facilitating M-RNA to be recognized as a template for the replicative proteins encoded by B-RNA. The requirement for the 58K protein in M-RNA replication implicates that only initiation of translation at nucleotide 161, resulting in the synthesis of the 105K polyprotein, will lead to replication of M-RNA. Replication of M-RNA will not follow upon initiation of translation at nucleotide 512, which results in the synthesis of the 95K polyprotein. The differential initiation of

translation at nucleotide 161 and 512 may provide a regulatory switch for replication of M-RNA at the one hand and the production of capsid proteins and 48K transport protein at the other hand. The relative amounts of the 58K and 48K proteins in CPMV-infected plants suggest that initiation of translation occurs more often at nucleotide 512 than at nucleotide 161 (Rezelman et al., 1989). Most translation events on M-RNA will thus not be followed by replication. This is not necessarily surprising as M-RNA must be translated 120 times to produce sufficient coat proteins to generate one progeny CPMV B- and one M-component. Also B-RNA needs to be translated more often than it is replicated because the production of one progeny genomic B- and M-RNA requires at least four molecules of VPg and consequently the synthesis of four 200K polyproteins.

IV. A MODEL FOR CPMV RNA REPLICATION

The principal conclusions of the preceding chapters have been incorporated in a model (FIG. 1) for CPMV RNA replication, that proposes an important role for proteolytic processing in the establishment of a functional replication complex and, accounts for the observed linkage between translation and replication.

A. Formation of the replication complex

When a B-RNA molecule is translated in the infected cell a 200K polyprotein will be synthesized, which is rapidly cleaved into the 32K and 170K proteins (FIG. 1a). The 32K and 170K proteins are associated with each other by interaction of the 32K protein with the hydrophobic domain of the 58K protein, that is contained within the 170K protein. The same protein-protein interaction inhibits further proteolytic processing of the 170K protein into smaller cleavage products (Peters et al., 1992). In these cells, the 60K domain of the 170K protein induces proliferation of membranes, possibly from the rough endoplasmic reticulum as in the case of poliovirus, which will lead eventually to the formation of the vesicular membranes characteristic of CPMV-infected cells. It should be noted that at this stage most, if not all, of the 32K and 170K proteins may not be associated with membranes but reside probably in the electron-dense structures, which appear in the cytoplasm of the cell early in infection.

