PROTEIN SYNTHESIS DIRECTED BY COWPEA MOSAIC VIRUS RNAs

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ter verkrijging van de graad van
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STELLINGEN

I De conclusie van Pelham dat op het CPMV M-RNA twee startplaatsen voor eiwitsynthese zijn is voorbarig.

Pelham, H.R.B. (1979), Virology 96, 463.
Fillipowicz, W., and Haenni, A.–L. (1979), Biochemistry 76, 3111.

II De conclusie van Thongmeearkom en Goodman dat beide manteleiwitten van CPMV gecodeerd worden door het M-RNA steunt niet op voldoende bewijsmateriaal.

Thongmeearkom, P., and Goodman, R.M. (1978), Virology 85, 75.

III De bewering dat het RNA2 van tabaksratelvirus (PRN stam) twee 'open' startplaatsen voor eiwitsynthese bevat is op onvoldoende bewijsmateriaal gebaseerd.

Fritsch, C., Mayo, M.A., and Hirth, L. (1977), Virology 77, 722.

IV De experimenten van Hoffman *et al.* rechtvaardigen niet hun conclusie dat de $Ph(NMe_2)_2$ oxidatietak van de ademhalingsketen van *Azotobacter vinelandii* niet bijdraagt tot de energieconservering.

Hoffman, P.S., Morgan, T.V., and DerVartanian, D.V. (1979), Eur. J. Biochem. 100, 19.
Laane, C., Haaker, H., and Veeger, C. (1979), Eur. J. Biochem. 97, 369.

V Waarnemingen dat zilverionen de productie van ethyleen remmen in sommige, maar niet in andere plantesystemen, zijn te verklaren uit het feit dat ethyleen zijn eigen productie 'autokatalytisch' kan versnellen.

Beutelmann, P., and Kende, H. (1977), Plant Physiol. 59, 888.
Veen, H. (1979), Planta 145, 467.
Beyer, Jr., E.M. (1979), Plant Physiol. 63, 169.

VI Bij het interpreteren van *in vitro* translatie experimenten wordt er vaak te snel vanuit gegaan dat de resultaten van deze experimenten in overeenstemming zullen zijn met de *in vivo* situatie.

VII Gezien het toenemend gebruik van kunststoffen bij de experimenten in het moleculair biologisch onderzoek zou de term *in vitro* vervangen dienen te worden.

VIII Gezien de steeds verder gaande tendens om de automatiese prijskompensatie te baseren op een zg. 'opgeschoond' prijsindexcijfer is het onbegrijpelijk dat ondernemers en regering nog van prijskompensatie durven te spreken.

IX Automatisering en andere verslechterende effecten op de arbeidsmarkt maken van werk*gevers* werk*nemers*.

X De koppeling van socialisme en pacifisme wordt door velen niet onderkend, getuige de vaak gebruikte uitdrukking 'pacifisten' als het gaat om vertegenwoordigers van de Pacifistisch Socialistische Partij (PSP).

XI De verkeersveiligheid zou enorm bevorderd kunnen worden door voor iedere auto een aparte rijstrook te reserveren; het snelst kan dit gerealiseerd worden door het aantal autos aan te passen aan het aantal beschikbare rijstroken.

XII De barkrukken in het Wageningse café 'Trust', voorheen ' 't Schuim', voorheen 'Troost', dienen -althans na 23.00 uur- te verdwijnen.

Proefschrift van Eugène Stuik 21 december 1979.

VOORWOORD

Ook dit proefschrift draagt slechts de naam van één auteur.
Velen echter hebben er toe bijgedragen dat dit proefschrift tot stand is gekomen. In de eerste plaats gaat mijn dank uit naar Jeffrey Davies voor zijn grote inbreng bij de *in vitro* translatie experimenten, zijn geduld en accuratesse bij het overzetten van het door mij gebezigde 'Engels' in Engels, en voor zijn kritieken op en diskussies over het werk.
Ook de stimulerende diskussies met en de adviezen van Ab van Kammen in de periode waarin de proeven uitgevoerd werden en tijdens het schrijven van dit proefschrift zijn van doorslaggevende betekenis geweest.
Bij het uitvoeren van de experimenten met tarwe kiemen extracten heb ik in de beginperiode veel hulp ondervonden van Andre Aalbers.
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CONTENTS

ABBREVIATIONS

A	Adenine
Ac	Acetate
AMV	Alfalfa mosaic virus
ATP	Adenosine triphosphate
B-	Bottom component
BBMV	Broad bean mottle virus
BMV	Brome mosaic virus
BP	Large polypeptide synthesized *in vitro* under direction of B-RNA
B-RNA	Ribonucleic acid from bottom component
CarMV	Carnation mottle virus
CCMV	Cowpea chlorotic mottle virus
C_cTMV	Cowpea strain of tobacco mosaic virus
CMV	Cucumber mosaic virus
CP	Coat protein
cpm	Counts per minute
CPMV	Cowpea mosaic virus
CTP	Cytidine triphosphate
d	Dalton
DTE	Dithioerythreitol
EDTA	Ethylene diaminetetraacetate
EGTA	Ethylene-glycol-bis (2 aminoethylether)-N, N'-tetra acetic acid
EMC	Encephalomyocarditis virus
F	Fast moving electrophoretic component of CPMV
G	Guanine
g	Centrifugal field (number times gravity)
GTP	Guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonate
LMC	Low molecular weight component RNA from TMV-RNA
M-	Middle component
MDL	Messenger-dependent (rabbit reticulocyte) lysate
MP	Large polypeptides synthesized *in vitro* under direction of M-RNA
M-RNA	Ribonucleic acid from middle component
mRNA	Messenger RNA
MW	Molecular weight
PEG	Polyethylene glycol
PMSF	Phenyl methyl sulphonyl fluoride
PMV	Papaya mosaic virus
PolyA	Polyriboadenylic acid
RNA	Ribonucleic acid

RNase	Ribonuclease
rpm	Rotations per minute
S	Svedberg, *unit of sedimentation*
SDS	Sodium dodecyl sulphate
T-	Top component
TBRV	Tomato black ring virus
TCA	Trichloroacetic acid
TMV	Tobacco mosaic virus
TNV	Tobacco necrosis virus
Tris	Tris(hydroxyl) aminomethane
tRNA	Transfer RNA
TRSV	Tobacco ringspot virus
TRV	Tobacco rattle virus
U	Uridine
UTP	Uridine triphosphate
v	Volume
w	Weight

I. INTRODUCTION

Most plant viruses have a genome that consists of single-stranded RNA. Plant viruses are the only RNA viruses known in which the division of genetic information over more than one RNA molecule, separately packed in different particles, is widely spread. The mechanism concerning the collaboration between these so called multipartite genome RNAs during virus multiplication in the infected cell is still obscure. Cowpea mosaic virus (CPMV), having a genome that is divided between two (single-stranded) RNA molecules, offers a good model system for the study of this mechanism. CPMV is readily propagated in *Vigna* plants, with a high yield of virus; the components and thereby the two RNAs are relatively simple to isolate and purify; also CPMV has been subject of study for many years in the laboratory, resulting in the knowledge of many aspects of CPMV. Evidence that the replication takes place in (virus) specific cytopathological structures, that can be isolated in a vesicle fraction, together with the finding of a virus specific replicase, bound to membranes, offers the oportunity to search for virus specific proteins in specific subcellular fractions. Furthermore the finding that the virion RNAs of CPMV act well as messengers in cell-free protein synthesizing systems made CPMV as a model even more advantageous. The aim of this study is to find out which proteins are encoded on the CPMV-RNAs; what is the possible function in the multiplication process; and how is the information for these proteins divided between the two RNA molecules. The finding that the structure of CPMV-RNAs is rather different from most other plant virus RNAs and closely resembles that of the RNAs from the animal picorna virus group made it very interesting to investigate if the structural properties of the RNAs were also reflected in their translation strategy.

I.1. Cowpea mosaic virus

CPMV is the typemember of the comovirus group (Van Kammen, 1972; Van Kammen and Mellema, 1977). CPMV produces three types of isometric particles of about 20 nm in diameter (Geelen, 1974). There are two nucleoprotein components; the third component consists of empty particles — protein capsids devoid of nucleic acid. The protein capsid of the three components is the same. It is composed of two proteins with approximate molecular weights of 37,000 and 22,000 (Wu and Bruening, 1971; Geelen, Van Kammen and Verduin, 1972; Rottier, Rezelman and Van Kammen, 1979). These two proteins occur in equal amounts of 60 copies each in the capsid and are arranged in an icosahedral symmetry (Geelen, 1974; Crowther, Geelen and Mellema, 1974). Besides the division of CPMV in three centrifugal components, CPMV also exsists in two different electrophoretical forms. When purified preparations of CPMV are analyzed by gel electrophoresis, two electrophoretic forms

are seen: a slow (S) moving and a fast (F) moving electrophoretic form (Geelen, 1974). All three centrifugal components show this phenomenon. It was found that proteolytic removal of a 2,500 d peptide from the smaller coat protein (*in vivo* and *in vitro*) was responsible for the conversion of the S-form in the F-form of the virus components (Geelen, 1974). The smaller of the two proteins therefore can be present as two proteins, differing 2,500 in molecular weight (Geelen, 1974). The centrifugal components can be separated by centrifugation in density gradients, due to the difference in RNA content, respectively 36%, 25% and 0% for the 115 *S* (B), 95 *S* (M) and 58 *S* (T) component (Van Kammen, 1967).
Each of the nucleoprotein components contain a single-stranded RNA molecule. The molecular weights of the RNAs are 2.02×10^6 for B-RNA and 1.37×10^6 for M-RNA (Reijnders *et al.*, 1974). Both nucleoproteins or RNAs are necessary for virus multiplication (Van Kammen, 1968) and Van Kammen and Rezelman (1972) also showed, using RNA-RNA hybridazation techniques, that there is no extensive sequence homology between the nucleotides of B- and M-RNA. Genetic experiments with mutant strains of CPMV, described by De Jager (1978), showed that some properties of the virus are located on B-RNA and some are located on the M-RNA. Taking all this results together it is clear that CPMV has a genome of which the genetic information is divided between the two RNA molecules, a conclusion already put forward by Van Kammen in 1971.
The CPMV-RNAs are replicated during virus multiplication by way of the synthesis of complementary chains, from which the virus RNAs are synthesized again. After extraction of RNA from infected leaves, double-stranded RNA molecules of the same lenght as the single-stranded virion RNAs can be obtained (Van Griensven and Van Kammen, 1969; Van Griensven, 1970; Van Griensven *et al.*, 1973). The majority of these double-stranded CPMV-RNAs was found after phenol extraction of a fraction containing virus specific structures as shown by Assink (1973). This so-called vesicle fraction, obtained by cell fractionation according to Assink (1973), contains the cytopathic structures in which the RNA replication takes place (De Zoeten *et al.*, 1971). The RNA replication is probably catalyzed by an RNA-dependent RNA polymerase (replicase) which is bound to membranes (Zabel *et al.*, 1974). This replicase can be isolated from infected *Vigna* leaves, but not from healthy leaves. This suggested that the enzyme activity is virus specific. From this and other observations (Zabel, 1978) it was suggested that the replicase or part(s) of it might be encoded on the virion RNAs. Since the total molecular weight of CPMV-RNAs is about 3.4×10^6, the genetic information on it is sufficient for coat proteins and replicase. There is also additional coding capacity for some other proteins. Part of this thesis concerns the search for these other proteins.
The RNAs of CPMV were the first plant virus RNAs shown to contain a polyA strech at their 3'-ends, about 100-200 nucleotides long (El Manna and Bruening, 1973; Steele and Frist, 1978). In having these polyA tails, the CPMV-RNAs resemble eukaryotic mRNAs.
Recently it was shown that a number of plant viruses from the nepovirus group also contain polyadenylated 3'-ends (Mayo *et al.*, 1979a). Another virus group shown to have polyA at the 3'-ends of their RNA is the animal picorna virus group. The 5'-ends of the RNAs of polio virus, encephalomyocarditis virus and foot and mouth disease

virus, three members of the picorna virus group, contain a small protein covalently linked to the RNA genome (Lee *et al.*, 1977; Flanegan *et al.*, 1977; Hruby and Roberts, 1978; Sangar *et al.*, 1977). Klootwijk *et al.* (1977) have demonstrated that the 5'-termini of the CPMV-RNAs do not contain a 'cap'-structure as found on many eukaryotic (and viral) messengers, nor that the 5'-end is phosphorylated or polyphosphorylated. Evidence was presented by Stanley *et al.* (1978) that a protein is linked to the 5'-ends of both CPMV-RNAs; a similar result was also found by Daubert *et al.* (1978).

On the RNAs of tobacco ringspot virus and tomato black ring virus (two members of the nepovirus group) a protein was also found at the 5'-end of the genome (Harrison and Barker, 1978; Mayo *et al.*, 1979b). In the case of the nepoviruses, infectivity was lost after removal of the protein (Harrison and Barker, 1978). Removal of the protein moiety of CPMV-RNAs seemed not to affect infectivity nor altered the *in vitro* translation results (Stanley *et al.*, 1978). The function of this 5'-end bound protein is still unclear.

The structure of the CPMV-RNAs (in regard to their 3'- and 5'-terminus) differs completely from the other plant virus RNAs, except for the nepoviruses, and strongly resemble the structure of the RNAs of the picorna viruses. To what extent the analogus structure of the RNAs of these virus is reflected in their translation strategy will be discussed in this thesis.

I.2. The translation strategy of plant viral RNAs.

The translation strategy of only the plus-strand RNa plant viruses is discussed here, plant viruses with double-stranded RNA, minus-strand RNA or DNA are not included.

From an examination of the literature concerning *in vitro* translation of plant virus RNAs, the (RNA) plant viruses can be divided into three kinds of viruses on the basis of their translation strategy.

1) Viruses that generate subgenomic messengers and of which all RNAs, including the virion RNAs, are translated as monocistronic mRNAs. As examples for this kind of viruses, tobacco mosaic virus (TMV), turnip yellow mosaic virus (TYMV), brome mosaic virus (BMV), alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), broad bean mottle virus (BBMV) and cowpea chlorotic mottle virus (CCMV) are described. From this kind of viruses the RNAs of TMV, TYMV, BMV, and CMV contain a m^7G (5')pp p(5')Np structure at their 5'-end (Keith and Fraenkel-Conrat, 1975; Zimmern, 1975; Dasgupta *et al.*, 1976; Symons, 1975; Pinck, 1975; Klein *et al.*, 1976); the RNAs of BBMV and CCMV might also contain this structure at their 5'-end. The RNAs of BMV, BBMV, CCMV, CMV, TMV and TYMV all have in common that their RNAs have a tRNA-like structure at their 3'-end, which is capable of accepting an amino acid (Hall *et al.*, 1972; Kohand Hall, 1974; Öberg and Phillipson, 1972; Pinck *et al.*, 1970).

2) Viruses that have one RNA molecule which possibly acts as polycistronic messenger RNA. As examples for this kind of viruses, carnation mottle virus (CarMV), papaya mosaic virus (PMV) and tobacco necrosis virus (TNV) are described.
3) Plant viruses that have RNAs that act as monocistronic messengers, containing a polyA strech at their 3'-end and a small protein at their 5'-end and are unlikely to generate subgenomic messengers. The virion RNAs are translated into large polypeptides from which the virus specific (functional) proteins are probably cleaved after translation. This kind of viruses show a considerable analogy with the animal picorna viruses. Examples are the nepoviruses and CPMV, as described in this thesis.
One of the most studied viruses of the first kind is TMV, a plant virus with a monopartite genome RNA. The early reports on the *in vitro* translation of TMV-RNA (molecular weight: 2.0×10^6) demonstrated that this RNA could stimulate the incorporation of amino acids in a cell-free system derived from wheat germ, leading to the synthesis of a heterogeneous mixture of polypeptides (Roberts and Paterson, 1973; Efron and Marcus, 1973; Roberts *et al.*, 1973; Roberts *et al.*, 1974). In none of these studies was the synthesis of TMV coat protein conclusively proven. The intact TMV-RNA in wheat germ extracts is translated into products ranging from 10,000 to about 140,000 molecular weight (Roberts and Paterson, 1973; Roberts *et al.*, 1973), whereas in frog oocytes the major translation product is a 140,000 molecular weight polypeptide (Knowland, 1974). In rabbit reticulocyte lysates, a polypeptide with a molecular weight of about 165,000 was also synthesized (Pelham and Jackson, 1976; Knowland *et al.*, 1975). The two large polypeptides synthesized under the direction of intact TMV-RNA were also claimed to be found *in vivo* (Zaitlin and Hariharasubramanian, 1972; Sakai and Takebe, 1974; Paterson and Knight, 1975; Siegel *et al.*, 1978). But, as will be shown below the nature of the about 160,000 molecular weight protein found *in vivo* is not clear. If it was however coded on the TMV-RNA together with the other large polypeptide, those two polypeptides must be read from overlapping genes. Recent evidence by Pelham (1978) shows that the 130,000 (there called 110,000) molecular weight polypeptide has peptide sequences in common with the 160,000 molecular weight polypeptide, indicating that the overlapping genes are read in the same 'reading frame'. Using suppressor tRNAs (amber and ochre) Pelham (1978) furthermore showed that the 160,000 molecular weight polypeptide is generated by read-through of a 'leaky' (UAG) termination codon. Taking this together with the findings that the 160,000 molecular weight protein found *in vivo* is probably a (tobacco) host protein (Huber, 1979), it can be concluded that the model presented by Knowland *et al.*, (1975) in which two initiation sites are located near the 5'-end of the TMV-RNA is not correct.
Coat protein could not be synthesized when full-size TMV-RNA was used as messenger in various translation systems. Hunter *et al.* (1976) discovered in tobacco leaves infected with TMV, a small RNA which could efficiently be translated into TMV coat protein. This so called low molecular weight component RNA (LMC) was also found by Siegel *et al.* (1976) and was proven to be the monocistronic messenger for coat protein. This coat protein gene is present on the intact TMV-RNA and located near the 3'-end and has a 'closed' initiation site (Hunter *et al.*, 1976). Coat protein can not be translated until this LMC is generated in the TMV infected cell. It is either cleaved from the intact TMV-RNA or separately replicated in the infected

cells. The situation with the cowpea strain of TMV (C_CTMV) is essentially the same with the difference that there more smaller RNAs exist, one coding for a 30,000 molecular weight polypeptide (Bruening *et al.*, 1976) and probably one coding for a 72,000 polypeptide (Huber, 1979).

From the findings described above it can be concluded that the TMV-genome acts as a monocistronic messenger, coding for a 130,000 molecular weight polypeptide, which is suggested as being the TMV-specific replicase (Scalla *et al.*, 1978). The other cistron(s) on the RNA have closed initiation sites. The other TMV-specific proteins as coat protein and the 30,000 and 72,000 molecular weight proteins (the latter in the case of C_CTMV) are only synthesized when the subgenomic (monocistronic) messenger molecules are generated.

A similar situation as with TMV is found with TYMV, another plant virus with a monopartite genome RNA. The intact RNA molecule of TYMV has a molecular weight of 2×10^6. Preparations of TYMV-RNA often show heterogeneities and small RNA fragments can also be generated *in vitro* from the intact RNA (Klein *et al.*, 1976; Pleij *et al.*, 1976). There is evidence that the smaller RNAs also occur in several nucleoprotein components of the virus (Matthews, 1960; Matthews, 1974); in this respect TYMV resembles to the multicomponent viruses. But since the large (intact) RNA molecule alone can give succesful infection and all the smaller RNAs seem to be generated from this intact RNA (*in vivo* and *in vitro*) it still is a monopartite genome RNA. All the smaller encapsidated RNAs have been isolated and can be translated *in vitro* into polypeptides with a similar range as the RNAs (Higgins *et al.*, 1978). It was proven by Mellema *et al.* (1979) that the initiation site of all these intermediate RNAs is the same and located near the 5'-end, except for the coat protein gene which is situated towards the 3'-end and has a closed cistron when it is present on a larger RNA. Like the case with BMV (see below) the mixture of all the TYMV-RNAs directs primarily the synthesis of coat protein (Davies, 1979). The smallest TYMV-RNA, also generated from the intact molecule, has a molecular weight of about 0.3×10^6 and has been shown to be the monocistronic messenger for coat protein (Klein *et al.*, 1976).

From the work described here it is concluded that TYMV has an encapsidated subgenomic RNA that codes for coat protein. Whether this RNA is replicated separately in the infected cell or cleaved from the intact RNA is still unknow. Like TMV-RNA, the large intact TYMV-RNA is translated in wheat germ extracts into a series of polypeptides with no coat protein among them. The largest polypeptide synthesized has a molecular weight of about 130,000 (Davies, 1979). In reticulocyte lysates a polypeptide of about 190,000 molecular weight was also synthesized (Benicourt *et al.*, 1978). These two large polypeptides probably have common sequences (as analyzed by *S. aureus* V8 protease disgestion) suggesting an 'equal reading frame'. It is possible that the same phenomenon as with TMV-RNA, *e.g.* a 'leaky' termination codon, is also observed here.