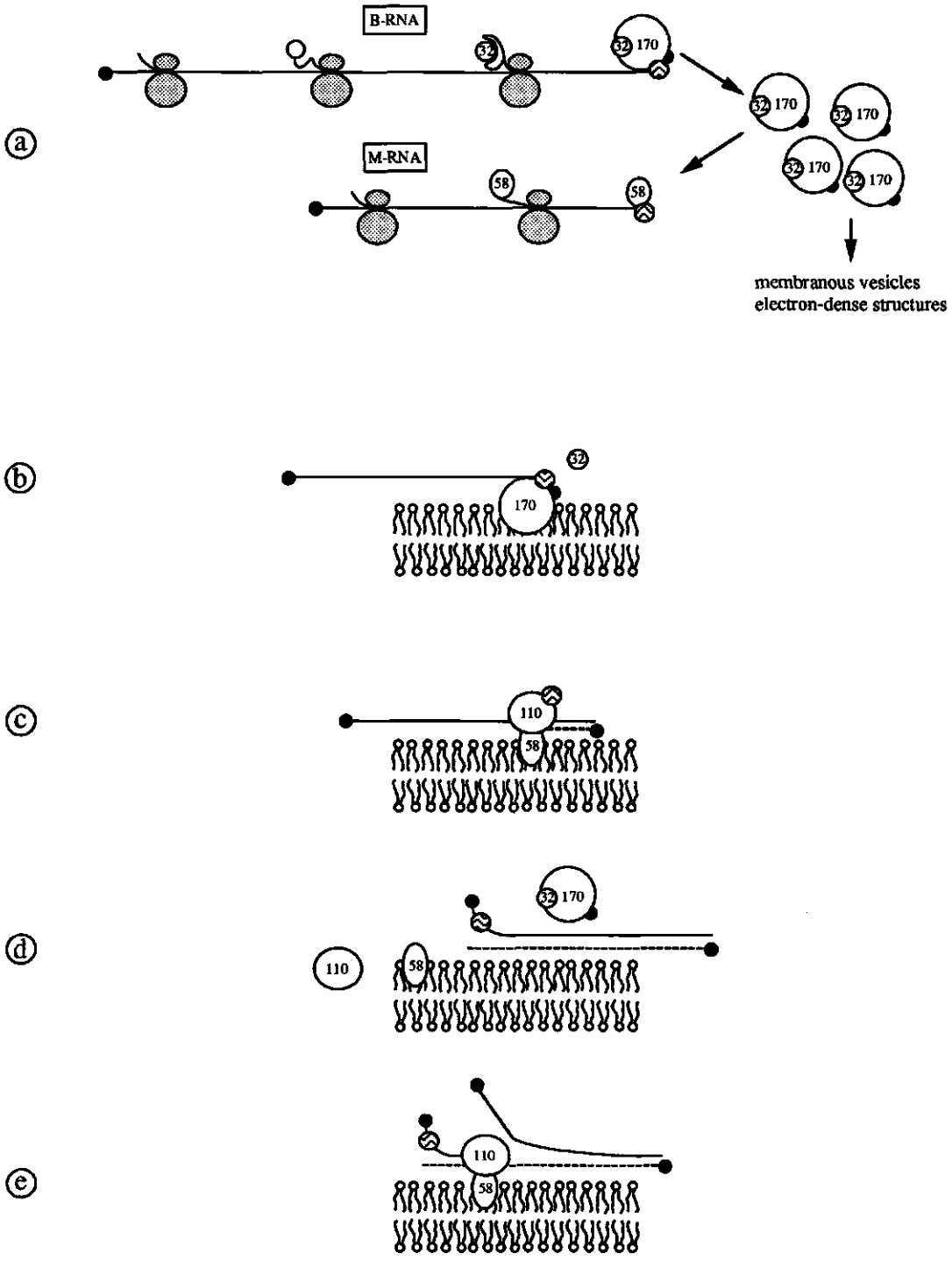
The replication of B-RNA was shown to be dependent on translation of the replicative proteins from the very same RNA molecule. Hence, the synthesized proteins must recognize the 3' end of the RNA from which they were translated. How this is accomplished is not known but it can be imagined that a ribosomal factor is involved in this process. The involvement of ribosomal proteins in viral RNA replication is also found for bacteriophage Qβ (Blumenthal and Carmichael, 1979) and possibly also for the plant virus cucumber mosaic virus (Quadt et al., 1991). Following translation of CPMV B-RNA a ribonucleoprotein complex may occasionally be formed, consisting of the interacting viral 32K and 170K proteins, viral RNA and possibly a ribosomal factor(s), which becomes attached to membranous vesicles (FIG 1b). The anchoring in membranes is mediated by the hydrophobic domain within the 58K protein of B-RNA, which is also the site of interaction with the 32K protein. It may be imagined that the association of the hydrophobic domain with membranes thus abolishes the 32K-58K interaction, thereby triggering further proteolytic processing of the 170K protein and enabling the completion of the formation of a replication complex (FIG 1c). The

functional membrane-bound replication complex consists of B-RNA template associated with viral replicative proteins (cleavage products) and possibly a (ribosomal) host factor(s).

The formation of a replication complex for M-RNA may be mediated in an analogous manner, requiring the binding of the 58K polypeptide at the 3' end of the RNA molecule. As for B-RNA a ribonucleoprotein complex may then be formed, consisting of M-RNA, the 58K protein (or its precursor) and possibly a ribosomal protein(s). This complex is recognized by the 32K-170K proteins of B-RNA and subsequently directed to the membranous vesicles. The formation of a functional M-RNA replication complex then proceeds identical as for B-RNA.

Although it is suggested by the model that a ribosomal host protein is involved in the formation of the membrane bound replication complex, this is speculative and needs to be proven. It has been found recently that CPMV RNA replication can occur in protoplasts of a wide variety of plant species (Wellink et al., in press), indicating that host proteins that are involved in viral RNA replication are well conserved during evolution. However, this observation does not preclude the possibility that host proteins may be engaged at other stages of viral RNA replication such as membrane proliferation or RNA initiation.

FIGURE 1. A model for CPMV RNA replication. a) Translation of the viral RNAs. The 200K polyprotein encoded by B-RNA is already cleaved during its synthesis into the 32K and 170K proteins. The 32K protein remains associated with the 170K protein by interaction with the hydrophobic domain of 58K protein, thereby inhibiting furher processing of the 170K protein. The B-RNA-encoded proteins are kept together in the cytoplasm of the infected cell and will induce the formation of electron-dense structures and membranous vesicles. Occasionally, the 32K-170K proteins are arrested at the 3' end of the B-RNA molecule, possibly by virtue of a ribosomal protein (represented by waved lines). Initiation of translation of M-RNA at nucleotide position 161 will result in synthesis of the 105K polyprotein. The 58K protein, which is contained in the 105K polyprotein, or the entire 105K polyprotein may bind to the 3' end of M-RNA, which possibly also requires the interference of a ribosomal protein. Subsequently, the 32K-170K protein complex recognizes the ribonucleoprotein complex at the 3' end of M-RNA. b) Docking of the pre-replication complex in vesicular membranes. The hydrophobic domain of the B-RNA-encoded 58K protein enables the template RNA and 32K-170K proteins to become membrane-bound. The membrane association of the 58K protein abolishes the 32K-170K interaction. The 170K protein is then further processed and a functional RNA replication complex is formed. c) Synthesis of complementary (-)-strand RNA. VPg, represented by a black dot, is likely to be involved in initiation of RNA synthesis. Elongation of the initiated (-)-strand RNA involves the RNA polymerase activity of the 110K protein and RNA helicase activity of the 58K protein. d) Termination of (-)-strand RNA synthesis and replication complex formation for (+)-strand RNA synthesis. A new set of 32K-170K proteins is recruited to the 5' end of the (+)-RNA strand, where they recognize a host protein bound to a 5' stem-loop structure. A membrane bound replication complex may then be formed, just as has been described for (-)-strand RNA synthesis. e) Synthesis of genomic RNAs. When (+)-strand RNA synthesis proceeds, the host protein may be translocated to the newly synthesized 5' stem-loop structure. A new set of 32K-170K proteins is attracted and formation of yet another replication complex occurs. Translocation of the host protein is repeated several times, thus allowing multiple (+)-strand RNAs to be synthesized on one (-)-strand RNA template.


a
B-RNA
32
170
M-RNA
58
membranous vesicles
electron-dense structures
b
c
110
d
e

B. CPMV RNA synthesis

The mechanism of initiation of CPMV RNA synthesis is still one of the most obscure steps in CPMV viral RNA replication. Since VPg is found at the 5' end of each progeny RNA, it is very likely that VPg or one of its precursors is involved in the initiation step. In analogy to poliovirus it is most likely in our view that uridylated VPg, or one of its precursors, functions as a primer in RNA synthesis (Takeda et al., 1986). However, the hairpin primer model of Flanegan and coworkers (Young et al., 1985; Tobin et al., 1989), which is based on the activity of a host terminal uridylyl transferase activity can not be excluded as yet. Once initiation of the CPMV RNA synthesis has occurred, RNA elongation proceeds, which very likely involves the RNA polymerase activity of the 110K protein (Dorssers et al., 1984). The putative RNA helicase activity of the 58K protein (or a precursor) encoded by B-RNA may be required also at this stage for unwinding secundary structures in the template RNA and/or preventing the formation of double strand structures between (+)- and (-) -sense RNAs.