All data together suggest that, like TMV, the RNA of TYMV generates (subgenomic) monocistronic messengers that are translated in the plant cells into the functional virus specific proteins. The difference with TMV (except probably for the cowpea strain of TMV, where smaller RNAs occur in small rods) is that these subgenomic mRNAs can be encapsidated. Whether or not this points to the direction of separate replication of

these RNAs can not be concluded yet.
One of the viruses of which the subgenomic mRNAs are encapsidated and present in the particles of the virus preparation is BMV. BMV consists of three nucleoprotein particles containing four RNAs. The two large RNAs (RNA1, MW 1.09×10^6 and RNA2, MW 0.99×10^6) are separatly encapsidated and the two others (RNA3, MW 0.75×10^6 and RNA4, mol. wt 0.28×10^6) are encapsidated in one component. RNA4 is not needed for succesful infection, yet it is regenerated in cells infected with the other three RNAs. RNA3 contains the coat protein cistron and has part of its sequence identical to the complete sequence of RNA4 (Shih, Lane and Kaesberg, 1972). When total BMV-RNA or an equal mixture of all four RNAs was translated in a wheat embryo extract, the major product synthesized was identified as authentic coat protein (Shih and Kaesberg, 1973). It was proven that RNA4 acts as monocistronic messenger for BMV coat protein. When this RNA4 is present in translation mixtures under standard conditions is prevents the translation of the other RNAs. At the time when these first investigations took place it was not known if the incubation conditions were favorable for the translation of the two larger RNAs. But inhibition or preferential translation was shown to be real for RNA3. When RNA3 is translated *in vitro*, the major product is a 34,000 mol. wt polypeptide (Davies, 1976). When RNA4 is added together with RNA3, the translation into the 34,000 decreases and coat protein is the major product (Shih and Kaesberg, 1973; Davies, 1976).
The 34,000 mol. wt product has no sequences in common with coat protein and could not be acetylated *in vitro* as could the coat protein (Shih and Kaesberg 1973). Therefore it was concluded that this polypeptide was synthesized under direction of RNA3, whereas the coat protein ciston present on this RNA has a 'closed' initiation site. It appears that RNA4, the functional (monocistronic) coat protein messenger arises by pretranslational cleavage or partial replication from RNA3. This also explains why RNA4 is not necessary for infection and is regenerated in cells infected with the three other RNAs.
For the *in vitro* translation of RNA1 and RNA2 the incubation conditions had to be altered. For translation of RNA1 suboptimal (in respect to the amino acid incorporation) RNA concentrations favored the synthesis of one large polypeptide; RNA2 translation *in vitro* was favored by a K^+ shift towards higher concentrations and yields also a large polypeptide (Shih and Kaesberg, 1976). The molecular weights of these two polypeptides are about 100,000 (Davies, 1979). Both products differ in their tryptic peptide pattern, suggesting two clearly different polypeptides. Comparing the molecular weight of the polypeptides with that of the RNAs, it can be concluded that almost full-size translation occurs (Shih and Kaesberg, 1976).
Taking all these results together it can be concluded that RNA1, RNA2 and RNA4 are monocistronic messengers and that RNA3 is dicistronic, but only one initiation site (at the 5'-end of the molecule) can be used. From this RNA3 the coat protein messenger RNA4 — located towards the 3'-end of the RNA3 — is generated.
Similar to BMV-RNA, in respect to their translation strategy is the RNA of AMV. This virus has also two large RNAs RNA1, 1.3×10^6 d and RNA2, 1.0×10^6d) and two smaller RNAs (RNA3, 0.8×10^6 d and RNA4, 0.3×10^6 d) all seperately encapsidated. As with BMV-RNA, the RNA4 of this virus acts as monocistronic messenger for coat protein (Davies, 1979; Mohier *et al.*, 1975; Van Vloten-Doting *et*

al., 1975; Thang *et al.*, 1975). RNA3 has the coat protein gene, but is *in vitro* translated mainly into a polypeptide of about 35,000 molecular weight and coat protein is hardly detected. In some cases however, initiation at this internal initiation site on AMV-RNA3 occurs, but this is probably an *in vitro* artefact, perhaps a result of the incubation conditions during translation. In some cases a read-through product from RNA3 can be synthesized *in vitro*, revealing a 65,000 molecular weight polypeptide which can be precipitated with anti-TMV serum, showing that it contains coat protein sequences (Van Vloten-Doting, 1976). The AMV-RNAs 1 and 2 are translated into large polypeptides corresponding to the translation of almost the entire lenght of the RNAs, behaving like monocistronic messengers (Mohier *et al.*, 1976; Rutgers, 1977; Van Tol and Van Vloten-Doting, 1979). Since all four RNAs seem to have only one initiation site as determined by Gerlinger *et al.* (1977) it can be concluded that all these RNAs behave like monocistronic messengers, the same situation as with BMV.

Purified CMV contains four major RNA species of molecular weight of respectively 1.35, 1.16, 0.85 and 0.35 $\times$ 10^6, designated as RNA1, 2, 3 and 4. The three large RNAs are necessary for infection, whereas RNA4 carries the gene for coat protein (Schwinghammer and Symons, 1975; Schwinghammer and Symons, 1977). RNA3 also carries the gene for coat protein as has been shown by genetic experiments (Habili and Francki, 1974) and sequence homology studies (Gould and Symons, 1977).

In the different translation systems used, *e.g.* wheat germ extracts, reticulocyte lysates and oocytes, it seems that the RNA3 and 4 can produce read-through products dependent on the system used (Schwinghammer and Symons, 1977). Despite these uncertainties about the results, it is clear that the RNAs 1, 2 and 4 of CMV act as monocistronic messengers, coding for polypeptides of respectively 120,000, 105,000 and 24,500, the last identified as coat protein.

RNA3 is here also a dicistronic messenger with one 'open' initiation site on the gene, coding for a 34,000 molecular weight polypeptide and a closed initiation site for the coat protein gene. The translation strategy for CMV-RNA is very similar to that of the other multipartite genome RNA viruses, described here. Translation studies with the RNAs of CCMV and BBMV (two other tripartite genome RNA viruses) show the same results as described above for the tripartite viruses BMV, AMV, and CMV (Davies and Verduin, 1979; Davies, 1979).

This translation strategy, in which all 'translatable' messengers are monocistronic and from the dicistronic ones only the initiation site at the 5'-end is used, may well be operative for all tripartite genome RNAs.

As described above, the viruses that generate subgenomic mRNAs or those that have multipartite genome RNAs show a translation strategy that is based on the translation of monocistronic messengers. From the RNA molecules that have two or more cistrons on the RNA (like the RNAs 3 of the tripartite viruses or the intact RNAs of the monopartite viruses) only the first initiation site at the 5'-end is used. For most of these 'monocistronic acting' messengers a 'cap' structure at the 5'-terminus has been shown (Keith and Fraenkel-Conrat, 1975; Zimmern, 1975; Dasgupta *et al.*, 1976; Symons, 1975; Pinck, 1975; Klein *et al.*, 1976). Recently is was shown by Paterson and Rosenberg (1979a) that this 5'-end structure was required for the efficient translation of prokaryotic mRNA in an eukaryotic cell-free system. The same authors showed in a subsequent paper that when this cap-structure was added to polycistronic mRNA, translation was resticted to the first gene (Paterson and Rosenberg, 1979b). These data suggest that the cap-structure plays a role in the recognition of the (80 *S*) ribosome of the initiation site near the 5'-end of the messenger. Furthermore it seems that when this cap is present, the initiation site nearby is preferentially used. Such a mechanism explains why mRNAs, having more cistrons, still act as monocistronic messengers in eukaryotic cell-free systems. If a similar mechanism occurs for the translation of the polycistronic messengers, described above, this offers a regulation system for the multiplication of these plant viruses: first a nonstructural protein is synthesized from these RNAs. This protein then regulates the synthesis (partial replication) or the cleavage of the coat protein gene from the polycistronic RNA. This mechanism also offers an explanation for the fact that the coatprotein gene on the polycistronic RNA is not translated.

In contrast to the above described viruses is the translation strategy of the RNAs of the viruses of the second kind: CarMV, PMV and TNV. When the RNA of CarMV (molecular weight: $1,4 \times 10^6$) is translated in a wheat germ cell-free system, three discrete polypeptides of respectively 77,000, 38,000 and 30,000 molecular weight are synthesized (Salomon *et al.*, 1978). The 38,000 molecular weight polypeptide was identified as authentic coat protein by gel electrophoresis, immunoprecipitation with antibodies against disrupted virus particles and peptide mapping. By peptide mapping analysis and the absence of cross-reaction of the antibodies with the two other polypeptides it could be concluded that the three polypeptides are encoded on three different cistrons on the CarMV-RNA. These observations might indicate that the genome of CarMV acts are as a polycistronic messenger in eukaryotic cell-free protein synthesis. Another monopartite RNA virus with a similar translation strategy as CarMV is PMV. It has an RNA with a molecular weight of about 2×10^6. When this RNA is translated in wheat germ extracts it directs the synthesis of several discrete polypeptides of which one was identified as coat protein (Bendena *et al.*, 1979). When the 5'-terminus was completely masked by way of limited *in vitro* reconstruction, coat protein was still synthesized when this nucleoprotein was used as messenger in a wheat germ cell-free extract. This implies that the coat protein was synthesized from an internal initiation site. The fact that this coat protein is also synthesized when 'naked' RNA is used as messenger excludes the possibility that this initiation site is normally closed and can only be made accessable when coat protein attachment to the RNA alters the secondary structure of the PMV-RNA. This suggests that PMV-RNA functions as a polycistronic messenger.

Another plant virus of which it has been suggested that the genome RNA acts as polycistronic messenger is TNV. *In vitro* translation of this RNA (1.4×10^6 d) in wheat germ extracts yields predominantly coat protein, but besides that two polypeptides (molecular weights 63,000 and 43,000) are synthesized in minor amounts (Salvato and Fraenkel-Conrat, 1977). The existence of subgenomic messengers (present in the RNA preparation used or in the wheat germ extract generated) can not be excluded in this case. In infected tobacco leaves replicative forms of such smaller TNV specific RNAs were found (Condit and Fraenkel-Conrat, 1979).

In the cases described above it could be concluded that the RNAs of CarMV, PMV and TNV function as polycistronic messengers in eukatyotic cell-free systems.

However the possibility that *in vivo* subgenomic messengers are generated or that the RNAs posses hidden breaks or are cleaved in the cell-free systems can not be ruled out completely. Furthermore it is necessary that more than one initiation site on the RNA is proven to exist and that read-through mechanisms are excluded. In the case of TNV-RNA it has been shown that the 5'-terminus has a diphosphate (Lesnaw and Reichman, 1970) which in view of the described mechanism for prokaryotic mRNAs (Paterson and Roberts, 1979a, 1979b) might indicate that this RNA can function as polycistronic messenger. PMV however, with a cap structure at the 5'-end does not fit in this picture. (Abou Haidar and Bancroft, 1978)

A plant virus that at this moment can not be classified in one of the three kinds of viruses described is tobacco rattle virus (TRV). TRV has a genome that consists of two RNA molecules, and is the typemember of the tobravirus group. The larger RNA, called RNA1 has a molecular weight of 2,5 $\times$ 10^6. The sizes of the smaller RNA (RNA2) are different for the different strains of the virus and vary from 1.1 $\times$ 10^6 (for strain PRN) to 0.65 $\times$ 10^6 (for the CAM strain). The RNA2 of the CAM strain directs the synthesis of coat protein in cell-free systems from animal cells (Ball *et al.*, 1973) as in wheat germ extracts (Mayo *et al.*, 1976). The RNA2 of the PRN strain can also be translated into coat protein, using wheat germ extracts and rabbit reticulocyte lysates; however with this RNA2 an additional polypeptide of higher molecular weight is also observed (Fritsch *et al.*, 1977). Altering the Mg^{2+} concentration changed the relative proportions of each polypeptide synthesized. Since some tryptic peptides were similar, it was suggested that there must be overlapping genes for these polypeptides with two 'open' initiation sites (Fritsch *et al.*, 1977). However a read-through mechanism offers a good explanation for these results, also in view of the dependence of the relative proportions of the polypeptides on the ionic conditions (Mg^{2+} concentration) of the translation mixtures. A similar situation occurs when RNA1 of the PRN stain was translated in wheat germ extracts containing spermidine or in rabbit reticulocyte lysates: two polypeptides are synthesized, molecular weights about 170,000 and 140,000 (Fritsch *et al.*, 1977). Also in this case the two polypeptides are synthesized from overlapping genes. Whether there are two 'open' initiation sites or two termination sites or another mechanism exists for the synthesis of these two polypeptides remains to be seen. It seems thus that TRV, at least the PRN strain shows a translation strategy quite different from the other plant viruses: one RNA is translated into large polypeptides and the smaller one possibly serve like a polycistronic mRNA. So in one respect (*e.g.* the smaller RNA) this virus shows analogy with the viruses described above, and on the other hand (the large RNA) the translation strategy shows analogy with the bipartite viruses from the nepovirus group and CPMV (as described below). However recent results of translation of TRV-RNAs in nuclease treated reticulocyte lysates suggest that the 170,000 molecular weight polypeptide is a read-through product of a leaky termination codon at the end of the smaller protein (Pelham, 1979b). Furthermore a 0.55 $\times$ 10^6 d RNA was found in the TRV (PRN strain) preparation which coded for a 31,000 molecular weight protein, which is probably the same as the 30,000 molecular weight protein, described by Fritsch *et al.* (1977) and has been shown to be, to be distinct from coat protein. This RNA3 is probably also located at the 3'-end of the large TRV-RNA. Pelham (1979b) suggests that the 30,000 protein is not synthesized from an internal initiation site on RNAs, but is the result of the monocistronic translation of a third RNA present in the TRV preparation, and is encapsidated. If this translation strategy for TRV is correct, this virus can be classified among the viruses of the first kind, showing a striking resemblence to TMV and TYMV.

One of the viruses of the third kind, having a bipartite genome RNA, is the nepovirus group of which tobacco ringspot virus (TRSV) is the typemember. Tomato black ring virus (TBRV) is one of the members of this group from which the translation of the RNAs has been studied. The viruses are in many respects very similar to CPMV. TBRV also has three isometric particles: an empty top component, a middle component containing a 1.5×10^6 molecular weight RNA and a bottom component containing an RNA of 2.5×10^6 molecular weight. There is evidence that TRSV contains a protein covalently linked at the 5'-terminus of both RNAs (Harrison and Barker, 1978; Mayo *et al.*, 1979b) and that several nepoviruses including TBRV and TRSV contain a polyA strech at the 3'-end of the RNA molecules (Mayo *et al.*, 1979a). The two RNAs of TBRV are translated *in vitro* (wheat germ extracts and rabbit reticulocyte lysates) into products with a maximum molecular weight of 220,000 (RNA1) and 160,000 (RNA2) (Fritsch *et al.*, 1978). The 160,000 molecular weight product reacted with antiserum against TBRV particles, whereas the the translation product of RNA1 dit not. This suggests that the RNA2, containing the coat protein gene, is translated into a large precursor protein from which the coat protein must be generated by a mechanism of 'post-translation' cleavage (Fritsch *et al.*, 1979).

CPMV-RNAs contain no m^7GpppNp cap, but posess a protein covalently linked to their 5'-end (Klootwijk *et al.*, 1978), similar to the RNAs of the nepovirus TRSV and the picorna virus group (Mayo *et al.*, 1979b; Lee *et al.*, 1977; Flanegan *et al.*, 1977; Hruby and Roberts, 1978; Sangar *et al.*, 1977) Whether this protein moiety is cleaved off in the host cell after infection, as found for polio virus (Nomoto *et al.*, 1977; Ambros *et al.*, 1978) or is also present in the mRNA of virus specific polyribosomes is not known. Since cap analogs as m^7Gp nor the removal of this protein seems to inhibit the translation of CPMV-RNA (Stanley *et al.*, 1978) it is conceivable that neither the messenger is being capped nor that the protein plays a role in translation. The latter is also suggested by the observation that the *in vitro* translation products are not altered when the protein is removed from the RNA (Stanley *et al.*, 1978). The structure of the CPMV-RNAs is in many aspects very similar to that of the RNAs of the picornavirus group and that of the nepovirus group. Nepovirus RNAs are probably translated into large polypeptides corresponding to (almost) the entire coding capacity of the RNAs, as described above. Poliovirus RNA (as example of the picornavirus group) is translated into large precursor proteins from which the virus specific proteins are cleaved after translation (also) *in vitro* (Shih *et al.*, 1979). If these similarities in RNA structure are reflected in the translation strategy as is suggested by the division of plant (plus-strand) RNA viruses in three kinds, is discussed in this thesis. Part of the work is also described in two papers (Pelham and Stuik, 1976; Davies *et al.*, 1977).

I.3. Protein synthesis in virus-infected plant cells

It is clear from the problems described above with the identification of polypeptides synthesized *in vitro* under direction of plant virus RNA, that investigations *in vivo* are necessary to verify the nature of the *in vitro* products. Most of the work concerning the search for virus specific proteins in infected plant cells has been performed with TMV. In 1971, Zaitlin and Hariharasubramanian reported that TMV-infected tobacco leaves synthesize several species of high molecular weight proteins in addition to viral coat protein. Using similar methods Singer (1971) however failed to detect these high molecular weight proteins, except when special infiltration techniques were used for the radioactive labelling of the proteins in the infected and uninfected leaf tissue. Although no specific radioactivity data of proteins are shown in those reports, it is clear from the gel analysis data that a very low amount of the added radioactive amino acids was incorporated into proteins in the leaves. The results of these analyses are therefore rather doubtful. A vast number of 'virus-related' proteins are reported in these publications (Zaitlin and Hariharasubramanian, 1971; Singer, 1971; Singer and Condit, 1974).

Studies with tobacco protoplasts showed that in this system proteins with molecular weights of approximately 130,000 and 160,000 could be detected after inoculation with TMV (Sakai and Tabeke, 1974; Paterson and Knight, 1975; Siegel *et al.*, 1978). Studies of Huber (1979) however showed that the so called 160,000 molecular weight protein could only be found when tobacco protoplasts were infected with TMV and not when the *Vigna* protoplast system was used. Furthermore it was shown that healthy tobacco protoplasts also contain this 160,000 protein, which indicates that this protein is rather a host protein, induced by the virus infection, than a TMV-specific protein (Huber, 1979). For the 130,000 protein it was proven that this protein is viral RNA coded (Scalla *et al.*, 1978; Huber, 1979) by comparing the *in vitro* synthesized polypeptide with the *in vivo* found protein.

The cowpea strain of TMV (C_CTMV) causes, besides the 130,000 molecular weight protein and coat protein, the synthesis of a 30,000 protein (Huber, 1979) which was also synthesized *in vitro* using C_CTMV-RNA with a smaller molecular weight than the intact genome RNA (Bruening *et al.*, 1976). Furthermore another protein with a molecular weight of about 72,000 was found after infection of either tobacco or *Vigna* protoplasts with C_CTMV (Huber, 1979). The translation strategy of TMV was elucidated by *in vitro* translation experiments using the different (C_C)TMV-RNA molecules as messengers as described above. The comparison of the *in vivo* and the *in vitro* results made this elucidation possible.

In tobacco plants infected with TNV, five 'virus-related' proteins, apart from coat protein, could be detected (Jones and Reichman, 1973), using the same techniques as Zaitlin and Hariharasubramanian (1971). Comparison with *in vitro* studies (Salvato and Fraenkel-Conrat, 1975) with TNV-RNA suggests that the three largest proteins found could be viral RNA coded *e.g.* the 63,000, 43,000 molecular weight proteins and coat protein. This again demonstrates that the comparison between the *in vivo*

synthesized polypeptides is necessary. It illustrates also that (as with TMV) care must be taken in assigning proteins only found in infected tissue as virus-specific before proof from *in vitro* translation studies is available.
In barley plants Hariharasubramanian *et al.* (1973) detected a protein of about 35,000 molecular weight after infection with BMV, a polypeptide also synthesized *in vitro* when RNA3 was translated in wheat germ extracts (Shih and Kaesberg, 1973). Working with tobacco protoplasts infected with BMV, Sakai *et al.* (1979) could detect, apart from coat protein, the same 35,000 molecular weight protein as mentioned above and also two large proteins that, within the limits of the gel electrophoresis techniques have a similar molecular weight as the *in vitro* translation products synthesized under direction of BMV-RNA1 and RNA2 (Shih and Kaesberg, 1976).
Also in tobacco protoplasts, virus specific proteins could be detected after infection with CCMV (Sakai *et al.*, 1977), being indentified as viral RNA coded by means of *in vitro* translation studies (Davies and Kaesberg, 1974; Davies and Verduin, 1979).
In this thesis it is described that in cowpea plants infected with CPMV, a high molecular weight protein (molecular weight approximately 170,000) can be detected apart from the coat proteins. In another thesis (Rottier, 1980) more virus specific proteins are described, all detected by working with the *Vigna* protoplast system. These proteins are compared with the polypeptides synthesized under direction of the CPMV-RNAs in eukaryotic cell-free systems.

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II MATERIALS AND METHODS

II.1. Growth and purification of CPMV

Cowpea mosaic virus (CPMV) was propagated in *Vigna unguiculata* (L.) Walp. var. Blackeye early Ramshorn. The plants were grown in a greenhouse and 9-12 days after sowing the primary leaves were inoculated with crude sap from leaves infected with CPMV. The CPMV used was the (Nigerian) virus isolate maintained in Wageningen (Van Kammen and De Jager, 1978). After inoculation the plants were kept in the greenhouse for another 10-14 days or transferred to a growth chamber and further grown at 30° with 75% relative humidity and continuous light (12,000 to 18,000 lux) for another 5-6 days. After these periods the primary leaves showed the typical chlorotic spots and the secondary leaves showed the mosaic symptoms, characteristic for the virus. The primary leaves were then harvested and CPMV was isolated by the PEG-NaCl method as described by Van Kammen (1967) and further purified by sedimenting through a 2.5 ml 40% (w/w) sucrose cushion in a Beckman Type 30 rotor at 30,000 rpm for 5 hrs at 4° C. The pellet of purified virus was resuspended in Tris-phosphate buffer (10 mM Na-phosphate in 15 mM Tris-HCl, pH = 8.8).

Separation of the components

The components of the virus were separated by CsCl equilibrium density gradient centrifugation. About 20 mg CPMV was layered on a CsCl gradient consisting of 8 ml 42%, 8 ml 49%, 8 ml 57% and 8 ml 65% CsCl in Tris-phosphate or Tris-HCl-buffer (pH = 8.8) and the gradient was centrifuged at 25,000 rpm in a Beckman SW27 rotor for 19 hrs at 4° C. The pH of 8.8 was chosen to get optimal separation between the M- and B-components (described by Geelen, 1974). The visible bands of separated M- and B-components were collected with a pasteur pipet and dialysed for 12 hrs against 15 mM Tris-HCl, pH = 8.8, with several changes. If necessary the CsCl centrifugation was repeated for further purification of the M- or B-component.

II.2. *In vivo* labelling of plant and viral proteins

Three types of *in vivo* labelling experiments were performed.

1. Experiments in which detached leaves were used. The plants were grown in a greenhouse and the primary leaves were inoculated as described above. The primary leaves were, at the indicated time, detached from the plants and cut comb-wise with a razor blade. The leaves were placed in petridishes (ϕ 19 cm) containing 20 ml phosphate buffer (0.01 M NaH_2PO_4 and 0.01 M Na_2HPO_4, pH = 7.0) with 10^{-6} M kinetine. Tritiated amino acids were added to the medium in the indicated amounts. Incubation was performed in a growth chamber under continuous light at 30° C and 75% relative humidity for 48 till 96 hrs, depending of the experiment. Phosphate buffer, containing kinetine, was administred during the incubation to avoid drying of the leaves.

2. Experiments in which plants were cut across the stem.

Vigna plants were grown and inoculated with CPMV as described above. At the indicated times the plants were cut across the stem and the secondary leaves were removed. The cuttings were separately placed in tubes, with with the cut stem immersed in 5 ml phosphate buffer containing 10^{-6} M kinetine. ^{35}S-methionine was added as indicated and the tubes were placed in a growth chamber and incubated as described for the detached leaves.

3. Experiments using whole plants.

Cowpea seeds were germinated in moisted Vermiculite (0.5 l H_2O per 1 Vermiculite) in the dark at 25° C for two days. About 15 seedlings were placed in pots containing 6 l Hoagland solution. The pots were put in a growth chamber and the plants were grown for 7 days with 14 hrs at 28° C in the light (10,000 to 18,000 lux) and 10 hrs at 22° C in the dark. About 10 plants were then transferred to smaller pots containing 50 ml Hoagland solution from which all sulphate compounds were omitted, and grown for another two days. After (mock-) inoculation the plants were put in a growth chamber and further grown as described. At the appropriate times for the experiments, the secondary leaves were removed and $^{35}SO_4^{2-}$ (0.5 mCi per plant) was injected into the culture medium.

II.3. Fractionation of *Vigna* leaf homogenates

Fractionation was performed as described by De Zoeten *et al.* (1973) and Assink (1974) with some modifications. The primary leaves were harvested and the midribs were removed. The leaf material was homogenized by chopping with a Yeda tissue chopper in a petridish containing 2 ml Honda buffer (0.25 M sucrose, 2.5% Ficoll, 5% Dextran T40 and 1 mM PMSF) per gram leaf tissue. The homogenate was pressed through two layers of Miracloth and the filtrate was centrifuged 15 min at 1,000 × *g* at 4° C. The 1,000 × *g* pellet was resuspended in a small volume of Honda buffer and portions of 2 ml were layered on discontinuous sucrose gradient consisting of 5 ml 60% (w/w), 15 ml 45% (w/w) and 10 ml 20% (w/w) sucrose in gradient buffer (10 mM Tris-HCl, 1 mM $MgCl_2$ and 1 mM PMSF; pH = 8.2). The gradient was centrifuged 2 hrs at 22,000 rpm in a Beckman SW27 rotor at 4° C. After this centrifugation, two green bands were visible on the boundaries of the different sucrose solutions of the gradients. The thick green band occurring on the boundary between the 20% and 45% sucrose layers, which is referred to as the chloroplast fraction, was collected with a pasteur pipet, diluted with gradient buffer and centrifuged 30 min at 18,000 × *g*, 4° C. The sediment of the chloroplast fraction was resuspended in Honda buffer and in portions of about 2 ml layered on discontinuous sucrose gradients consisting of 5 ml 45% (w/w), 5 ml 43% (w/w), 10 ml 41% (w/w), 7ml 39% (w/w) and 5 ml 37% (w/w) sucrose in gradient buffer. These gradients were centrifuged 15 min at 22,000 rpm in a Beckman SW27 rotor at 4° C. After this centrifugation green material was present in bands on the four boundaries of this gradient. The material on the boundary between the 37% and 39% sucrose (fraction I), 39% and 41% (fraction II) and between 41% and 43% combined with that between 43% and 45% (fraction III) was collected, diluted with gradient buffer and sedimented by centrifugation, 30 min at 18,000 × *g*, 4° C. After washing two times with gradient buffer, followed by sedimentation (30 min centrifugation at 18,000 × *g*) the resulting pellet was resuspended in TGSP-buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS and 1 mM PMSF: pH = 8.3) and stored at -20° C.

Replicase assay of subcellular fractions

For replicase activity tests, the pellets were resuspended in about 1 ml R-buffer (50 mM Tris-HCl, pH = 8.2, 25% glycerol, 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM DTE and 0.5 mM PMSF), giving a concentration of about 1 mg protein per ml, and immediately tested according to Zabel *et al.* (1974). The assay mixture (total volume of 0.240 ml) contained 0.05 M Tris-HCl, pH = 8.2, 10% glycerol, 0.01 M $MgCl_2$, 0.025 M KCl, 0.013 M $(NH_4)_2SO_4$, 0.001 M EDTA, 0.25 μM each of ATP, GTP and CTP, 0.01 μM of UTP, 5 μci of ^{3}H-UTP (specific activity 12 to 14 Ci/mmol), 1 μmol of phosphoenol pyruvate, 10 μg of pyruvate kinase, 5 μg of actinomycin D, 0.004 M DTE, 25 μg of CPMV-RNA and 25 μl of the resuspended pellet. Assay mixtures were incubated for 30 min at 22° C and the reaction was terminated by the addition of 3 ml ice-cold 10% TCA containing 4% $Na_2P_2O_7$ and 4% NaH_2PO_4. After the addition of bovine serum albumine (350 μg per sample) the mixture was left on ice and then the acid insoluble precipitates were collected on Whatman GF/A filters, washed five times with 5% TCA containing 2% $Na_4P_2O_7$ and 4% NaH_2PO_4, five times with 1 N HCl containing 0.1 M $Na_4P_2O_7$, twice with 80% ethanol and finally with ether. The filters were treated with 0.75 ml of a soluene-water mixuture (9:1) for two hrs at 50° C to solubilize the precipitates and were subsequently counted with 10 ml of toluene-permablend scintillation cocktail containing acetic acid (2 ml/l). Under these conditions the counting efficiency for tritium was 40% in a Tricarb scintillation counter.

II.4. RNA preparation

CPMV-RNA

Two different methods for the preparation of CPMV-RNA were used. The most used procedure was the standard phenol extraction method. This method was used when RNA was extracted from separated virus components. The purified particles were disrupted by addition of an equal volume detergent mixture (0.1 M glycine-NaOH, pH = 9.0, 0.1 M NaCl, 0.01 M EDTA, containing 4% sarkosyl NL 97, 1% SDS and 2% Na-triisopropylnaphtalene sulphonate) followed by 2 min incubation at 60° C. The solution was then extracted once by a phenol-chloroform mixture (1:1) saturated with Tris-HCl, pH = 9.0. The water layer with the RNA was extracted twice with Tris-buffer saturated phenol. The RNA was precipitated by adding two volumes of ethanol in the presence of 0.25 M Na-acetate. The pellet of precipitated RNA was washed with ethanol and dried. The dried RNA pellet was dissolved in double-destilled water. CPMV-RNA was also isolated from purified virus by the method of Klootwijk *et al.* (1977). After disruption of the virus particles, as described above, the mixture was layered in portions of about 2 -4 mg RNA onto 34 ml linear 15-30% (w/v) sucrose gradients in TNES buffer (0.01 M Tris-HCl, pH = 7.2, 0.1 M Nacl, 0.01 M EDTA and 0.5% SDS). Centrifugation was performed in a Beckman SW27 rotor at 22,500 rpm for 16 hrs at 20° C. After this centrifugation the separated B- and M-RNA were collected and precipitated and treated as described above. If necessary the B- or M-RNA was further purified by repeating the sucrose gradient centrifugation procudure.

tRNA

Mouse liver tRNA was a gift from Hugh Pelham. Transfer RNA was prepared from wheat germ or *Vigna* leaves. The homogenate of *Vigna* leaves (prepared as described above) was centrifuged 15 min at 30,000 × *g*. The 30,000 × *g* supernatant was centrifuged for 4 hrs at 40,000 rpm in a Beckman Ti75 rotor to remove the ribosomes. The supernatant was extracted several times with phenol, saturated with TNES-buffer. After precipitation with ethanol (containing Na-acetate), the RNA pellet was washed twice with ethanol, dried and dissolved in a buffer containing 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH = 7.5 and 0.5 mM DTE (Smish-buffer). The supernatant of centrifugation in a Homef centrifuge was layered on a Sephadex G-50 column, equilibrated with Smish-buffer. The column was eluted with Smish-buffer and the void-volume containing the tRNA was collected. The RNA was precipitated, washed with ethanol, dried and dissolved in double-destilled water. tRNA from wheat germ was prepared by the same method, starting from a 'S-30' wheat germ extract (see II.6), that was centrifuged to remove the ribosomes.

II.5. Preparation of and translation in the messenger-dependent lysate (MDL)

Preparation

Reticulocyte lysates were prepared according to Hunt and Jackson (1974). Rabbits weighing 2 to 3 kg were made anaemic by daily subcutaneous injections of 0.9 ml/kg of a 1.25% solution of acetyl-phenyl hydrazine in saline (0.13 M NaCl, 5 mM KCl and 7.5 mM $MgCl_2$) over a period of 4 days. Five days after the last injection the rabbits were killed and bled. The blood was collected in tubes (kept on ice) containing heparin as anti-coagulant. Reticulocytes were sedimented by centrifugation 1,000 × *g* for 10 min at 4° C. They were washed three times with ice-cold saline and lysed by addition of 1.5 volumes of ice-cold water per volume of packed cells. The debris was removed by centrifugation at 30,000 × *g* for 15 min at 4° C. The supernatant was stored in 1-2 ml aliquots under liquid nitrogen. The procedure for making this lysate messenger-dependent was as described by Pelham and Jackson (1976).
An aliquot of 1 ml lysate, which had been stored under liquid nitrogen, was supplemented immediately on thawing with 15 μl haemin (1 mM, in 90% ethylene glycol, 20 mM Tris-HCl, pH = 8.2) and 10 μl creatine kinase (5 mg/ml in 50% aqueous glycerol). To 800 μl of this lysate was added 50 μl KM (2 M KCl and 10 mM $MgCl_2$), 50 μl creatine phosphate solution (0.2 M), 50 μl amino acid mixture (1 mM of each amino acid, excluding methionine), 10 μl $CaCl_2$ solution (100 mM) and 10 μl Micrococcal nuclease (16,000 U/ml). After 10 min incubation at 20° C, 20 μl EGTA solution (100 mM) was added to inactivate the nuclease activity. The messenger-dependent lysate was put on ice when immediately used for translation experiments or frozen under liquid nitrogen in 200 μl aliquots.

In vitro translation in MDL

For the *in vitro* translation experiments with MDL the only further additions were mRNA and heterologous tRNA, in the appropriate amounts for the experiments, and ^{35}S-methionine which was usually added in a concentration of 10% (v/v). Following incubation at 30° C, 5 μl samples were withdrawn and added to 0.5 ml of distilled water. To this was added 0.5 ml 1 N NaoH containing 0.5 M H_2O_2 (to decolourize the sample) and roughly 1 mg/ml methionine. After incubation at 90° C for 5 min, protein was precipitated by the addition of 1 ml 25% TCA. The precipitate was collected by filtration on glass fibre discs (Whatman GF/C), washed twice with 5% TCA, twice with ethanol, once with ether and dried. Radioactivity was determined in a Packard Tricarb scintillation counter.

II.6. Preparation of and translation in wheat germ extract

Preparation

Wheat germ (General Mills, Vallejo, Cal.) was floated on a mixture of CCl_4 and cyclohexane (500 : 150 ml) and dried on filter paper. Obvious non-embryo material was removed. One gram of dry embryo was ground with crushed glass for a few seconds in a mortar. After addition of 2 ml homogenization buffer (120 mM KAc, 5 mM $MgAc_2$, 1 mM DTE and either 5 mM HEPES or 6 mM $KHCO_3$) this was ground for 2 min and a further 8 ml of homogenization buffer was added for further extraction. The homogenate was centrifuged 10 min at 30,000 × *g* at 4° C. The pH of the homogenate should be 6.4-6.8 (as checked on the supernatant after the 30,000 × *g* centrifugation). If the pH is lower, preparations of reduced activity are obtained. A higher pH results in release of endogenous messenger, giving correspondingly less dependence on the exogenous mRNA. Adjustment of the pH, if necessary, is done by increasing or decreasing the HEPES or $KHCO_3$ molarity in the homogenization buffer (Marcus *et al.*, 1974). To this supernatant 1/50 × volume 500 mM HEPES-KOH (pH = 7.6) was added and the extract was centrifuged again at 30,000 × *g* for 10 min at 4° C. The resulting (clear) supernatant was dialyzed for 16-24 hrs against 1 l of buffer containing 120 mM KAc, 5 mM $MgAc_2$, 20 mM Tris-Ac (pH = 7.6) and 1 mM DTE, changed after 12 hrs. Small aliquots of the final wheat germ 'S-30' extracts were rapidly frozen in liquid nitrogen and stored at -70° C or in liquid nitrogen. *In vitro* translation with wheat germ extracts.
The incubation mixture (100 μl) for translation contained 50 μl S-30, 20 mM HEPES (pH = 7.6 with KOH), 10 mM Tris (pH = 7.6, with acetic acid), 0.025 mM of each amino acid (exept for the radioactive one), 2.5 mM ATP (K^+-salt), 0.375 mM GTP (tri-Li-salt), 10 mM creatine phosphote (Tris-salt), 0.5 mM DTE; further more 3.0 mM Mg^{2+}, 0.4 mM spermidine (HCl), 130 mM K^+ (120 mM as KAc and approx. 10 mM from KOH and ATP) and 20-70 μg/ml mRNA depending on the type of experiment. The radioactive amino acids used were ^{35}S-methionine or ^{3}H-leucine. Following incubation at 30° C, 5 μl samples were spotted on Whatman 3 MM paper discs. The discs were washed batch-wise one time with 5% TCA containing about 1 mM methionine or leucine at 90° C, two times with cold TCA, two times with ethanol and two times with ether. After drying the filters, radioactivity was determined in a Pacard Tricarb scintillation counter.

II.7. Polyacrylamide gel electrophoresis

Preparation of the samples

The subcellular fractions (described in II.3.) containing about 2 mg protein per ml were thawed and mixed 1 : 1 with 2 × sample buffer. The composition of the sample buffer is: 1 ml Tris-HCl (0.5 M), pH = 6.8, 0.2 gram SDS, 0.1 ml β-mercaptoethanol, 4 ml glycerol, 0.001 gram bromophenol blue and 4.9 ml H_2O. The mixtures were heated at 100° C for 10 min and centrifuged 10 min in an Eppendorf centrifuge, to remove any debris. From the incubation mixture of MDL, a 5 μl sample was withdrawn and mixed with 55 μl sample buffer and heated 10 min at 100° C. After centrifugation in an Eppendorf centrifuge the total amount of the supernatant was used for gel electrophoresis. The incubation mixtures with wheat germ extracts were mixed with an equal volume of 2 × sample buffer and heated for 10 min at 100° C. The supernatant of centrifugation in an Eppendorf centrifuge was used for gel electrophoresis.

Gel electrophoresis

Polyacrylamide gel electrophoresis of proteins extracted from *Vigna* leaves was performed on 10% polyacrylamide gels according to Laemmli (1970). Samples containing about 80-100 μg protein were layered on cylindrical gels. Electrophoresis was performed at 5 mA per gel until the bromophenol blue marker reached the end of the gel. Then the gels were frozen and sliced with a Mickle gel slicer in 1 mm slices. The slices were incubated 12 hrs at room temperature in 10 ml toluene-permablend scintillation liquid containing 0.75 ml soluene. Radioactivity was determined in a Packard Tricarb scintillation counter. Slab gel electrophoresis was performed in a Studier apparatus (Studier, 1973) with the buffer system according to Laemmli (1970) or with the phosphate buffer system according to Weber and Osborne (1969). In general 60 μl samples from MDL incubations (with the added sample buffer) were layered. This volume (5 μl of the original incubation mixture) was the maximum amount that could be layered on a gel slot, because with larger samples the big amount of globin present in the MDL would disturbe the electrophoresis as result of overloading the gel with to much protein. Samples up to 100 μl from incubation mixtures with wheat germ extract could be layered on the gels. Electrophoresis was performed at about 150 V until the bromophenol blue marker reached the end of the gel. The gels were stained with Coomassie brilliant blue, destained with an ethanol-acetic acid-water mixture (50:75:1000) and then cut lenght-wise. One half was dried down on Whatman 3 MM paper and exposed to Kodak-X-omat film for autoradiography.

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III. PROTEIN SYNTHESIS *IN VIVO*

III.1. Radioactive labelling of leaf proteins

Except for the two coat proteins (MW: 37,00 and 22,000) of CPMV, no other virus specific proteins were well characterized at the start of these investigations. Because the coat proteins are produced in vast amounts, starting from the third day after inoculation, these proteins can be easily detected in homogenates of CPMV-infected *Vigna* leaves, by various methods. From the work of Zabel (1978) it has become reasonable to suppose that the RNA-dependent RNA polymerase (replicase) enzyme, as a whole or in part, is encoded by the CPMV genome. Apart from these, no other proteins that may play a role in the virus multiplication were known. Since the genome of CPMV is about 3.4×10^6 d, there is a great possibility that there are more proteins encoded by these RNAs.

Proteins that play a catalytic role in the cell — and the virus specific non-coat proteins expected to exist are presumably such proteins — are usually present in low amounts. The concentrations of these proteins will probably be low and therefore not easy so distinguish among the leaf proteins, as the viral coat proteins are. Another difficulty is that in *Vigna* leaves, inoculated with CPMV, not all the cells are infected with virus and even after spreading of the virus infection a great deal of the leaf cells stay healthy and contribute to the synthesis of host proteins. Furthermore host protein synthesis can be enhanced as result of wound reaction processes, caused by the mechanical way of inoculation of CPMV. From the work with protoplasts, where about 90% of the cells are infected, we also know that in virus infected cells host protein synthesis is not inhibited by infection with CPMV (Rottier, 1980). Also the fact that cytopathic structures occur in CPMV infected cells (De Zoeten *et al.*, 1974) point out that some host protein synthesis processes are even enhanced by CPMV infection.