Once the synthesis of the complementary (-)-strand RNA has been completed the (-)-strand can be used as a template to produce multiple progeny (+)-strands RNAs. However, at some point during synthesis of the (-)-strand, probably during initiation, VPg has to be attached to the 5' end of the newly synthesized (-)-strand and, consequently, proteolytic processing of a new 200K polyprotein has to occur to synthesize VPg-linked (+)-strand RNA. Hence, for each new strand of VPg-linked RNA to be synthesized an entire new set of replicative proteins is produced. Therefore, it has been proposed previously (Eggen and van Kammen, 1988; Eggen et al., 1988) that the viral replicative proteins are used only once and that an entire new set of B-proteins (32K-170K) must be recruited at the 3' end of the (-)-strand RNA template. Since translation of the (-)-strand RNA template does not occur, the formation of a replication complex for (+)-strand RNA synthesis must proceed differently than for (-)-strand RNA synthesis. This raises the question how the replicative proteins encoded by B-RNA recognize the (-)-strand RNA as a template for RNA synthesis. A clue how this might be accomplished is provided by the remarkable observation by Andino et al. (1990), who demonstrated that the synthesis of poliovirus (+)-strand RNA was dependent on a stem-loop structure at the 5' end of the (+)-sense RNA. It was found that the association of a host protein(s) with this 5' stem-loop structure and the subsequent binding of poliovirus proteins 3C and 3D, or their precursor protein 3CD, was required for (+)-strand RNA synthesis. Likewise, CPMV (+)-strand RNA synthesis may also be dependent on a host protein, which may have been used already for (-)-strand synthesis, that recognizes a structure at the 5' end of the (+)-RNA strand (FIG 1d and 1e). This ribonucleoprotein structure is then recognized by the 32K-170K proteins of B-RNA and, upon proteolytic processing of the 170K protein, a functional replication complex can be formed. Initiation of (+)-strand RNA synthesis may then proceed similar as (-)-strand synthesis, templated by the (-)-RNA that is in close proximity with the activated replicative proteins. Evidence that the 5' end of (+)-sense RNA is involved in viral (+)-strand RNA synthesis has also been obtained recently for brome mosaic virus, a member of the alpha-like supergroup (Pogue and Hall, 1992). It was suggested by these authors that the binding of host proteins to structures at the 5' end of (+)-sense RNA may be a general feature in the (+)-strand RNA synthesis of many other viruses. Interestingly, structural comparison of the 5' non coding regions of the viral RNAs of the comoviruses CPMV, RCMV, BPMV and CPSMV reveals a striking conservation of two 5 ' terminal stem-loop structures, whereas other parts of the 5' non coding regions have widely diverged (C. Pleij and J. Wellink, personal communications).

V. CONCLUSION

Sofar, RNA-synthesizing activity *in vitro* for the CPMV polymerase has been obtained only upon isolation of the viral RdRp activity from CPMV-infected plants. This activity is independent of exogenous RNA and is capable only of elongating nascent RNA strands, of which the initiation had already occurred *in vivo*. As proposed by the model of CPMV RNA replication, activity of the CPMV polymerase may be restricted to endogenous template RNA due to the dependency of activation of the CPMV polymerase by proteolytic processing of a precursor molecule that is already bound to template RNA. In turn, binding of precursor molecules to the RNA template is dependent on the synthesis of (functional) proteins from the same RNA molecule, being the 58K protein for M-RNA and the entire 200K polyprotein for B-RNA. It is therefore proposed that an appropriate assay system to test CPMV polymerase activity *in vitro* should facilitate translation of the added template RNA molecules. At the other hand, the experimental observations do not as yet preclude the possibility that the CPMV polymerase is just not able to initiate RNA synthesis *in vitro*, and therefore cannot show RNA synthesizing activity on an exogenous template. In that case, conditions need to be found that mimick the initiation process. A possible means of achieving this is by offering the CPMV polymerase an RNA template that is annealed to uridylylated VPg or one of its precursors.

Research will be required to resolve the speculations that are included in the current replication model. By the development of a transient expression system for cowpea protoplasts some of the questions raised are now amenable to further investigations.