The presumed virus specific proteins, necessary for virus multiplication, are not easy to discover among the bulk of leaf proteins. Therefore methods of radioactive labelling of proteins, *de novo* synthesized during the replicative cycle of the virus, were used. Since the aim was to detect these proteins by comparing proteins from healthy and

infected *Vigna* leaves by means of polyacrylamide gel electrophoresis, it was necessary to obtain high specific radioactivity levels in the newly made proteins. Otherwise these proteins (present in very small quantities) would be not visible among the other more abdundant proteins, since there is a limited amount of protein that can be analyzed by gel electrophoresis methods (about 100 μg protein as maximum).
For radioactive labelling, we aimed at a period of time in which the expected virus specific non-coat proteins are synthesized and active in the virus synthesizing machinery. The multiplication of CPMV in *Vigna* leaves starts about one and a half days after inoculation. After 5 to 6 days the rate of virus multiplication decreases. Then the symptoms on the leaves are clearly visible and many virus particles are present (Van Kammen and de Jager, 1978). Replicase is detectable on day 1 after inoculation and increases rapidly to reach a maximum after 4 days, so proceeding the virus multiplication (Zabel *et al.*, 1974). From these facts it was reasonable to label between the first day after inoculation till 4 to 5 days after inoculation, since the processes of virus multiplication, in which we expect the non-structural virus specific proteins to be synthesized, take place then. Therefore this time-period was chosen for the administration of the radioactive protein precursors. Different radioactive protein precursors were investigated.

Tritiated amino acids

Amino acids, administered to plants by addition to nutrient solution, when plants are kept on water culture, will probably not reach the leaves in high quantities. Several pathways in the roots and stem and the internal pools of amino acids will prevent a high enough concentration of labelled amino acids *in* the leaves to give enough radioactive label into protein. Therefore several attempts were made to label leaf proteins by incubation of detached leaves or plants cut by the stem. The specific radioactivity of the viral coat proteins is in these experiments considered as representative for the *de novo* synthesized proteins, and thus also for the virus specific proteins for which we are looking.
For these experiments several types of incubation were used:
a) The leaves were detached and cut comb-shaped with a razor blade and incubated in petri dishes containing buffer and the radioactive amino acids.
b) Plants cut across the stem were placed with their stem in tubes containing buffer and label.
The results of these different types of incubation is shown in Table III.1. Defined conditions for obtaining optimum labelling of leaf proteins could not be derived from these determinations. The highest specific activity of virus reached in these experiments was 140,000 cpm/mg. This specific activity is by far not high enough to expect any succes in distinguishing the low amounts of *de novo* synthesized (virus specific) proteins of the multiplying virus from the normal plant proteins synthesized. Therefore it was concluded that this labelling method was not suitable.

TABLE III.1. LABELLING ATTEMPTS WITH TRITIATED AMINO ACIDS.

Added ^{3}H label μCi/gr. leaves	Start of labelling (days p.i.)	Labelling period (hours)	Total cpm in CPMV	Specific activity (cpm/mg CPMV)	Method used (see text)
5	1	96	215,000	< 10,000	detached leaves
17	1	72	418,000	50,000	detached leaves
7	2	48	461,000	51,000	detached leaves
4	3	48	452,000	33,800	detached leaves
20	4	60	175,000	140,000	detached leaves
10	1	72	115,000	38,000	cut across the stem
17	3	48	6,000	< 10,000	cut across the stem

^{35}-*methionine*

Since the specific radioactivity of ^{*5}S-methionine, commercially available, is much higher than that of tritiated amino acids, this was tried next as protein precursor. The first labelling attempt with ^{35}S-methionine was indeed more succesfull than in all previous experiments with tritiated amino acids. Two days after inoculation leaves were detached and, after cutting comb-shaped, incubated in petri dishes containing buffer and 15 μCi ^{35}S-methionine per gram leaf tissue. After 50 hrs incubation, the specific radioactivity of the isolated CPMV was 400,000 cpm per mg virus. This was a better result than the best labelling result with tritiated amino acids (140,000 cpm/mg). Despite the fact that the results of this labelling method with detached leaves seemed promising, the further investigations were performed with plants, cut across the stem, because of the following reasons: when detached leaves were incubated in petri dishes over a long period, the leaf material showed symptoms of aging (especially on the edges where they were cut with the razor blade). Also the cutting of the leaves might provoke to many wound reactions that could obscure the events as result of the viral infection. With plants cut across the stem these problems can be somewhat overcome and the natural situation is approximated. The specific activities of the proteins, labelled this way, were similar to those achieved with detached leaves.
Kinetics of uptake and incorporation was measured. Plants cut across the stem were put into tubes containing phosphate buffer with 10^{-6} M kinetine and 2.5 μCi ^{35}S-methionine. This was done one day after inoculation and every cut plant, with only the two primary leaves, was separately placed in a tube. At the indicated time, one infected and one healthy plant was taken out and of each plant the leaves were gathered and homogenized in a mortar containing 10 ml homogenization buffer. After pressing this homogenate through a double layer of Miracloth, the total radioactivity and the TCA precipitable counts in the sap were determined. A typical experiment is

shown in Fig. III.1. The time course was not identical in all experiments as in these examples shown here. The reason for these differences could be variation in uptake in the plants, notwithstanding the fact that plants of the same age were used. Also the mechanical way of CPMV or mock inoculation causes wounds on the leaves. Not all leaves are wounded to the same extent, so differences in wound reaction can give differences in the amount of ^{35}S-methionine that is taken up and/or incorporated. It is clear however, that the label is taken up and incorporated rapidly in the first 8 hrs. After this time the amounts of label in the leaves decreased. The reason for this phenomenon was not further investigated.

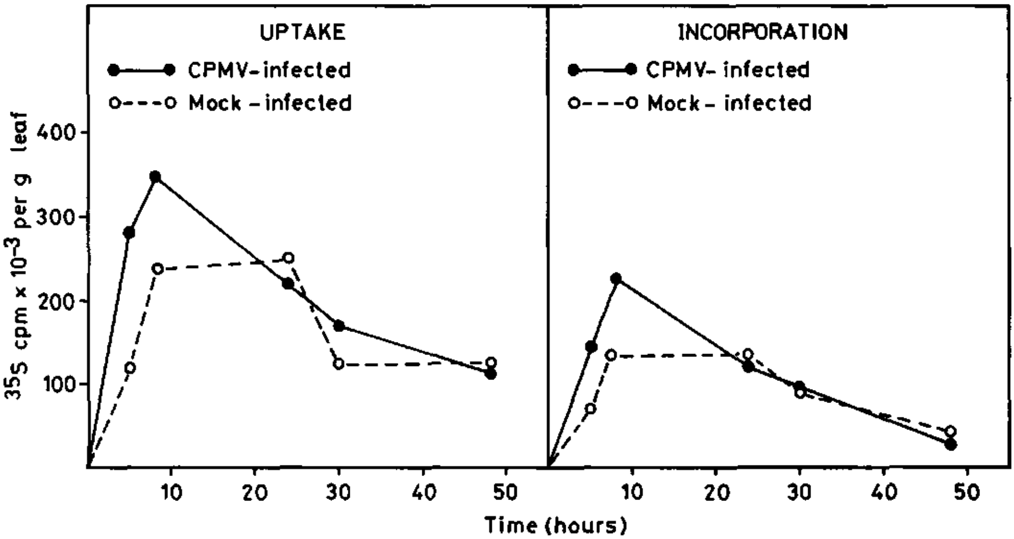


Fig.III.1. *Uptake and incorporation of ^{35}S-methionine into CPMV-infected and mock-infected Vigna leaves.*
One day after inoculation the plants were cut across the stem and placed in tubes containing phosphate buffer with 10^{-6} M kinetine and 2.5 μCi ^{35}S-methionine per plant. At the indicated times the primary leaves were detached and homogenized in a mortar. The homogenate was pressed through two layers of Miacloth. The total radioactivity (left panel) and the TCA precipitable radioactivity (right panel) in the sap was determined: •——• CPMV-infected leaves; o——o mock infected leaves.

Time periods of 8 hrs were chosen to determine the optimal time period after inoculation for labelling. Fig. III.2. shows the result of this experiment. This figure indicates that the uptake of ^{35}S-methionine is rather constant in the 8 hour periods starting with 24 hrs till 72 hrs after inoculation. The incorporation into TCA precipitable material is however maximal during the 8 hrs labelling period 24 hrs after inoculation and becomes lower in the later periods. Also in this experiment (not shown in the figure) the phenomenon, observed in the previous experiments, that after 8 hrs no more ^{35}S-methionine was incorporated, was investigated. About 26% of the input ^{35}S-methionine was incorporated into TCA precipitable material and 43% of the input was taken up by the leaves. So this means that about 60% of the methionine that reaches the leaves is incorporated into protein. When we assume that the other 40% is used in side reactions like methylation, this would mean that all the methionine available for incorporation into protein is exhausted after 8 hrs. In order to test this,

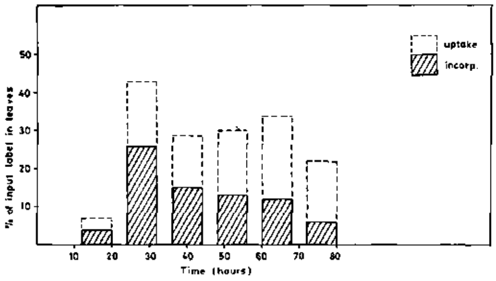


Fig.III.2. *Uptake and incorporation of ^{35}S-methionine during 8 hrs periods at different times after inoculation.*
At the indicated times CPMV-infected *Vigna* plants were cut across the stem and incubated during 8 hrs in tubes containing phosphate buffer with 10^{-6} M kinetine and 2.5 μCi ^{35}S-methionine. After 8 hrs the primary leaves were harvested and homogenized in a mortar. The homogenate was pressed through two layers of Miracloth and the total radioactivity (uptake) and TCA precipitable radioactivity (incorporation) in the sap was determined.

an identical dose of ^{35}S-methionine was added to the medium after the first labelling period of 8 hrs, starting 24 hrs after inoculation. This extra addition after 8 hrs resulted in a negligible increase of the incorporation: only 1% of the secondly added amount of ^{35}S-methionine was incorporated and 2% was taken up by the leaves. This result indicates that exhaustion of ^{35}S-methionine can not be the reason for the cessation of incorporation after 8 hrs, but that the uptake and transport through the stem to the leaves is stopped.
Although labelling with ^{35}S-methionine was more succesful than with tritiated amino acids, a maximum specific activity of 400 cpm/μg protein gave little hope that the proteins sought could be resolved from the bulk of host proteins, since the aim was to obtain specific activities in the order of at least 10,000 to 20,000 cpm/μg. Therefore another labelling procedure was tried out.

$^{35}SO_4^{2-}$

In the next experiments ^{35}S as sulphate was used as protein precursor. The advantages of using sulphate as precursor are:

1. Plants can be kept on Hoagland solution, so one can use intact plants for the experiments.
2. The plants used for these experiments can be starved for sulphate before addition of the label, resulting in a rapid uptake (and incorporation) of the administred sulphate.
3. Since the major pathway for the sulphate metabolism in plants leads to incorporation into methionine or cysteine, most of the administred sulphur might be incorporated into protein.

The results of the time course of incorporation of ^{35}S into proteins of CPMV- and mock-infected leaves (measured as TCA precipitable material) are shown in Fig. III.3. The label was added 24 hrs after inoculation. As can be seen in this figure the incorporation reaches a plateau after about 3 days. Further it is clearly seen in this figure that the host protein synthesis in the mock infected leaves show the same kinetics as in the virus infected leaves. The amounts in the virus infected leaves are higher, suggesting that the effect of viral protein synthesis is superimposed on the host protein synthesis. Since the sulphate was administred 24 hrs after inoculation, this labelling takes place during the intended period. With this method the specific radioactivity of the virus

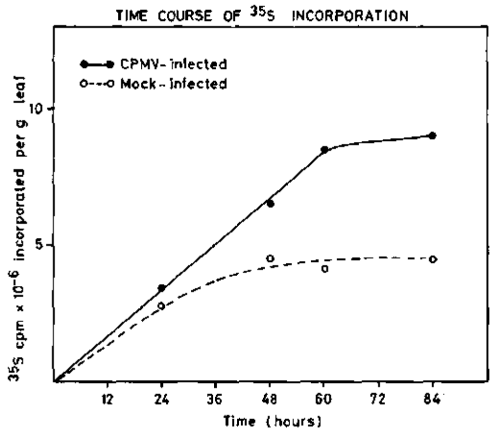


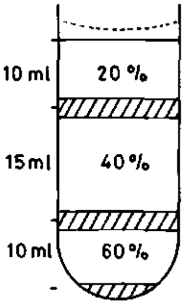
Fig.III.3. *Time course of incorporation of* ^{35}S *in CPMV-infected and mock-infected Vigna leaves.*
Vigna plants were grown on Hoagland solution as described in Materials and Methods. Nine days old plants were inoculated and one day later 0.5 mCi $^{35}SO_4^{2-}$ per plant was administered. At the indicated times the primary leaves were harvested and homogenized. After pressing the homogenate through two layers of Miracloth the TCA precipitable radioactivity was determined: •——• CPMV-infected leaves; o——o mock infected leaves.

reached values of about 10,000 to 30,000 cpm/μg.
Since the best labelling results were obtained by labelling with $^{35}SO_4^{2-}$, and the specific activities of the newly synthesized proteins were high enough, this method was further used to label the proteins in *Vigna* leaves for the fractionation and gel electrophoresis experiments.

III.2. Fractionation of leaf homogenates.

An approach to detect virus specific proteins in infected cells is the isolation of fractions enriched in virus specific structures. In these fractions the virus specific proteins, necessary for the multiplication of the virus, can be expected in higher concentrations than in extracts of whole leaf cells. The comparison with proteins extracted from a similar fraction obtained from mock-infected cells might indicate which proteins from the CPMV infected leaves are virus specific. CPMV multiplication occurs in specific cytopathological structures, induced by the CPMV infection (De Zoeten *et al.*, 1974). These structures have been shown to contain the replicative forms of CPMV-RNA (Assink, 1973). These structures can be isolated in a so called vesicle fraction. Since the majority of the double-stranded CPMV-RNA is present in this vesicle fraction, it was of interest to analyse these fractions for the proteins synthesized during CPMV multiplication and to compare them with proteins from a similar fraction, extracted from mock infected leaves. As a biochemical probe for the fractions where CPMV replication is supposed to take place, the replicase activity assay as described by Zabel *et al.* (1974) was used.
The leaf homogenate (see Materials and Methods) was centrifuged 10 min at 1000 × *g*. The 1000 × *g* pellet containes the structures in which the dsRNA (Assink, 1973) is located. After resuspension, this material was fractionated by sucrose density gradients (discontinuous 60-20% w/w sucrose). The fractions resulting from these gradients and the distribution of replicase activity, compared with the distribution of dsRNA in a similar procedure as determined by Assink (1973), is shown in Fig. III.4. From this it is clear that most of the material of interest is present in the so called chloroplast

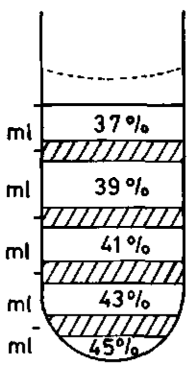
DISTRIBUTION OF 1000 x g FRACTION OVER A SUCROSE GRADIENT



	Replicase activity pmoles ^{3}H-UTP incorp. per 30 min	%	pmoles ^{3}H-UTP incorp. per 30 min	%	ds RNA %
Rest I fraction	2	0.8	2	0.3	-
Chloroplast fraction	160	60.6	587	75	72-93
Rest II fraction	102	38.6	136	17.4	rest I, rest II } 6-25
Nuclear fraction	-	-	59	7.5	1-3

Fig.III.4. *Distribution of the 1000 × g fraction of Vigna leaves over a sucrose gradient.*
The 1000 × *g* pellet of a leaf homogenate was resuspended in Honda buffer and layered on a discontinuous sucrose gradient as described (Materials and Methods). After centrifugation for 2 hrs at 22,000 rpm in a Beckmann SW27 rotor the fractions on the boundaries of the different sucrose layers of the gradient were collected. Replicase activity in these fractions was determined according to Zabel *et al.*, (1974). the percentages of dsRNA hybridizable to CPMV-RNA were taken from Assink (1973).

DISTRIBUTION OF CHLOROPLAST FRACTION OVER A SUCROSE GRADIENT



	Replicase activity pmoles ^{3}H-UTP incorp. per 30 min	%	ds RNA %
Fraction I	381	59	61-70
Fraction II	109	17	19-25
Fraction III	159	24	11-14

Fig.III.5. *Districution of the chloroplast fraction of Vigna leaves over a sucrose gradient.*
The chloroplast fraction (see Fig.III.4.) was diluted with gradient buffer and pelleted by centrifugation during 30 min at 18,000 × *g*. The pellet was resuspended in Honda buffer and layered on a discontinuous sucrose gradient as described (Materials and Methods). After centrifugation for 15 min at 22,000 rpm in a Beckmann SW27 rotor the fractions on the boundaries of the different sucrose layers of the gradient were collected. Replicase activity in these fractions was determined according to Zabel *et al* (1974). The percentages of ds RNA hybridizable to CPMV-RNA were taken from Assink (1973).

fraction: CPMV-RNA hybridizable dsRNA (72-93%) and replicase activity (54-75%); these last data come from all experiments performed; in the figure two examples are shown.

The chloroplast fraction was subjected to further fractionation over a second discontinuous sucrose gradient (45-37% w/w). The resulting fractions from this second gradient are called fraction I, II and III. The division of dsRNA hybridizable with CPMV-RNA (taken from Assink, 1973) and the distribution of replicase activity is shown in Fig.III.5. as typical example for such an experiment. From this it is clear that most of the dsRNA (61-70%) and the majority of the replicase activity (53-59%) is present in fraction I. Since electron microscopy had also shown that this fraction I is the fraction enriched in vesicle structures (De Zoeten *et al.*, 1974; Assink, 1973), this fraction was considered to contain virus specific structures from which the proteins could be extracted. Attention was therefore focussed on this fraction for the search for CPMV specific proteins.

III.3. Analysis of the proteins by polyacrylamide gel electrophoresis

As described in the two previous paragraphs, the conditions for detecting CPMV specific proteins *e.g.* sufficient radioactive labelling of *de novo* synthesized proteins with ^{35}S and a well characterised fractionation, were established. The next step was the separation of the proteins of the fractions from CPMV and mock infected *Vigna*leaves. This was done by electrophoresis in polyacrylamide gels, containing SDS. The proteins in the fractions were dissolved by addition of sample buffer followed by a 10 min incubation at 100°C. The same amount of protein was layered on each gel. After electrophoresis in cylindrical gels, the gels were sliced in 1.0 mm slices and the radioactivity was determined. The results of these analyses are presented in Fig.III.6. The pattern of proteins from fraction I are presented in panel a: proteins from CPMV-infected leaves by the solid lines and from mock-infected leaves by the dotted lines. Fig.III.6. panel b shows the patterns of proteins from fraction II from CPMV-infected (solid lines) and mock-infected (dotted lines) leaves.