The need for polyprotein processing to obtain a functional RNA replicase may be investigated in complementation experiments. For example, it would be interesting to know whether the separate expression of the coding sequences of the 32K and 170K proteins (or other combinations) in one cell could support the replication of M-RNA, just as was observed upon expression of the complete 200K coding region.

Other experiments may be designed to further examine the linkage between translation and synthesis of (-)-strand RNAs. If the transient expression vectors are linearized prior to transfection mRNA transcripts will be generated that can be used as templates for synthesis of the complementary (-)-strand. Introduction of defined mutations in the coding region as well as in the 3' non coding region of these vectors may result in the identification of cis-acting sequences that are involved in the formation of replication complexes for (-)-RNA synthesis.

Finally, the cis- and trans-acting factors in (+)-strand RNA synthesis need to be resolved. One question to be answered is whether or not there is also an indirect linkage between translation and replication for the synthesis of (+)-RNA, mediated by the re-cycling of proteins that have been used for (-)-strand RNA synthesis. This can be assayed by determining the ability of (-)-sense RNA transcripts of M-cDNA to be replicated by wild type helper B-RNA.

Verfication of our current model of CPMV RNA replication by experiments like those devised above will undoubtedly reveal new aspects of the viral RNA replication process and will thus pave the way to fully comprehend the molecular pathways leading to multiplication of the genome of CPMV and of related viruses

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SAMENVATTING

Veel virussen die planten of dieren infecteren hebben enkelstrengs RNA moleculen met een positieve polariteit als drager van hun genetische informatie. Genomische RNA moleculen met positieve polariteit, of (+)-strengs RNA, betekent dat deze RNA moleculen in de cel van de gastheer gebruikt worden als boodschapper RNA voor de productie van virale eiwitten. Deze eiwitten maken dan de verschillende stappen in de levenscyclus van het virus mogelijk, zoals de vermenigvuldiging van het virale RNA (replicatie), het inpakken van dit RNA in eiwitmantels en de verspreiding van het virus van geïnfecteerde cellen naar andere, nog niet geïnfecteerde cellen. Verschillende groepen (+)-strengs RNA virussen volgen bij het tot stand brengen van deze stappen elk hun eigen strategie. Een groot aantal van de (+)-strengs RNA virussen zijn op grond van overeenkomsten in organisatie van de genetische informatie en de manier waarop die informatie tot expressie komt en wordt gerepliceerd ingedeeld in twee supergroepen; de picorna-achtige virussen en de Sindbis- of alpha-achtige virussen (hoofdstuk 1). Tot de supergroep van picorna-achtige virussen behoren onder andere het humane poliovirus en mond- en klauwzeer virus, -beide picornavirussen-, en de plantevirussen van de comovirus groep. Tot die laatste groep behoort cowpea mosaic virus (CPMV), dat als gastheer de cowpea (kouseband) plant heeft. Het doel van het onderzoek in dit proefschrift was meer inzicht te krijgen in de moleculaire mechanismen die optreden bij de RNA replicatie van CPMV. Gedurende het onderzoek werd regelmatig een vergelijking gemaakt tussen het mechanisme van RNA replicatie van CPMV en van poliovirus.