The protein extracts from healthy and infected leaf material were layered on separate cylindrical gels. A comparison between to separate cylindrical gels can give difficulties in identifying differences between the patterns, because variations in the gels themselves might cause some heterogeniety in the patterns. Nevertheless it is clear that at least three peaks can be seen (indicated by arrows) in the protein pattern of infected material which are not presented in the pattern of proteins extracted from mock infected leaves. These proteins seem to be present in fraction I and fraction II, but not in the same amounts. The molecular weight of the paec indicated by the arrows 1a and 1b cannot be estimated in these gels, but probably exceeds the 150,000. The peaks designated 2a and 2b represent a protein with a molecular weight of about 70,000. The proteins indicated as 3a and 3b migrate towards the place where the large coat protein (mol. wt 37,000) of CPMV also migrates and probably represents this coat

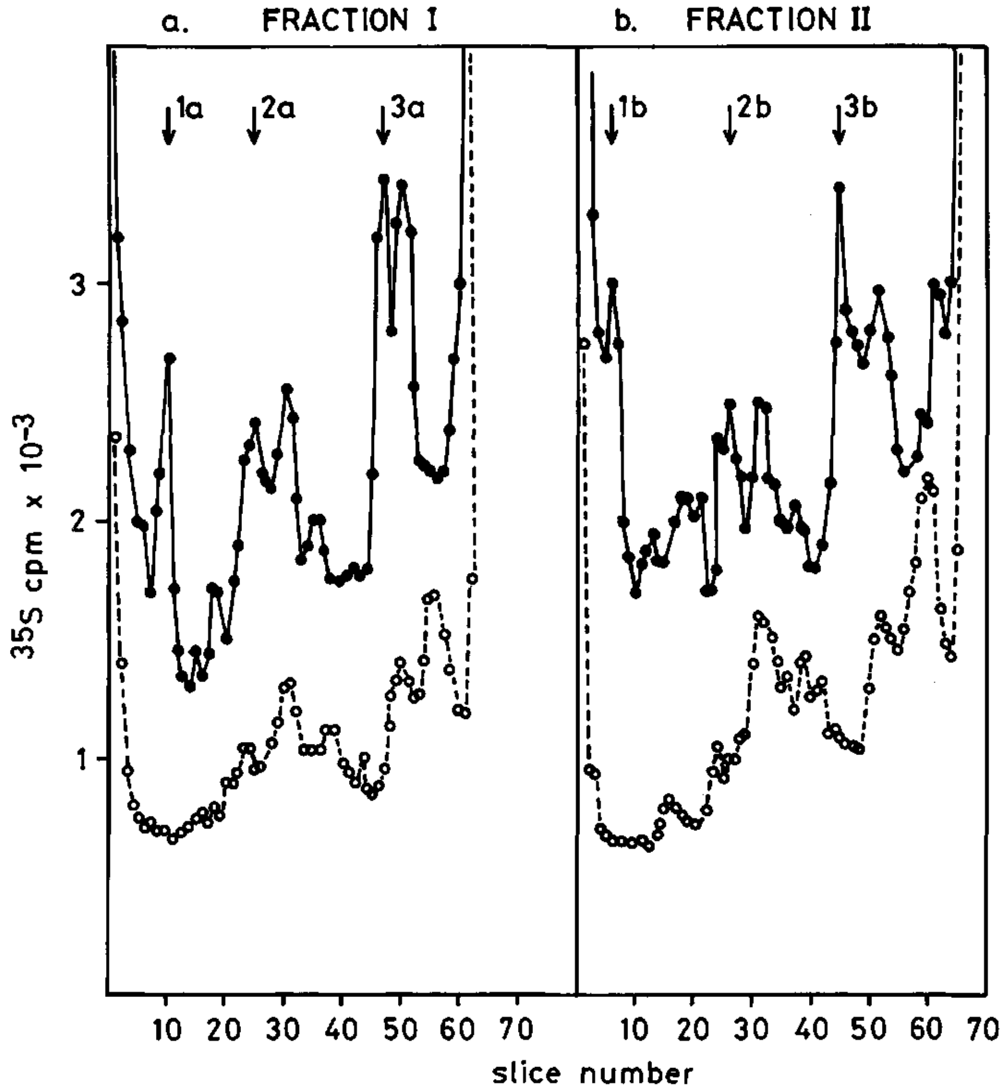


Fig.III.6. *Polyacrylamide gel electrophoresis of proteins from fractions I and II of the chloroplast fraction of Vigna leaves.*
After the additon of sample buffer the fractions were 10 min incubated at 100° C to dissolve the proteins. An equal amount of protein (about 70-80 μg) was layered on each gel. Electrophoresis was performed on 10% polyacrylamide gels in Laemmli buffer. The dectrophoresis at 5 mA per gel was continued until the bromophenol blue marker reached the end of the gel. The gel was soaked in acetic acid solution, water and then frozen. After slieing the gel into 1.0 mm slices with a Mickle gel slicer, the radioactivity in the gel slices was determined as described in Materials and Methods. Panel a: protein from fraction I; panel b: proteins from fraction II. •——• proteins from infected leaves; o——o proteins from mock infected leaves.

protein. The small coat protein (22,000) is not detectable on these gels because it probably migrates in the region where a lot of host proteins are present. Since comparison of protein patterns is more reliable when performed by slab gel electrophoresis, this technique was also used. An example of an autoradiogram is shown in Fig.III.7. Again a very large protein (indicated by the arrow) only present in the extracts from CPMV infected leaves, was observed. Its molecular weight is much higher than the β-galactosidase marker (116,000) and probably represents the same protein that was indicated by the peaks 1a and 1b in Fig.III.6. The molecular weight is approximately 170,000. The 70,000 molecular weight protein was not observed, but may have been obscured by the large amounts of host proteins present. The two coat proteins of CPMV (indicated by CP in the figure) are readily detectable on this autoradiogram.

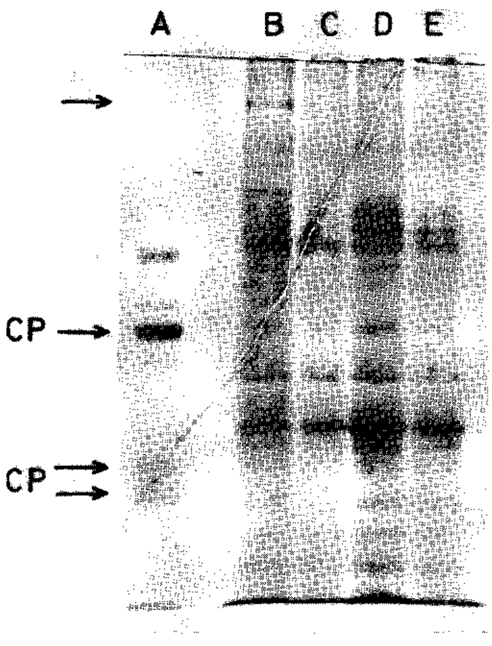


Fig.III.7. *Polyacrylamide slabgel electrophoresis of proteins from fraction I and II of the chloroplast fraction of Vigna leaves.* After the addition of sample buffer the fractions were incubated for 10 min at 100° C to dissolve the proteins. Electrophoresis was performed on a 10% polyacrylamide slabgel in Laemmli buffer. Electrophoresis was continued until the bromophenol blue marker reached the end of the gel. After staining with coomassie brilliant blue and destaining with an ethanol — acetic acid — water mixture (50:75:1000) the gel was cutted length-wise and one half was dried down on Whatmann 3 MM paper. For autoradiography the dried gel was exposed to Kodak-X-omat film. The autoradiogram shows in lane A: coat proteins (indicated by CP); lane B: proteins from fraction I from infected leaves; lane C: proteins from fraction I from mock-infected leaves; lane D: proteins from fraction II from infected leaves; lane E: proteins from fraction II from mock-infected leaves.

III.4. Discussion

Radioactive labelling of *Vigna* leaf proteins *in vivo*, to give a high enough specific radioactivity, was only possible when ^{35}S as sulphate was used as precursor for the proteins. Administration of radioactive amino acids to detached leaves or plants cut across the stem resulted in a too low specific radioactivity in the proteins. A reason for this can be the fact that the internal amino acid pools of *Vigna* leaves might be high. Another thing is that as soon as the leaves are detached or the plants cut across the stem, processes like aging are started. By the breakdown of proteins, which accompagnies this process, the amino acid concentrations in the leaf tissue are enlarged. So the effective concentration of the added (radioactive) amino acids *in* the leaves is very low. A third reason might be that by cutting the leaves or stems the tissue on the edges dies rapidly and these dead edges might fail to take up any substances after a short period and so prevent the uptake of the majority of the label.

Only by labelling *intact* plants with ^{35}S as sulphate could these difficulties be overcome, since the administration of amino acids through the roots seemed hardly possible. As can be seen in the results (section III.2.), the fractionation method used was effective in that the isolation of a vesicle fraction (fraction I) in which most of the CPMV replicase activity is located and therefore in which CPMV specific proteins can be expected, was succeeded. As a result of the labelling and fractionation, two proteins were detected (apart from coat protein), present in extracts from CPMV infected *Vigna* leaves and absent in extracts from mock infected leaves. The molecular weight of the largest protein found was not determined exactly. The estimation of the molecular weight on basis of the mobility in the gels used is approximately 170,000. The molecular weight of the other non-structural protein found in these experiments was about 70,000. This protein has the same size as a protein found in the membrane fraction of fractionated (CPMV infected) *Vigna* protoplasts, as described by Rottier (1980). The very large protein found resembles on size basis the 170,000 molecular weight protein found in infected protoplasts (Rottier et al., 1979).

From this work it was concluded that at least one very large polypeptide is synthesized in plant cells, as result of virus infection. If this protein is coded by the viral RNA, it must (on basis of its size) be encoded on the B-RNA. At least one other protein, intermediate in size between the large protein and the coat proteins, is also synthesized. By the time these proteins were discovered, the protoplast system with advantages over work with plants (see Rottier, 1980) was developed. This system proved to be more succesful in the search for virus specific proteins; so it was decided to discontinue the work with whole plants at that time to await further investigations of *in vivo* protein synthesis with protoplasts.

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IV CHARACTERIZATION OF THE *IN VITRO* SYNTHESIZING EXTRACTS

As described in the introduction, *in vitro* translation of viral RNA appears to be a useful tool for the elucidation of the translation strategy of plus-stranded RNA viruses. In the case of a multicomponent virus, *in vitro* translation gives a particular application in identifying which polypeptides are encoded on each of the RNAs. CPMV lacks the properties normally associated with the so called minus-strand RNA viruses, like an RNA-dependent RNA polymerase within the virus particles as for instance: vesicular stomatitis virus, newcastle disease virus, influenza virus and sendai virus (Moyer and Bannerjee, 1975; Baltimore *et al.*, 1970; Baltimore and Pons, 1975; Simpson and Bean, 1975; Kingsbury, 1974; Shatkin, 1974). The CPMV-RNAs both contain a strech of polyA at their 3'-end (El Manna and Bruening, 1973; Steel and Frist, 1978) and in that respect strongly resemble messenger RNA molecules. Since the RNAs occurring in the virions of CPMV were, in the translation experiments described here, proven to be of the plus-strand type, these RNAs — after extraction from purified virus components — could be used as messenger RNA in *in vitro* protein synthesizing systems. This made the preparation of well defined virus messenger RNA relatively simple.

The messenger-dependent rabbit reticulocyte lysates as described by Pelham and Jackson (1976) and the wheat germ extracts, originally described by Marcus (Marcus *et al.*, 1968; Marcus, 1970) for embryos from seeds and modified by Davies (Davies and Kaesberg, 1973; Davies *et al.*, 1977) for wheat germ, were used as eukaryote cell-free systems for the translation studies.

The use of two different systems was chosen because comparison of the results could give indications of the reliability of the *in vitro* results. Another reason was, that possible post-translational features of interest may occur in one system and not in the other and could be overlooked when only one system was used. For instance: the *in vitro* translation products of poliovirus and encephalomyocarditis virus RNA can be processed in reticulocyte lysates (Shih *et al.*, 1978; Shih *et al.*, 1979); a phenomenon as yet not observed in wheat germ extracts.

IV.1. The messenger-dependent lysate of rabbit reticulocytes (MDL)

The preparation of the MDL was described in detail in the Materials and Methods section (Chapter II.5.). The characteristics and the optimal conditions for the translation of CPMV-RNAs in the MDL were examined by measuring the incorporation of ^{35}S-methionine into TCA precipitable material.

Time course

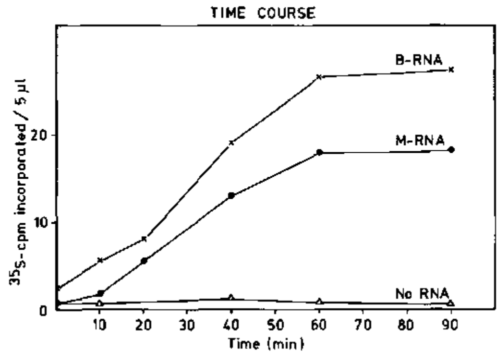

Fig.IV.1. *Time course of ^{35}S-methionine incorporation in MDL.* At the indicated times 5 µl samples were withdrawn from the incubation mixtures (under standard conditions). After precipitation with TCA, the samples were decolourized and the precipitate was collected on Whatman 3 MM paper discs and washed as described in Materials and Methods. ×——× 100 µg/ml B-RNa added; •——• 100 µg/ml M-RNA added; Δ Δ no RNA added.

Fig.IV.1. shows the time course of ^{35}S-methionine incorporation, without or with M- or B-RNA at concentrations of 100 µg per ml. It is clear that there is indeed no endogenous messenger RNA activity left in the MDL. The incorporation of ^{35}S-methionine, when no RNA was added, did not exceed 1000 cpm in 5 µl samples of the incubation mixture. Addition of M- or B-RNA gives a large increase of the amino acid incorporation: in this experiment up to about 30,000 cpm/5 µl; however in other experiments activities of more than 300,000 cpm/5 µl were obtained after 60 min incubation with more added ^{35}S-methionine (with higher specific activity). The incorporation increases linearly for about 60 min with both M- and B-RNA and stops then after this time to reach a plateau. A similar result was obtained with the natural mixture of CPMV-RNAs at a concentration of 100 µg/ml. An incubation time of 60 min was routinely used in the further experiments.

Messenger RNA concentration

The second parameter studied was the concentration of added CPMV-RNA. From Fig.IV.2. it can be seen that optimal amino acid incorporation occured at RNA concentrations of 100 µg/ml for both B- and M-RNA. For the natural mixture of CPMV-RNAs this optimum was the same *i.e.* the maximal incorporation was obtained with 100 µg RNA per ml. In the further translation studies this concentration of 100 µg viral RNA per ml was used.

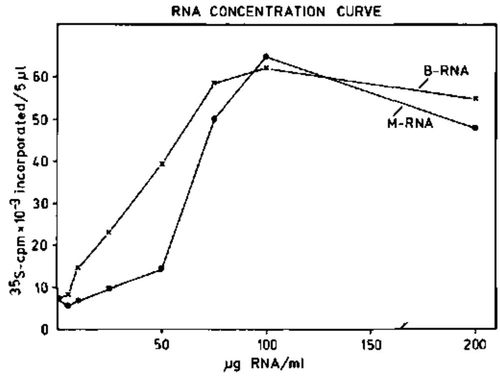

Fig.IV.2. *RNA concentration curve.*
Increasing amounts of CPMV-RNA were added to the MDL. The mixture was incubated under standard conditions for 60 min. 5 µl aliquots were withdrawn and processed as described in Materials and Methods. ^{35}S-methionine cpm on the filters was determined.
x——x B-RNA; •——• M-RNA.

Potassium ion concentration

The dependence of amino acid incorporation under direction of B-RNA on the K^+ concentration is shown in Fig.IV.3. The curve has a broad plateau between 75 and 125 mM potassium. This was also the case with M-RNA or the natural mixture of CPMV-RNAs. In the MDL KCl was used. The optimum and the amount of incorporated ^{35}S-methionine was not altered when potassium acetate was used instead of KCl, as is the case in wheat germ extracts (see IV.2.). The concentration used in the further translation studies was 125 mM KCl.

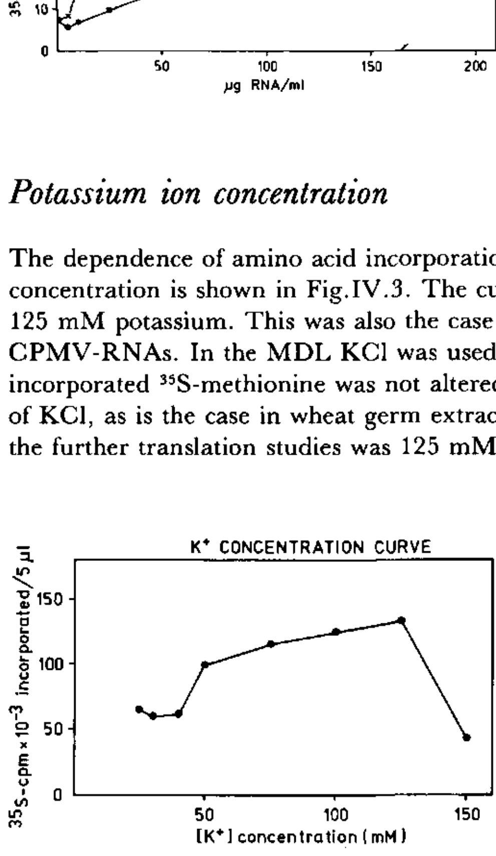

Fig.IV.3. *Potassium concentration curve.*
M-RNA (100 µg/ml) was added to the MDL. The mixture was incubated under standard conditions with varying amounts of K^+. ^{35}S cpm incorporated in 5 µl aliquots were determined as described.

Heterologous tRNA

As shown (Pelham and Jackson, 1976; Pelham and Stuik, 1977) the addition of heterologous transfer RNA is necessary for efficient translation of CPMV-RNA in the MDL. The optimal concentration of tRNA, extracted from mouse liver, was therefore determined. Fig.IV.4. shows the result of such an experiment. Very small amounts of mouse liver tRNA stimulate the amino acid incorporation in the MDL considerably.

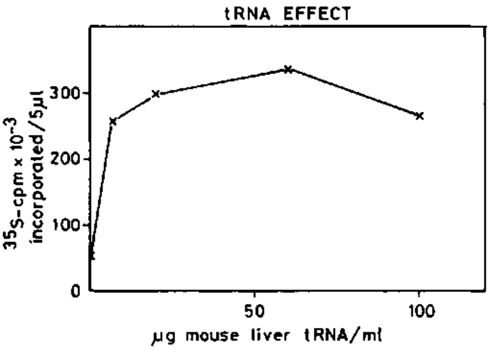

Fig.IV.4. *Effect of the addition of mouse liver tRNA.* Increasing amounts of mouse liver tRNA were added to the MDL containing 100 µg/ml CPMV-RNA. Incubation was under standard conditions for 60 min. ^{35}S cpm incorporated in 5 µl aliquots were determined as described.

The maximum incorporation is reached if tRNA was added in a concentration of about 60 µg/ml. This concentration was further routinely used. Transfer RNA, prepared from wheat germ could be used instead of the mouse liver tRNA, with a similar effect on the amino acid incorporation. In chapter V.1. will be shown that the addition or omission of heterologous tRNA has a dramatic effect on the *in vitro* translation products.

Standard conditions

The standard incubations for the further translation studies in the MDL had an incubation time of 60 min and contained, considering the investigated parameters, 100 µg mRNA and 60 µg tRNA per ml MDL, and 125 mM KCl.

IV.2. The wheat germ extract.

CPMV-RNAs were first tested in wheat germ extracts under conditions that were known to be suitable for several other plant virus RNAs (see for a review: Davies, 1979). These RNAs are translated in the presence of 50 mM K^+ and without spermidine or spermine. Higher K^+ concentration and addition of the polyamines might help these RNAs to be more efficiently translated, but such conditions are not obligatory. With CPMV-RNAs very little translation occurs under these conditions. Since these two parameters (spermidine addition and the K^+ concentration) were that important, the other parameters could not be thoroughly investigated until these requirements were characterized.
The preparation of the wheat germ extract was described in detail in the Materials and Methods section (II.6.). The characteristics and the optimalization of the translation of CPMV-RNAs in the wheat germ extract were studied by measuring TCA precipitable radioactivity of the added amino acids (^{35}S-methionine or ^{3}H-leucine).

Potassium ion concentration

As shown in Fig.IV.5. the potassium acetate concentration curve indicates that optimal incorporation is reached at K^+ concentrations between 80 and 110 mM. This curve was obtained by incubating 100 µg/ml CPMV-RNA (natural mixture); the results with separate M- or B-RNA were similar.
At first a concentration of 110 mM K^+ was used in the further translation studies; Later this was changed to 130 mM on basis of results from the product analyses (see V.2.), which shows that optimal incorporation of amino acids does not necessarily coïncide with efficient translation.
It is stressed that the results were obtained using potassium acetate. When KCl was used the optimum was 40-50 mM ánd the incorporation was less (Fig.IV.5.). So we conclude that Cl-ions are probably inhibitory in the wheat germ extract.

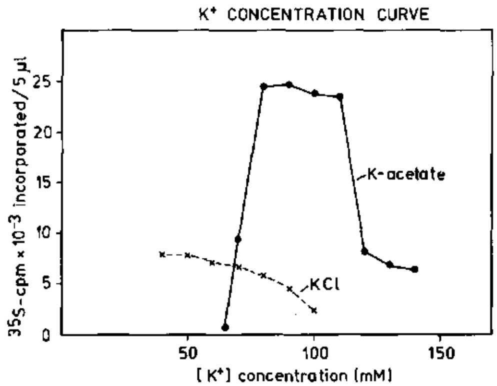

Fig.IV.5. *Potassium concentration curve.*
CPMV-RNA (60 µg/ml) was added to the wheat germ extract. The mixture was incubated under standard conditions with varying amounts of either K-acetate or KCl. ^{35}S cpm incorporated in 5 µl aliquots were determined as described (*Materials and Methods*).
o——o Potassium acetate; x——x Potassium chloride.

Spermidine

Amino acid incorporation in wheat germ extracts under direction of CPMV-RNA was almost fully dependent on addition of spermidine. The concentration curve for spermidine (Fig.IV.6.) shows a sharp optimum at a concentration of 0.4. mM. Spermidine also affected the Mg^{2+} optimum as will be shown below; the optimum for spermidine was determined with a Mg^{2+} concentration of 3.0 mM. In further translation studies the spermidine concentration was 0.4 mM. The effect of spermidine on the translation products in wheat germ extracts will be discussed in chapter V.2.

Mg^{2+} concentration

The Mg^{2+} optimum was different in the absence or presence of spermidine. Fig.IV.7. shows that without spermidine the Mg^{2+} optimum is found between 3.5 and 4.0 mM. With 0.4 mM spermidine the optimum shifted to 3.0 mM and became sharper. Further it is clearly visible in this figure that addition of spermidine stimulates the amino acid incorporation considerably.

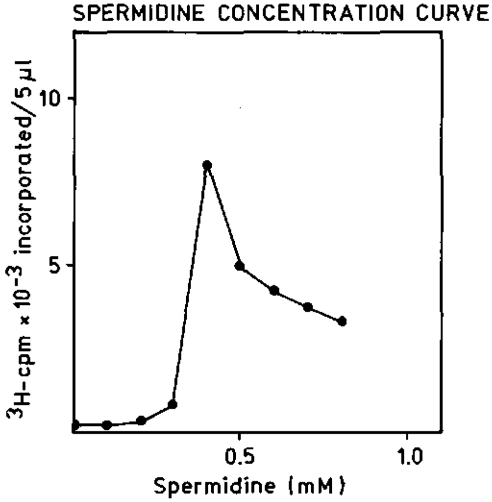

Fig.IV.6. *Spermidine concentration curve.*
CPMV-RNA (60 µg/ml) was added to the wheat germ extract. The mixture was incubated under standard conditions with varying amounts of spermidine. ^{35}S cpm were determined as described.

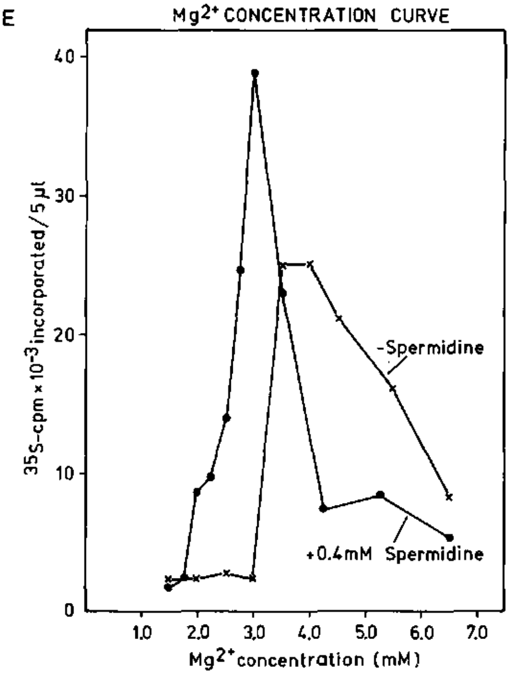

Fig.IV.7. *Magnesium concentration curve in the presence and absence of spermidine.*
Increasing amounts of Mg^{2+} were added to the wheat germ extracts containing 60 µg/ml CPMV-RNA and either no spermidine (x——x) or 0.4 mM spermidine (•——•). ^{35}S cpm in 5 µl were determined as described.

Time course

As in the case with the MDL (IV.1.) the kinetics of incorporation of amino acids were studied. The result is shown Fig.IV.8. It is clear that the wheat germ extract itself has hardly any messenger activity: without addition of mRNA there is almost no incorporation. Addition of CPMV-RNA however gives a large increase of the amino acid incorporation. The incorporation with added CPMV-RNA (natural mixture) increased for about 60 min. and levels off to give a plateau in the time curve. The results with separate M- or B-RNA or when methionine was used are the same as shown here. For further translation studies an incubation time of 60 min was routinely chosen.

Messenger RNA concentration

The dependen of amino acid incorporation on the CPMV-RNA concentration is shown in Fig.IV.9. the concentration that causes maximum amino acid incorporation was between 40 and 60 µg/ml. In the figure is shown the result for the natural mixture of CPMV-RNAs; separate M- or B-RNA show an identical optimal concentration. The concentration of 60 µg virus RNA per ml wheat germ extract was used in the further translation studies.

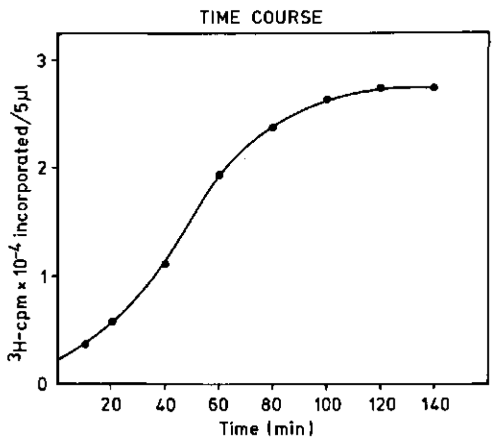

Fig.IV.8. *Time course of ^{3}H-amino acids incorporation in wheat germ extracts.*
At the indicated times 5 µl samples were withdrawn from the incubation mixtures (under standard conditions). After precipitation with TCA, the precipitate was collected on Whatman 3 MM paper discs and washed as described in Materials and Methods.
^{3}H cpm were determined as described.

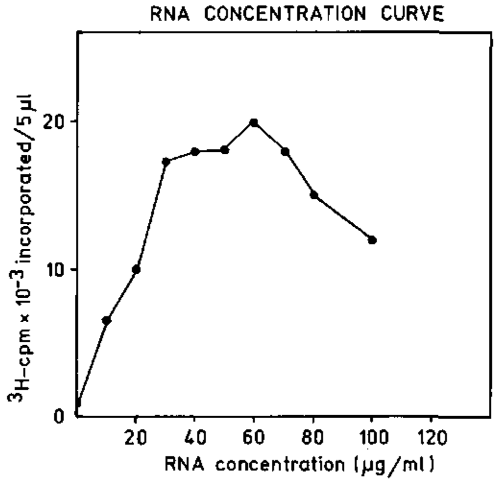

Fig.IV.9. *RNA concentration curve.*
Increasing amounts of the natural mixture of CPMV-RNAs were added to the wheat germ extracts. The mixture was incubated under standard conditions for 60 min. 5 µl aliquots were withdrawn and processed as described in Materials and Methods. ^{35}S cpm were determined as described.

Standard conditions

The standard incubations for the further translation studies in wheat germ extracts had an incubation time of 60 min. and contained, considering the investigated parameters, 60 µg RNA/ml, 110 mM (later 130 mM) K-acetate, 3.0 mM Mg-acetate and 0.4 mM spermidine.

IV.3. Discussion

CPMV-RNA seems to be a good messenger in both sorts of extract we used; *viz.* messenger dependent rabbit reticulocyte lysate and the wheat germ extract. We may conclude this from the fact that there is a large stimulation of the incorporation of amino acids when either M-, B- or natural mixture CPMV-RNA was added to the extracts. The kinetics of incorporation of amino acids is in both systems comparable: a linear incorporation for about 60 min. In both systems there is a short lag-period of about 5 min.
The messenger concentration showed a marked difference between the two systems. In the MDL the optimum is 1u0 µg/ml and in the wheat germ extract the optimal mRNA concentration is 40-60 µg/ml. This difference is probably caused by a difference in ribosome concentration of both extracts. The wheat germ extracts used here are deliberately diluted to minimize RNase activity, resulting in a ribosome concentration of about 2-3 mg/ml. In the MDL however the ribosome concentration is about 5-6

mg/ml. From this one may conclude that saturation of mRNA ribosomes is reached at lower mRNA concentrations in the wheat germ extract, which causes the lower optimal concentration for the CPMV-RNAs. The optimal concentration in µg per ml is the same for both M- and B-RNA in the system used. This means that on molar basis there are less B-RNA molecules present in the extract. This could indicate that B-RNA is more effective as messenger than M-RNA. From the incorporation data shown here this impression is corroborated; but since the incorporation data are strongly dependent on the RNA preparation used this does not hold true for all experiments: sometimes a M-RNA preparation gave better incorporation than a B-RNA preparation.

The potassium optimum is almost the same in both extracts and the values are about the same as found for other plant viral RNAs used as messengers in these extracts (see review of Davies, 1979). That Cl^- ions give a lower optimal concentration for the K^+, and are inhibitory in the wheat germ extract is not understood. A similar deletorious effect of Cl^- ions have not been observed in the MDL.

The effect of spermidine and K^+ concentration (in the wheat germ extracts) and that of heterologous tRNA (in the MDL) will be discussed in the next chapter.

It should be stressed that not all the possible parameters influencing translation of CPMV-RNA in these two systems are investigated and that the parameters, investigated were not thoroughly studied. For instance: the investigation of the dependence on Mg^{2+} in the MDL was not performed, the concentration as used by Pelham and Jackson (1976) was used, nor the possible effects of spermidine in the MDL was studied. Also the dependence on ribosome concentration or the amount of added radioactive amino acids were not studied in both systems. The reason for this is that the aim was not to study the mechanisms of the *in vitro* translation in these systems, but to use them, when reliable translation took place, for the elucidation of the translation strategy of cowpea mosaic virus RNA.

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V ANALYSIS OF THE *IN VITRO* TRANSLATION PRODUCTS

The results, shown in the previous chapter indicated that the CPMV-RNAs acted well as messengers in the MDL and the wheat germ extract. In this chapter the analysis of the products made in the *in vitro* protein synthesizing extracts is described. For these analyses we used polyacrylamide slabgel electrophoresis in SDS containing gels as described by Laemmli (1970). Apart from characterisation of the products, we also examined the effect of added heterologous tRNA (in the case of MDL) and the effects of spermidine and K^+ concentration (in the case of wheat germ extracts) on the nature of the *in vitro* translation products of CPMV-RNA. Furthermore an estimation of the molecular weights of the polypeptides synthesized is described.

V.1. Productanalysis of the polypeptides synthesized in the MDL

The first analyses of the products, synthesized in the standard incubation mixture (as described in IV.1.) under direction of either M-, B- or natural mixture CPMV-RNA, were performed on 15% polyacrylamide slabgels. A typical autoradiogram of such a gel is shown in Fig. V.1. As can be seen in this figure (lane A) the largest polypeptide, synthesized under direction of B-RNA (indicated by BP), electrophoresed a bit slower than the myosine marker (MW app. 200,000). M-RNA (lane B) directed mainly the synthesis of two large polypeptides (indicated by MP_1 and MP_2). The smaller one electrophoresed somewhat faster and the bigger one somewhat slower than the β-galactosidase marker (MW 116,000). The natural mixture of CPMV-RNAs directed the synthesis of the same polypeptides (lane C) as M- and B-RNA separately did. Besides these large polypeptides, synthesized under direction of CPMV-RNAs, there was always a number of smaller products present in the gels. However among these smaller products no coat proteins could be detected. In Fig.V.1. the position where the

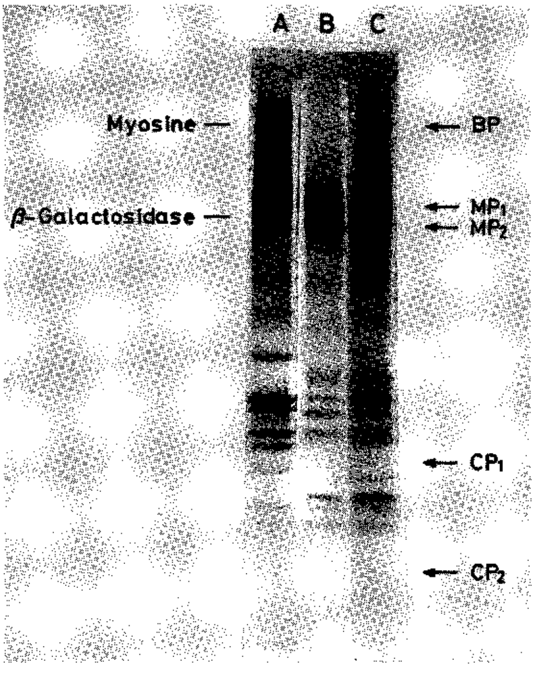

Fig.V.1. *Electrophoretic patterns of the polypeptides synthesized in MDL.*
Electrophoresis was performed in a 15% polyacrylamide gel in Laemmli buffer.
The place where the marker proteins and CPMV-coat proteins migrate to is indicated.
A: products from B-RNA.
B: products from M-RNA.
C: products from the natural mixture of CPMV-RNAs.

coat proteins migrate to is indicated by CP_1 and CP_2. The origin of these smaller products that appear on the gels is not known. Since the amount and the patterns of these smaller polypeptides varied with different experiments, we considered them as not the 'real' translation products of CPMV-RNAs.

Heterologous tRNA effect

The products as shown in Fig.V.1. were synthesized in a standard incubation mixture; so mouse liver tRNA was added. When this heterologous tRNA was omitted no large products showd up in the gels, as can be seen in Fig.V.2.: lane A, products of CPMV-RNA with added tRNA; lane B, products made under direction of CPMV-RNA when no tRNA was added to the incubation mixture. Clearly shown here is the phenomenon that the tRNA addition not only stimulates the incorporation of amino acids, as was shown in IV.1., but is absolutely necessary for the translation of CPMV-RNA into well defined products in the MDL. Transfer RNA from other sources, like wheat germ or *Vigna* leaves served in the same way. A possible explanation will be presented in the discussion (V.4.). Also shown in this Fig.V.2. (lane C) are the 'products' of the incubation when no messenger RNA was added to the incubation mixture. It is clear that the observation already made from the incorporation studies — there is no endogenous activity left in the MDL — is confirmed by this analysis.

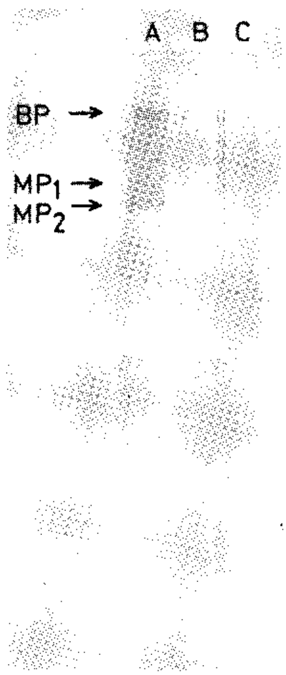

Fig.V.2. *Effect of heterologous tRNA on the translation of CPMV-RNA in MDL.*
Electrophoresis was performed in a 10% polyacrylamide gel in Laemmli buffer.
A: CPMV-RNA and mouse liver tRNA added.
B: CPMV-RNA added.
C: no RNA added.

V.2. Productanalysis of the polypeptides synthesized in wheat germ extracts

As with the products made in the MDL, the productanalysis of polypeptides synthesized in wheat germ extracts under direction of CPMV-RNAs was performed on polyacrylamide slabgels according to Laemmli (1970). Fig.V.3. shows an autoradiogram of such a gel. The products made under standard conditions are like those found in the MDL programmed with CPMV-RNAs: one large polypeptide made under direction of B-RNA (indicated by BP in lane A) and two large polypeptides from M-RNA (indicated by MP_1 and MP_2 in lane B). The natural mixture of CPMV-RNAs directed the synthesis of all the products made by the separate RNAs (lane C). The electrophoretic behaviour in relation to the markers (myosine and β-galactosidase) is approximately the same as described in the previous paragraph. The results of an incubation when no RNA was added, is shown in lane D: confirming the incorporation data, almost no products are visible on the gel. In wheat germ extracts, programmed with CPMV-RNAs a number of smaller products, similar to the MDL system, were detected.

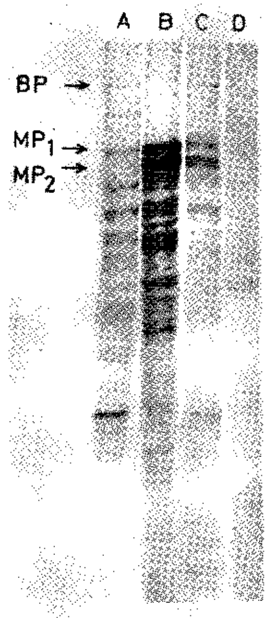

Fig.V.3. *Electrophoretic patterns of the polypeptides synthesized in wheat germ extracts.*
Electrophoresis was performed in a 5-12% polyacrylamide gradient gel in Laemmli buffer.
A: products from B-RNA.
B: products from M-RNA.
C: products from the natural mixture of CPMV-RNAs.
D: no RNA added.

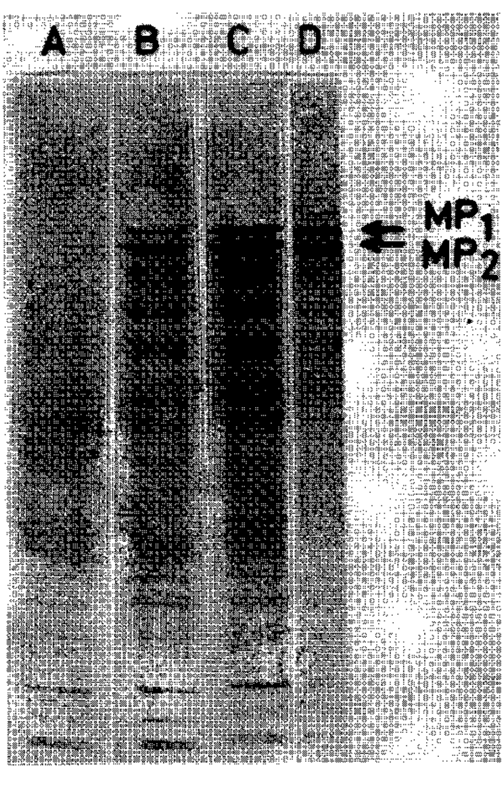

Fig.V.4. *Effect of K^+ concentration on the translation of CPMV-RNA in wheat germ extracts.*
M-RNA was translated in wheat germ extracts with different K^+ concentrations.
Electrophoresis was performed in a 10% polyacrylamide gel in Laemmli buffer.
A: 65 mM K^+.
B: 70 mM K^+.
C: 110 mM K^+.
D: 130 mM K^+.

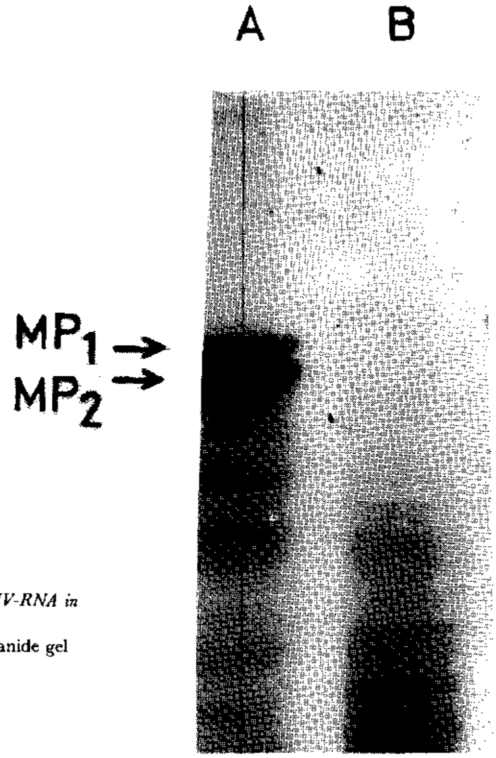

Fig.V.5. *Effect of spermidine on the translation of CPMV-RNA in wheat germ extracts.*
Electrophoresis was performed in a 10% polyacrylanide gel in Laemmli buffer.
A: 0.4 mM spermidine and 3.0 mM Mg^{2+}.
B: 4.0 mM Mg^{2+}, no spermidine.

Effects of potassium acetate concentration

The number of smaller products could be deminished when the K^+ concentration was increased. This is shown in Fig.V.4. for M-RNA. With a potassium acetate concentration of 65 mM only some smaller products were made (lane A), when 70 mM K^+ was used, the high molecular weight products are synthesized besides a lot of smaller material (lane B). Under conditions of maximal incorporation (110 mM) indeed the MP_1 and MP_2 bands on the autoradiogram are more intense (lane C), but a lot of smaller products are still made. However highering the potassium concentration at 130 mM K^+, a concentration where the incorporation is about a third of the maximum, causes a considerable diminishing of these smaller products (lane D). In other experiments with 130 mM K^+, sometimes more but also sometimes less till almost no smaller products are made. The discussion deals with this problem further.