Het genoom van CPMV bestaat uit twee RNA moleculen met een lengte van 5805 en 3299 nucleotiden, respectievelijk B- en M-RNA genaamd, die afzonderlijk zijn verpakt in een eiwitmantel. Beide RNAs bezitten aan het 5' eind een klein eiwit, VPg, en een poly(A) staart aan het 3' einde. Translatie van deze RNAs leidt tot de synthese van grote poly-eiwitten, die proteolytisch in verschillende functionele eiwitten geknipt worden. Voor een succesvolle infectie van planten zijn eiwitten afkomstig van beide genomische RNA moleculen van CPMV noodzakelijk, maar er is een duidelijke verdeling van taken die de B- en M-RNA-gecodeerde eiwitten vervullen in het infectie proces. Zo codeert M-RNA voor alle functies die nodig zijn voor de verspreiding van het virus door de plant, en B-RNA voor alle virale eiwitten die nodig zijn voor de replicatie van de virale RNA moleculen. Translatie van M-RNA geeft, afhankelijk van het gekozen start codon, een poly-eiwit van 105K of 95K, waarbij de aminozuur volgorde van het 95K eiwit volledig identiek is aan het carboxy-terminale einde van het 105K eiwit. Volledige klieving van deze M poly-eiwitten geeft de 58K/48K transport eiwitten en de 37K en 23K manteleiwitten. Het primaire translatieproduct van B-RNA, het 200K poly-eiwit, wordt onmiddelijk na of zelfs al tijdens de synthese gekliefd in de 32K en 170K eiwitten. Het 170K eiwit kan vervolgens op verschillende manieren verder gekliefd worden en geeft dan de 60K en 110K, 84K en 87K of 58K en 112K eiwitten. Volledige klieving van het B-RNA-gecodeerde 200K poly-eiwit geeft de 32K, 58K, VPg, 24K en 87K eindproducten, die in deze volgorde in het 200K poly-eiwit bevat zijn.

De replicatie van (+)-strengs RNA virussen volgt in grote lijnen eenzelfde schema. Eerst worden de genomische RNAs als matrijs gebruikt voor de synthese van een complementaire streng (de (-)-streng), die vervolgens weer als matrijs dient voor de synthese van nieuwe (+)-strengen. De exacte functie van de B-RNA-gecodeerde eiwitten in het RNA replicatie proces is echter maar ten dele bekend of is alleen gebaseerd op aminozuur sequentie homologie met eiwitten waarvan de functie reeds bekend is. Het 32K eiwit is een co-factor in de klieving van de M-poly-eiwitten en is ook betrokken in de regulatie van de klievingen van het B-poly-eiwit. Het 24K eiwit is het actieve protease dat alle klievingen van de CPMV eiwitten uitvoert. Het 87K eiwit bezit homologie met RNA polymerases; enzymen die in staat zijn nieuwe RNA strengen te synthetiseren. Het 87K eiwit vormt samen met het 24K eiwit het 110K eiwit, dat vermoedelijk de actieve vorm van het polymerase is in RNA replicatie complexen. Het 58K eiwit, dat tesamen met VPg het 60K eiwit vormt, zou op grond van homologie in aminozuur volgorde een RNA helicase kunnen zijn, noodzakelijk voor het

ontwinden van dubbelstrengs RNA structuren tijdens de replicatie. Het 60K eiwit wordt ook een functie toegedacht in het verankeren van CPMV RNA replicatiecomplexen aan de membranen waar de virale RNA synthese plaats vindt. VPg, tenslotte, wordt een rol toegedacht als primer in het starten van de RNA synthese (initiatie).

Om meer te weten te komen over de functie van de verschillende door het B-RNA gecodeerde eiwitten bij de CPMV RNA replicatie werden de afzonderlijke B-eiwitten gesynthetiseerd in insektecellen door gebruik te maken van baculovirus expressievectoren (hoofdstukken 2 en 4). De 87K, 110K, 170K en 200K eiwitten, die allen het polymerase motief van het 87K eiwit bevatten, werden gemaakt om te bepalen welk van deze eiwitten het actieve RNA polymerase is, en om na te gaan of proteolytische klievingen van voorlopers van het 87K eiwit noodzakelijk zijn om een actief polymerase te verkrijgen.

Na expressie in insektecellen werden CPMV eiwitten verkregen die, onder denaturerende condities, immunologisch niet te onderscheiden waren van de CPMV eiwitten die in CPMV-geïnfecteerde planten gevonden worden. Daarnaast vertoonden de uit insektecellen afkomstige eiwitten ook een aantal kenmerken van functioneel actieve CPMV eiwitten. Zo bleek dat eiwitten die het 24K protease domein bevatten, op juiste wijze gekliefd werden tot kleinere CPMV eiwitten. Daarnaast werd gevonden dat het 32K eiwit een regulerende rol had bij de klievingen van het 170K eiwit, hetgeen duidt op een correcte vouwing van de in insektecellen geproduceerde eiwitten. Met electronen-microcospie werd verder aangetoond dat in insectecellen waarin het 200K poly-eiwit gemaakt wordt structuren voorkomen die lijken op de zogenaamde cytopathologische structuren die in CPMV-geïnfecteerde cowpea cellen gevonden worden (hoofdstuk 4). Deze cytopathologische structuren bestaan uit ophopingen van membraanblaasjes, waarmee de virale RNA replicatie geassocieerd is, met daarnaast grote hoeveelheden electronendicht materiaal, waarvan de functie nog onbekend is maar waarin het merendeel van de B-RNA-gecodeerde eiwitten zijn samengepakt. De vesiculaire membranen worden ook gevonden in insektecellen waarin alleen het 60K eiwit geproduceerd wordt, hetgeen aangeeft dat het 60K eiwit betrokken is bij de vorming van deze structuren. In overeenstemming met de voorgestelde rol van het 60K eiwit als membraan-anker voor CPMV replicatiecomplexen, bleek bovendien dat het 60K eiwit in deze cellen gebonden aan de vesiculaire membranen voor komt. Tenslotte werd gevonden dat de vorming van de electronendichte structuren alleen dan plaats vindt wanneer, naast het 170K eiwit, ook het 32K eiwit in de insektecellen wordt geproduceerd.

Hoewel de hierboven beschreven waarnemingen erop duiden dat de B-RNA-gecodeerde eiwitten verkregen uit insektecellen functioneel actief zijn, bleek het niet mogelijk om in extracten van insektecellen *in vitro* RNA polymerase activiteit voor deze eiwitten aan te tonen (hoofdstukken 3 en 4). Dit in tegenstelling tot de ook in insektecellen geproduceerde polymerases van de picornavirussen poliovirus en mond-en klauwzeer virus, die wel RNA kunnen synthetiseren op een toegevoegde poly(A)-bevattende RNA matrijs, mits die voorzien is van een oligo(U) primer. Het lijkt er dus op dat de polymerases van CPMV en van picornavirussen, ondanks homologie in aminozuur volgorde, beschikken over verschillende eigenschappen.

Eén van de verklaringen voor de afwezigheid van polymerase activiteit voor de CPMV eiwitten zou kunnen zijn dat het CPMV polymerase een plant-specifieke component nodig heeft voor de RNA synthese. Om dit te onderzoeken werd een expressievector systeem gebruikt dat het mogelijk maakt om de verschillende door het B-RNA-gecodeerde eiwitten in cowpea protoplasten te synthetiseren (hoofdstuk 5). De in de protoplasten geproduceerde eiwitten bleken ook nu weer dezelfde eigenschappen vertoonden als die van de authentieke virale eiwitten. Door de expressie in cowpea protoplasten uit te voeren was het bovendien mogelijk om direct na te gaan of de virale eiwitten ook alle activiteiten bezaten die nodig zijn voor RNA replicatie. Hiertoe werden protoplasten geïnoculeerd met de verschillende expressievectoren, tesamen met M-RNA, dat voor replicatie afhankelijk is van de eiwitten van B-RNA. Replicatie van M-RNA bleek inderdaad plaats te vinden in protoplasten die getransfecteerd waren met de expressievector die codeerde voor het gehele 200K eiwit, maar niet in

protoplasten waarin slechts een deel van dit 200K eiwit werd aangemaakt. Dit laat zien dat alle eiwitten van B-RNA noodzakelijk zijn voor de virale RNA replicatie. Hoewel het mogelijk was om polymerase activiteit *in vivo* aan te tonen, lukte het niet om deze replicase activiteit *in vitro* te reconstitueren. Ook in extracten van protoplasten was het CPMV polymerase niet in staat om toegevoegde RNA moleculen, al dan niet voorzien van een RNA primer, te gebruiken als matrijs voor RNA synthese. Het poliovirus polymerase, dat eveneens in cowpea protoplasten aangemaakt was, was wel tot deze activiteit in staat (hoofdstuk 5). Het lijkt er dus op dat het polymerase van CPMV, in tegenstelling tot het poliovirus polymerase, niet in staat is om toegevoegde RNA moleculen te herkennen als matrijs voor de RNA synthese.