Effect of spermidine

Spermidine not only stimulates amino acid incorporation (as been shown in IV.2.) but is an absolute requirement for the synthesis of high molecular weight products under direction of CPMV-RNA. Fig.V.5. shows clearly that the MP_1 and MP_2 products of M-RNA are only synthesized when spermidine is present in the incubation mixture

That the percentage of the gels or the buffer system are not the only parameters that influence the behaviour of the translation products on gels, can be illustrated by next experiment. Translation mixtures (wheat germ extract) were prepared for gel electrophoresis in an identical way. Aliquots of these mixtures were layered on two gels in phosphate buffer, differing only in concentration, namely 4% and 5%. Electrophoresis was performed in an identical manner and the marker proteins on both gels were identical. Fig.V.7. shows the autoradiograms of both gels and the position of the marker proteins. Clearly visible is that in the case of the 4% gel the MP_1 and MP_2 are migrating faster than the β-galactosidase marker, whereas on the 5% gel they are migrating a bit slower. This results in molecular weight estimations for MP_1 resp. 123,000 (5% gel) and 114,000 (4% gel). A similar phenomenon is observed for the MP_2.

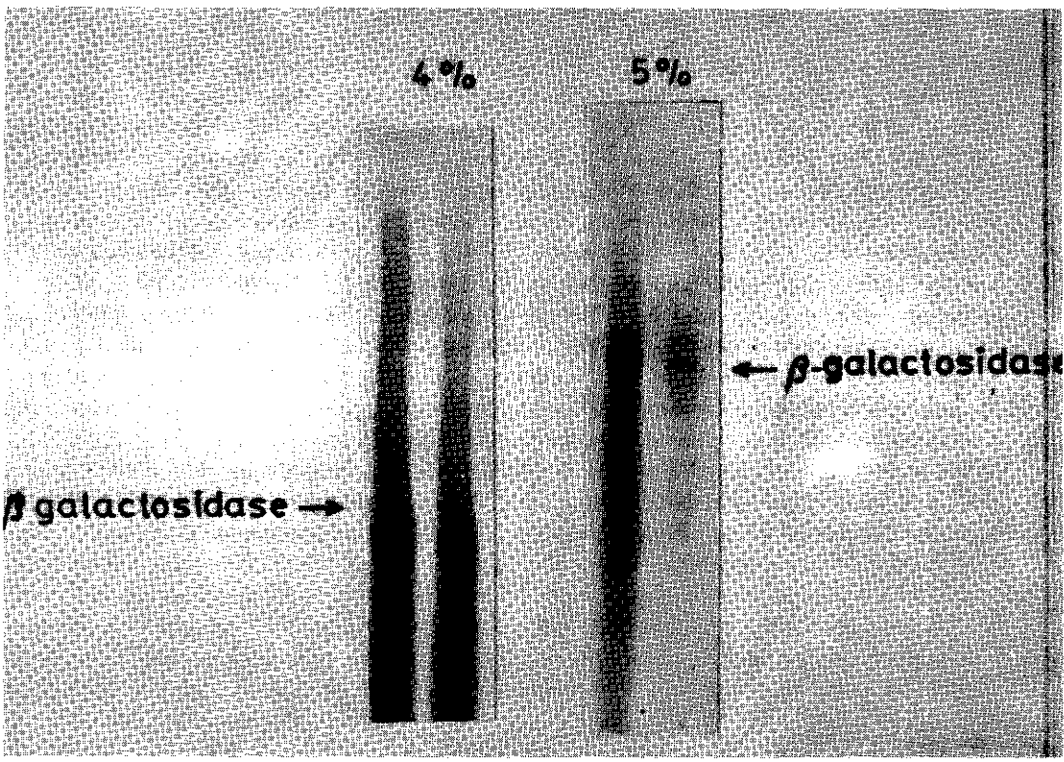

Fig.V.7. ***Comparison of the in vitro translation products from M-RNA in two gels differing in polyacrylamide percentage.***
Two different polyacrylamide gels (4 and 5%) in phosphate buffer were used to compare the electrophoretic behaviour of the products from M-RNA with that of β-galactosidase.

V.4. Discussion

The results shown in this chapter confirm the conclusion from the previous chapter: *i.e.* virion RNAs serve well as messengers in both *in vitro* systems. Furthermore it is shown here that specific products are synthesized. The product analyses in this chapter show that both B- and M-RNA are translated into large polypeptides, that are similar in both translation systems used.

Except for these large polypeptides, there were always a number of smaller products visible on the gels. The number and appearance of these smaller products was variable in different experiments. Also differences in the patterns of those smaller proteins (on gels for the analysis), synthesized in both systems were observed. Since the large proteins were reproducible, in what ever system we used (when intact CPMV-RNAs were used as messengers), we consider those as the 'real' translation products. However among these smaller polypeptides some functional virus specific proteins might be present, but the variaty in the appearance of these smaller polypeptides makes this assumption unlikely. The origin of the smaller products may come from translation of CPMV-RNAs that have hidden breaks showing up during translation causing the synthesis of smaller polypeptides. Another explanation might be that there is residual RNase activity left in the translation systems, *e.g.* the Micrococcal Nuclease is not completely inactivated and in the wheat germ extracts some RNase activity is already present. Also premature termination cannot been excluded as reason for the synthesis of smaller polypeptides in both systems. Some of the smaller products synthesized may come also from the translation of endogenous mRNA (this in the case of wheat germ extracts) that might be enhanced by the addition of the CPMV-RNAs (Davies, unpublished results).

In the MDL, the additon of heterologous tRNA was an absolute requirement for the repreducable translation of CPMV-RNAs into large polypeptides. For the *in vitro* translation of globin messenger (the homologous messenger for this system) this addition was superfluous (Pelham and Jackson, 1976). When heterologous tRNA was omitted from the incubations with CPMV-RNA in the MDL, no large products were synthesized, but only a limited number of smaller polypeptides. Similar results were obtained with TMV-RNA (Pelham and Jackson, 1976). An explanation for this phenomenon can be that reticulocytes are deficient in some tRNAs, necessary for the translation of other mRNAs than globin messenger RNA. Reticulocytes are differentiated cells in which the primary function is to synthesize globin. During the differentiation, tRNAs for codons not existing in globin mRNA, will be lost and therefore these codons will not been recognized. The result is then that these codons will act like stop codons giving rise to the artificial production of smaller polypeptides. A proof for this hypothesis comes from the work of Sharma *et al.* (1976) where it has been shown that the tRNA from reticulocytes participated efficiently only in the translation of globin mRNA whereas translation of oviduct mRNA and encephalomyocarditis viral RNA led to an accumulation of smaller polypeptides. One can also not rule out the possibility that the nuclease treatment will destroy some tRNA molecules.

In the translation experiments with wheat germ extracts there were two important parameters for repreducable translation of CPMV-RNAs into large polypeptides. Spermidine was absolute necessary for the synthesis of high molecular weight products. Without addition of spermidine, combined with a lower Mg^{2+} concentration, wheat germ extracts programmed with CPMV-RNA synthesized only smaller products. Addition of spermidine causes a dramatic change in the pattern of the synthesized proteins in that the large molecular weight products appear. Similar results were obtained with spermine. The exact role of polyamines in protein synthesis is not completely understood. In wheat germ extracts programmed with other plant viral RNAs this requirement is not always necessary. Smaller mRNAs seeme to be efficiently translated in wheat germ extracts without the addition of polyamines; for instance the RNA of satellite tobacco necrosis virus (Leung *et al.* 1976), the satellite-like RNA 5 of cucumber mosaic virus (Owens and Kaper, 1977) and also the small RNAs of multicomponent viruses like brome mosaic virus RNA4 (Davies and Kaesberg, 1973), and RNA3 (Shih and Kaesberg, 1976), the alfalfa mosaic virus 17S and 12S RNAs (Thang *et al.*, 1976; and Rutgers, 1977) and RNA4 from cowpea chlorotic mottle virus (Davies and Kaesberg, 1973). For *in vitro* translation of larger mRNAs in wheat germ extracts the addition of polyamines stimulates the incorporation of amino acids into protein, and therefore gives a higher yield of proteins synthesized, but is in most cases not a prerequisite for the translation. Examples for this are turnip yellow mosaic virus RNAs (Benincourt and Haenni, 1976), cucumber mosaic virus RNAs (Schwinghammer and Symons, 1977), tobacco rattle virus RNA (Fritsch *et al*, 1977) and tobacco mosaic virus RNA (Hunter *et al.*, 1977); in this last case has been shown that polyamines don't affect the initiation, but are stimulatory on the elongation process. The large brome mosaic virus RNA1 and RNA2 (Shih and Kaesberg, 1976) seeme not to need polyamines at all. In stead of that translation of RNA1 needed suboptimal (in respect to the amino acid incorporation) RNA concentrations, while RNA2 translation was favored by a K^+ shift towards higher concentrations. Only for some large mRNAs the presence of polyamines was absolutely required. For example the translation of the 20S and 24S RNAs of alfalfa mosaic virus (Thang *et al.*, 1976) and the mouse interferon in RNA (Thang *et al.*, 1975) and as described here and previously (Davies *et al.*, 1977) the CPMV-RNAs. Polyamines are present in eukaryote cells in nearly the same concentration as Mg^{2+} (Cohen, 1971). Since by the preparation of wheat germ extracts a prolonged dialysis step is included, the polyamines are removed from the extract. Addition of spermidine and/or spermine therefore restores a more natural situation for the *in vitro* translation in wheat germ extracts. This explains also why it was not necessary to add polyamines to the MDL, since the intracellular polyamines are still present in the lysate. Removal of polyamines from rabbit reticulocyte lysates (by gel filtration) resulted in a lower rate of protein synthesis that could be restored by addition of polyamines (Hunter *et al.*, 1977).
One of the proposed explanations for the role of polyamines in protein synthesis is that the increased rate of elongation caused by the presence of polyamines (the precise mechanism is still unknown) increases the probability of the ribosomes to complete the translation before the messenger is damaged by nucleolytic attacks (Hunter *et al.*, 1977). This would also give an explanation why smaller mRNAs are less effected when polyamines are omitted. Also the fact that spermidine and spermine are naturally in

relatively high quantities of resp. 5.05 μg and 0.17 μg per mg virus (Bruening *et al.*, 1968; Nickerson and Lane, 1977) suggests that, especially for CPMV-RNAs, these polyamines are important. It might also be that they protect the RNAs *in vivo* from nucleolityc attack in the plant cells.
The second important parameter for the translation of CPMV-RNAs in wheat germ extracts was the potassium concentration. As clearly visible in the results (Fig.V.4.), at higher concentrations of K^+ (130 mM), when the amino acid incorporation is sub-optimal, the elongation rate of the peptide chain seems to be increased resulting in the synthesis of more high molecular weight products. In this case either inhibition of RNase activity or the effect of a faster synthesis by higher K^+ concentrations might be the reason for this. Effects like this are described for several eukaryote mRNAs, *viz*, hepatic mRNA (Astel and Ganoza, 1974), collagen mRNA (Beneviste *et al.*, 1976), type I and type II procollagen mRNAs (Harwood *et al.*, 1975), immunoglobulin mRNAs (Schmeckpepper *et al.*, 1974), for the RNAs of cucumber mosaic virus (Schwinghammer and Symons, 1977) and for the RNAs of alfalfa mosaic virus (Rutgers, 1977) and polio virus RNA (Villa-Komaroff *et al.*, 1975).
In all translation experiments with both systems, the virion RNAs *never* directed the synthesis of proteins with the same electrophoretic behaviour as the CPMV coat proteins. This leads to the conclusion that the coat proteins are not synthesized in these *in vitro* systems when virion RNAs were used as messengers. The question how the coat proteins are synthesized then will be answered in chapter VII.
The first molecular weight estimations of the large polypeptides synthesized as published before (Pelham and Stui, 1977, Davies *et al.*, 1977), were 220,000 for BP and 140,000 for MP_1 and 120,000 for MP_2. The genome of B-RNA (2.02×10^6 d) consists of about 5800 bases. About 100-200 of these bases are part of the polyA strech at the 3'-end, which does not code for protein (El Manna and Bruening; Steele and Frist). Furthermore the initiation site is probably not situated directly at the 5'-end of the RNA molecule. Observations by Stanley and Van Kammen (1979) show that the first initiation site is not within the first 17 bases from the 5'-end and might well be further of. The coding capacity of B-RNA is therefore probably maximal 5650 bases and minimal 5450 bases. This means that when full size translation occurs, a protein of maximal 225,000 and minimal 210,000 molecular weight could be synthesized (assuming a average molecular weight of 120 for amino acids. based on a normal distribution for amino acids in protein, where the distribution of amino acids in the β-galactose protein is taken as typical example (Fowler and Zabin, 1977). Calculating the same for the M-RNA (1.37×10^6), where the 3'-end contains the same polyA strech and the 5'-end is similar to that of B-RNA, this would result in a protein between 160,000 and 150,000. Comparing these calculations of the coding capacity of the RNAs with the molecular weight estimations of 220,000 (BP) and 140,000 (MP_1), it would imply that almost the whole coding capacity of B-RNA and about 90% of that of M-RNA was used for the translation into these large polypeptides, leaving no other cistrons on the RNAs (exept overlapping genes). From table V.1. it is clear that accurate determination of the molecular weights of the synthesized proteins was not so easy to perform. The variation in the molecular weight estimations of the proteins on different gels made impossible to assigne an exact value for the proteins synthesized. One thing still was clear: the initial molecular weight determinations as published by

Pelham and Stuik (1977) and Davies *et al.* (1977) were too high. Further analyses showed that the molecular weight of BP is about *180,000* to *190,000*, MP_1 is about *110,000* to *125,000* and MP_2 is about *100,000* to *105,000*. This could indicate that there is still room on the RNAs for regions coding for coat protein, *e.g.* the large coat protein (37,000) on the M-RNA and the small coat protein (22,000) on the B-RNA. This situation is discussed in chapter VII.
The explanation for the two large molecular weight products that are synthesized under direction of M-RNA is still unknown. There are no conditions found (not shown in the results) in which differential synthesis of one polypeptide is enhanced. Both polypeptides seem to be produced in about the same amounts, in both MDL and wheat germ extracts. The two polypeptides must be read from overlapping genes, since the M-RNA molecule is not large enough to code for both polypeptides separately. The question if the reading frame is the same for both proteins can not be answered here; although the work of Pelham (1979) indicates that the two polypeptides have peptide sequences in common, suggesting an identical reading frame. One explanation might be that the largest polypeptide is a read-through product. This could arise by the same mecahnism as is described by Pelham (1978) for TMV-RNA, and by Phillipson *et al.* (1978) for Moloney murine leukemia virus and mouse sarcoma virus RNAs. when suppressor tRNA (amber and ochre), isolated from the appropriate yeast strains, was added to the MDL, the synthesis of read through products was enhanced in these cases. Also with $Q\beta$ amber mutant RNA this RNA can be translated *in vitro* into full-size $Q\beta$ proteins, when suppressor tRNA was added (Weiner and Weber, 1971).
A similar situation could exist here with CPMV-RNA where the assumed termination codon is not to be recognised as such by the tRNAs of mouse liver or wheat germ. However it is rather unlikely that the tRNAs from *Vigna* leaves causes a similar effect. It can not be excluded that these two polypeptides are indeed functional virus specific proteins. Also the existance of two initiation sites ('in-frame') can not be ruled out. The existence of two ribosome binding sites (and from that two postulated initiation sites) on the M-RNA has been claimed by Pelham (1979). However analysis of the N-terminal ends of the polypeptides show identical peptides. Furthermore ribosome binding experiments have to be reconsidered in view of the finding that leader sequences are capable of binding ribosomes (Filipowicz and Haenni, 1979). CPMV-RNAs also have such leader sequences since the elucidation of the sequence of the first 17 nucleotides from the 5'-end do not include the start codon for protein synthesis (Stanley and Van Kammen, 1979).
It should not be overlooked that in *in vitro* systems artificial enlarged polypeptides can be synthesized. Using two different protein synthesizing systems that produce the same results gives some indications that the *in vitro* translation results might reflect the *in vivo* situation; but the comparison with *in vivo* found virus specific proteins can give further indications in how far the results of the *in vitro* experiments are indeed reflecting the *in vivo* situation.

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VI COMPARITIVE STUDIES OF THE CPMV-SPECIFIC PROTEINS

VI.1. comparison of the *in vitro* products from MDL and wheat germ extract

From the data concerning the molecular weight estimations, described in the previous chapter (V.3.), the impression is given that the polypeptides, synthesized in both types of extract, differ in molecular weight. Since there is a large variation in these estimations, only *direct* comparison by analysis on slabgels can give an answer to the problem if these polypeptides have the same electrophoretic mobility (or not). Fig. VI.1. shows the direct comparison of BP, MP_1 and MP_2 synthesized in either wheat germ extracts or MDL on a slabgel in Laemmli buffer. Lanes A and B show the products synthesized under direction of B-RNA in respectively MDL and wheat germ extracts; lanes C and D those of M-RNA in respectively MDL and wheat germ extracts. Analysis in a different gel system (phosphate buffer) gives the same results as described above. fig. VI.2. shows the autoradiogram of such a gel: BP synthesized in MDL (lane A) coëlectrophorezed with BP synthesized in wheat germ extract (lane B). Similarely the M-RNA directed polypeptides (MP_1 and MP_2) showed no difference in electrophoretic mobility if they were synthesized either in MDL or in wheat germ extracts (lanes C and D).
Sometimes a larger polypeptide with a molecular weight of about 200,000 was synthesized under direction of B-RNA in MDL, in addition to BP. The synthesis of a similar sized polypeptide is described by Pelham (1979), when amino acid analogs were added to the MDL translation mixture. Goldbach and Keus also detected this polypeptide in wheat germ extracts.

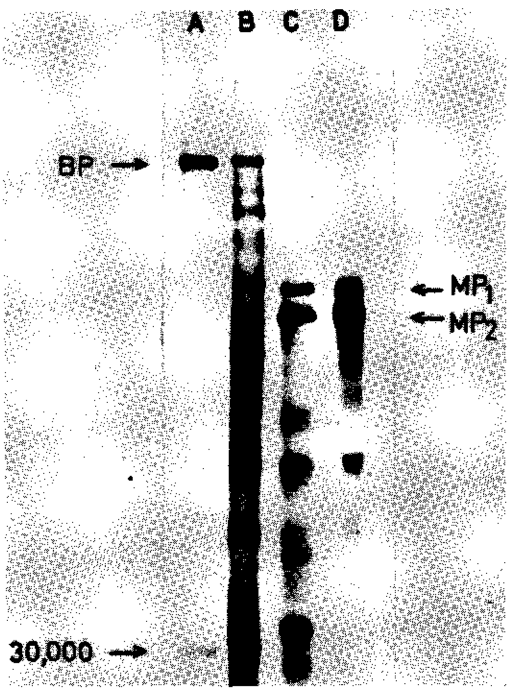

Fig.VI.1. *Comparison of the translation products from CPMV-RNAs.*
B- and M-RNA were translated in wheat germ extracts and MDL.
Electrophoresis was performed in a 7.5% polyacrylamide gel in Laemmli buffer.
A: B-RNA translated in MDL.
B: B-RNA translated in wheat germ extract.
C: M-RNA translated in MDL.
D: M-RNA translated in wheat germ extract.

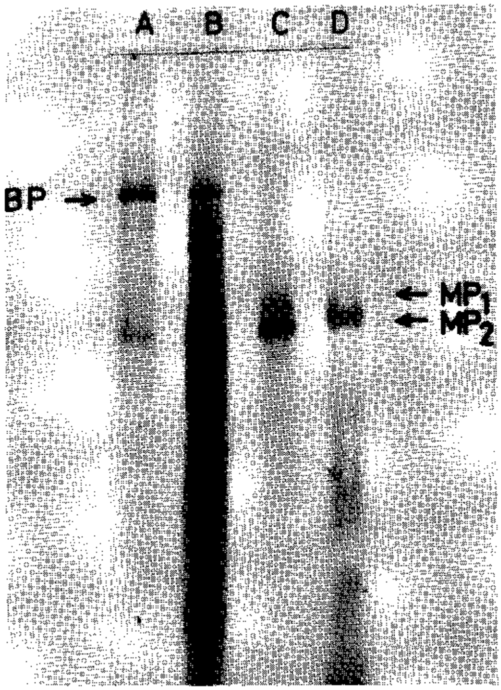

Fig.VI.2. *Comparison of the translation products from CPMV-RNAs.*
B- and M-RNA were translated in wheat germ extracts and MDL.
Electrophoresis was performed in a 5% polyacrylamide gel in phosphate buffer.
A: B-RNA translated in MDL.
B: B-RNA translated in wheat germ extract.
C: M-RNA translated in MDL.
D: M-RNA translated in wheat germ extract.

In some experiments a 30,000 molecular weight polypeptide also appeared after translation of B-RNA in MDL and wheat germ extracts (Pelham, 1979; Goldbach and keus, 1979). This 30,000 molecular weight polypeptide was not noticed before (see chapter V and above). The reason for these different results is not clear.