De experimenten die in hoofdstuk 6 beschreven zijn hadden tot doel die eigenschappen van de genomische RNAs van CPMV te vinden die verantwoordelijk zijn voor de herkenning van deze RNA moleculen door het virale replicase. Hiertoe werden cowpea protoplasten getransfecteerd met mutante B- en M-RNA moleculen en werd gekeken of deze RNAs gerepliceerd werden door de eiwitten afkomstig van normale (wild-type) B-RNA moleculen. Mutanten van B-RNA, die niet in staat zijn om zichzelf te vermenigvuldigen, werden ook niet gerepliceerd in aanwezigheid van het wild-type B-RNA. Hieruit blijkt dat de replicatie van B-RNA plaatsvindt *in cis*, wat wil zeggen dat de herkenning van een B-RNA molecuul als matrijs voor replicatie alleen plaats vindt door eiwitten die door vertaling van datzelfde B-RNA molecuul zijn gemaakt. Er lijkt dus sprake te zijn van een sterke koppeling van de translatie en de replicatie van het B-RNA. De replicatie van M-RNA is afhankelijk van de door B-RNA gecodeerde eiwitten en M-RNA wordt dus *in trans* gerepliceerd. Merkwaardig genoeg werd ook voor M-RNA gevonden dat er een koppeling was tussen translatie en replicatie. Replicatie van M-RNA moleculen vond alleen plaats indien translatie plaats vond van het 5' eind van het open leesraam, dat codeert voor het 58K eiwit. Dit wijst er op dat het 58K eiwit van M-RNA betrokken is bij de herkenning van datzelfde M-RNA molecuul als matrijs voor het B-RNA-gecodeerde replicatiecomplex. Deze waarneming houdt tevens in dat replicatie van een M-RNA molecuul alleen op kan treden indien initiatie van translatie plaats vindt op het eerste start codon, waarmee de synthese van het 105K poly-eiwit begint.

Op grond van de in dit proefschrift beschreven resultaten is een nieuw model voor de RNA replicatie van CPMV opgesteld (hoofdstuk 7). In dit model wordt een verband gelegd tussen de translatie van de virale genomische RNAs, de proteolytische klieving van poly-eiwitten, de vorming van een replicatiecomplex aan een membraan en tenslotte de synthese van nieuwe RNA strengen, van zowel (+)- als (-)-polariteit. Het expressie systeem beschreven in hoofdstuk 6 biedt mogelijkheden verschillende onderdelen van dit model te toetsen, om aldus ons inzicht in het RNA replicatiemechanisme van CPMV en dat van nauw verwante RNA virussen nog verder te vergroten.

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CURRICULUM VITAE

Hans van Bokhoven werd op 9 mei 1963 te Vlijmen geboren. In 1981 behaalde hij het Atheneum-B diploma aan het Dr. Mollercollege te Waalwijk en begon hij met zijn studie planteziektenkunde aan de Landbouwuniversiteit te Wageningen. In de doctoraalfase verrichtte hij onderzoek bij de vakgroepen Virologie (Prof. Dr. R. Goldbach), Organische Chemie (Prof Dr. Æ. de Groot) en Biochemie (Prof. Dr. C. Veeger). In het kader van zijn stage werkte hij 6 maanden op de afdeling Biologie (Prof. Dr. T.C. Hall) van de Texas A & M Universiteit in de Verenigde Staten. In november 1987 werd het ingenieursdiploma behaald. Van 1 december 1987 tot 1 december 1991 was hij als onderzoeker in opleiding werkzaam op de vakgroep Moleculaire Biologie van de Landbouwuniversiteit (Prof. Dr. A. van Kammen), waar hij het in dit proefschrift beschreven onderzoek verrichtte. Vanaf 1 april 1992 is hij als wetenschappelijk medewerker verbonden aan het Anthropogenetisch Instituut van de Katholieke Universiteit te Nijmegen.