VI.2. Comparison of *in vitro* and *in vivo* synthesized CPMV-specific proteins

As described in the Introduction (chapter I) the comparison of *in vitro* translation products with *in vivo* detected viral related proteins may laed to the identification of some virus-specific proteins. In Fig. VI.3. the polypeptides synthesized *in vitro* under direction of the CPMV-RNAs are compared by SDS gel electrophoresis with the CPMV-related proteins detectable in a 30,000 × *g* supernatant of a homogenate of infected protoplasts. As can be seen, the 170,000 molecular weight protein found in CPMV-infected protoplasts (lane A) has no counterpart among the *in vitro* products from B-RNA (lanes C and D). A minor band with the same mobility can be seen among the translation products from B-RNA in wheat germ extracts (lane D). In the results shown here, the B-RNA specific polypeptide BP is definitely larger than the virus-specific protein found in infected protoplasts. The 30,000 molecular weight polypeptide synthesized under direction of B-RNA migrates in these gels with the same mobility as the 30,000 molecular weight protein found *in vivo* (not clearly visible in this figure). This finding is in agreement with that of Rottier (1980).
By comparing the *in vivo* detectable proteins with the *in vitro* M-RNA products, it might be concluded that a 110,000 molecular weight protein has a similar electrophoretic mobility as MP_1 (cf. lanes A and E, F). However this result is doubted by observations of Rottier (1980).

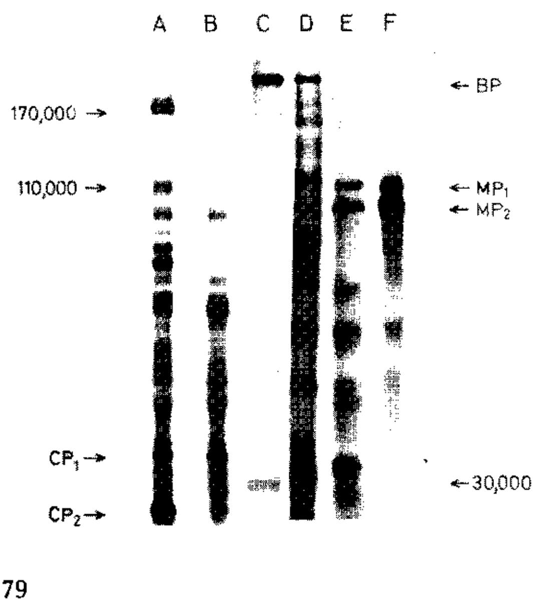

Fig. VI.3. *Comparison of the in vitro synthesized polypeptides with in vivo found virus-specific proteins*
Electrophoresis was performed in a 7.5% polyacrylamide gel in Laemmli buffer.
A: 30,000 × *g* supernatant of a homogenate of CPMV-infected protoplasts.
B: 30,000 × *g* supernatant of a homogenate of healthy protoplasts.
C: B-RNA translated in MDL.
D: B-RNA translated in wheat germ extract.
E: M-RNA translated in MDL.
F: M-RNA translated in wheat germ extract.

VI.3. Discussion

The comparison of the *in vitro* products synthesized in MDL and wheat germ extracts shows that there is little or no difference between the results in both types of cell-free protein synthesizing systems. Although some variation in the results can be observed, the 'real' translation products (see chapter V) have an identical electrophoretic mobility in two different types of gel system.

The comparison of the *in vitro* and *in vivo* detectable CPMV-specific proteins gives no clear answers as to whether the *in vivo* found CPMV-related proteins are really coded on the CPMV-RNAs, or if the *in vitro* synthesized polypeptides reflect an *in vivo* situation. Only a 30,000 molecular weight protein detected in CPMV-infected protoplasts is electrophoretically identical with a polypeptide detectable among the *in vitro* translation products from B-RNA (see also: Rottier, 1980). The nature of this protein is still unclear, but it is claimed to be a virus-specific protease for the specific cleavage of the precursor polypeptides synthesized from the CPMV-RNAs (Pelham, 1979), a phenomenon not observed here (see chapter VII).

The reason for the difference between the large protein found in CPMV-infected protoplasts and the *in vitro* B-RNA product BP can be, that *in vivo* the large precursor (identical to BP) is cleaved very rapidly. The 170,000 molecular weight protein found *in vivo* is coded by B-RNA as judged by its size, and proven to be so in an experiment in which protoplasts were infected with B-component only (Rottier, 1980). *In vitro* processing of possible precursor polypeptides could not been shown here (see chapter VII).

The nature of the 110,000 molecular weight protein, in these experiments identical to MP_1, is still unclear.

The conclusion from this work is that there is as yet little agreement between the *in vivo* and *in vitro* translation results. Further experiments (like peptide mapping and processing *in vitro*) have to be performed to elucidate the true origin of the 'CPMV-specific' proteins.

By all this one has to keep in mind that there is a big time difference between to two situations we are looking at. *In vitro* one is looking at *immediate de novo* synthesized products. *In vivo* however the first proteins detectable appear after at least 8 hrs (Rottier, 1980) in protoplasts; virus is then made already which indicates that the presumed processing has already occurred. It is therefore not completely suprising that the *in vitro* synthesized polypeptides show a different pattern to the *in vivo* detectable virus-specific proteins.

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VII THE TRANSLATION STRATEGY OF CPMV-RNAs

When the virion RNAs of CPMV were translated *in vitro*, coat proteins could not be detected among the synthesized products, but translation in almost full-size products occurred. These translation results are similar to those obtained with TMV-RNA and with the RNAs of the nepoviruses, respectively viruses of the first kind and third kind (see Introduction). The possibility however that the coat proteins are *in vivo* translated from an internal initiation site (like the viruses of the second kind), not accessible for ribosomes in *in vitro* translation, is not excluded. Therefore three different mechanisms are still possible for the synthesis of CPMV coat protein.

1. The coat proteins are synthesized from separate subgenomic mRNA(s) as with TMV.
2. The coat proteins are synthesized starting from an internal initiation site, not accessible for ribosomes in an *in vitro* translation system programmed with the viral genome RNAs.
3. The large polypeptides, found *in vitro*, are precursor molecules from which *in vivo* the coat proteins (and other virus specific proteins) are cleaved by posttranslational processing of the precursor.

In this chapter experiments concerning the translation strategy of CPMV-RNAs are described.

VII.1. Experimental results

Vigna leaves, infected with CPMV, were harvested five days after inoculation. On basis of the growth curve of CPMV in *Vigna* leaves (Van Griensven, 1970) the leaves were harvested at the moment that the coat protein production is believed to be maximal. Total RNA was extracted from the leaves and fractionated on a sucrose density gradient. Fig.VII.1., upper panel, shows the sedimentation pattern of the RNA in this gradient.

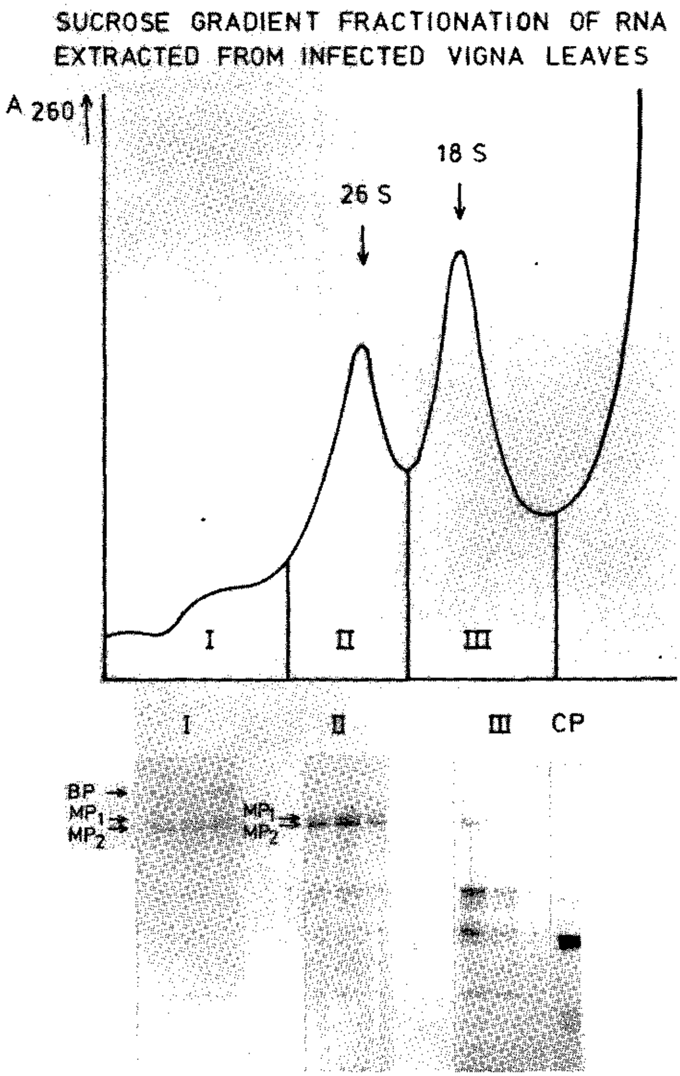

Fig. VII.1. *Translation of RNAs extracted from CPMV-infected leaves.*
RNA was extracted from CPMV-infected *Vigna* leaves and fractionated by sucrose density centrifugation. The upper panel shows the pattern of the RNA in the gradient. From different parts of the gradient RNA fractions were collected (indicated by I, II and III) and translated in MDL. Electrophoresis was performed in 15% polyacrylamide gel in Laemmli buffer. The lower panel shows the patterns of the translation products and coat proteins. Increasing amounts of RNA were added to the MDL.
From left to right:
I: 50, 95 and 185 μg RNA per ml.
II: 80, 150 and 300 μg RNA per ml.
III: 100, 215 and 435 μg RNA per ml.

From different parts of the gradient the RNA was collected (as indicated by I, II and III) and used as mRNA for *in vitro* translation. As can be seen in the lower panel of Fig.VII.1., RNA isolated from the part of the gradient were the virion RNAs are found (Fraction I and II) is translated into the well known polypeptides BP MP_1 and MP_2. Subgenomic messengers for CPMV coat protein would have a molecular weight of approximately 0.2×10^6 for the small coat protein and 0.35×10^6 for the large coat protein or 0.55×10^6 if both coat proteins are synthesized as one precursor protein. RNA molecules of that size are expected to sediment in that part of the gradient indicated by III. The RNA fraction from that part of the gradient is however not translated into products with the same electrophoretic mobility as coat proteins nor a product with a molecular weight of about 60,000 (the molecular weight of a hypothetical precursor for the coat proteins) can be detected. Experiments in which a similar procedure was used with RNA extracted from tobacco leaves infected with TMV, showed that TMV coat protein was synthesized when an RNA fraction, sedimenting betwee 6 *S* and 18 *S*, was translated *in vitro* (Hunter *et al.*, 1976). Further analyses of the RNA from TMV-infected leaves showed the LMC (0.25×10^6) to be the subgenomic messenger for TMV coat protein (Siegel *et al.*, 1976).

In another approach to reveal possible CPMV-specific messenger RNA molecules other than the virion RNAs, polysomes were isolated from infected and healthy leaf tissue according to Jackson and Larkins (1976). The polysomes were fractionated by sucrose density gradient centrifugation in linear (65-12,5%) gradients. The different size classes of polysomes collected from these gradients were translated in wheat germ extracts. The elongation (and translation) products were analyzed by polyacrylamide gel electrophoresis. The products from the polysomes were compared with the polypeptides synthesized under direction of B- or M-RNA. Shown here is the result of the translation of the smallest size-class of polyribosomes; similar results with the larger polysomes are not shown.

In Fig. VII.2. it can be seen that the polysomes from healthy tissue produce a number of polypeptides (lane C) of which a great deal is probably host specific. the polysomes from CPMV-infected leaf tissue synthesized these polypeptides too, but in addition to it the CPMV-specific BP, MP_1 and MP_2 are synthesized (lane B). Lanes A and D show the polypeptides synthesized under direction of B- and M-RNA. Again no coat proteins, of which the position in the gel is indicated by CP, could be detected among the translation products, indicating that there are no subgenomic messengers for coat protein present in this polysomal fraction.

Work describedelsewhere (Rottier, 1980) shows that analyses of the RNAs synthesized in CPMV-infected protplasts could not reveal any other CPMV-specific RNAs than the virion RNAs at any time during virus multiplication.

From all this it was concluded that it is highly unlikely that there exist subgenomic messenger(s) from CPMV-RNA.

The synthesis of large polypeptides, that utilize almost entirely the coding capacity of the RNAs, suggested that those RNAs act as monocistronic messengers for these large polypeptides, except perhaps for M-RNA where the two large polypeptides can be synthesized from two overlapping genes. The possibility can not be excluded that the *in vivo* situation could be different and that the CPMV-RNAs in cells act as polycistronic messengers: that is, the coat proteins are *in vivo* initiated at internal sites.

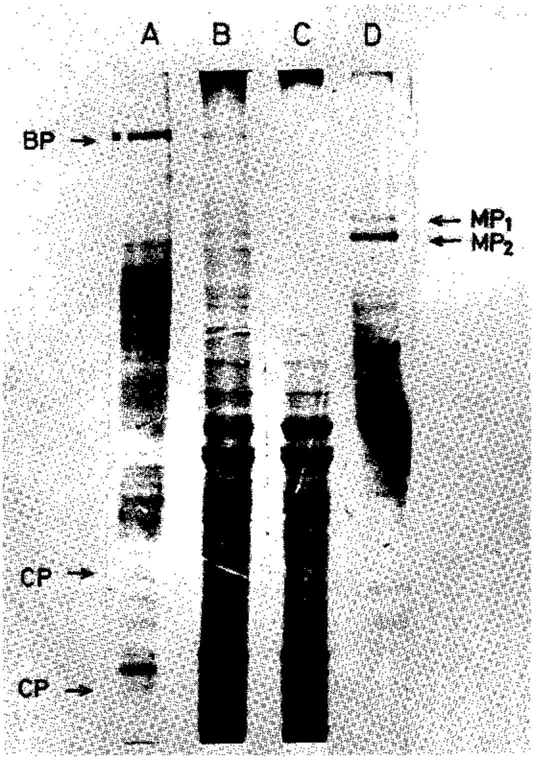

Fig.VII.2. *Translation of polysomes extracted from CPMV-infected and healthy Vigna leaves.*
Polysomes and CPMV-RNA were translated in wheat germ extracts.
Electrophoresis was performed in 7.5% polyacrylamide gels in Laemmli buffer.
A: B-RNA.
B: polysomes from CPMV-infected leaves.
C: polysomes from healthy leaves.
D: M-RNA.

Translation or replication of the RNAs might might alter the secondary structure of the RNAs, resulting in a situation in which internal initiation sites on the RNAs are made accessable for the ribosomes, whereas in the *in vitro* systems this does not occur. The experiment above described, in which polysomes were used to programme the *in vitro* translation system, indicated that polycistronic translation of the CPMV-RNAs (*in vivo*) is unlikely, since the same polypeptides as synthesized *in vitro* under direction of B- and M-RNA are synthesized from the virus specific polysomes (see Fig.VII.2.). Another possibility for revealing internal initiation sites, is to perform translation experiments with CPMV-RNA fragments that are smaller than the genomic RNAs. In experiments in which CPMV-RNA with a few random breaks was used, coat protein was never synthesized (results not shown). Also experiments in which partially digested (T_1-RNase) CPMV-RNAs were used as messengers did not reveal any coat protein (Stuik, unpublished results; Pelham, 1979a), but a number of smaller polypeptides were synthesized. These smaller polypeptides did not arise only by early termination because of the smaller RNA molecules, but were also synthesized from different — normally 'closed' initiation sites — as shown by Pelham (1979a). The nature of these other 'initiation sites', that are not used on intact RNAs, is unknown, but could be the result of false initiation or accidental or normal occuring internal AUG sequences, normally not used as start codons.

The results reported here show firstly that *in vitro*, coat proteins are not directly translated from the virion RNA, CPMV specific polysomes or smaller RNAs, *in vitro* generated from the intact CPMV-RNAs. Secondly the existence of subgenomic messengers could not be demonstrated.

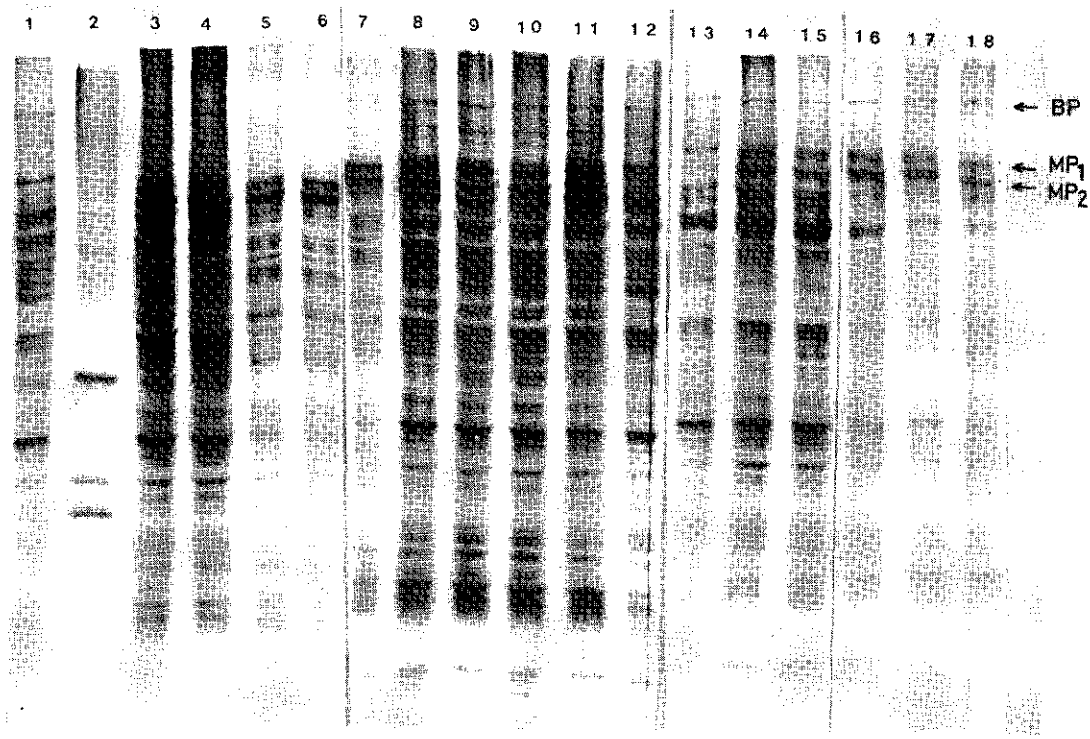

Fig. VII.3. *'Processing' in wheat germ extracts.*
B- and M-RNA were translated in wheat germ extracts for different times.
Mixtures of the translation products or the separate incubations were further incubated.

1: B-RNA incubated for 6 hrs.
2: Marker proteins, β-galactosidase (116,000), phosphorylase A (92,000), transferrine (80,000), BSA (68,000) and CPMV coat proteins.
3: B-RNA incubated for 6 hrs, M-RNA incubated for 3 hrs; mixed and further incubated for 1 hr.
4: B-RNA incubated for 6 hrs, M-RNA incubated for 6 hrs; mixed and further incubated for 1 hr.
5: M-RNA incubated for 6 hrs.
6: M-RNA incubated for 3 hrs.
7: M-RNA incubated for 1 hr.
8: M-RNA and B-RNA separately incubated for 1 hr, mixed and further incubated for 1 hr.
9: M-RNA and B-RNA separately incubated for 1 hr, mixed and further incubated for 3 hrs.
10: M-RNA incubated for 1 hr, B-RNA incubated for 6 hrs; mixed and further incubated for 1 hr.
11: M-RNA incubated for 1 hr, B-RNA incubated for 6 hrs; mixed and further incubated for 3 hrs.
12: B-RNA incubated for 3 hrs.
13: B-RNA incubated for 1 hr.
14: B-RNA incubated for 1 hr, M-RNA incubated for 3 hrs; mixed and further incubated for 1 hr.
15: B-RNA incubated for 1 hr, M-RNA incubated for 6 hrs; mixed and further incubated for 1 hr.
16: CPMV-RNAs (B + M) incubated 1 hr.
17: CPMV-RNAs (B + M) incubated for 3 hrs.
18: CPMV-RNAs (B + M) incubated for 6 hrs.

proposed to be a virus specific protease, responsible for the processing events, was also observed in some (but not all) experiments. If such a protease is synthesized from B-RNA it is very strange that the activity can only be observed when MDL is used. Furthermore, the cleavage products observed by Pelham in MDL do not agree with the virus specific polypeptide pattern from infected protoplasts (Rottier, 1980).

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SUMMARY

The study described here concernes the proteins that are synthesized under direction of CPMV-RNAs.
In chapter III it is described that sufficient radioactive labelling of proteins was achieved when ^{35}S as sulphate was administered to intact *Vigna* plants, cultivated in Hoagland solution. After fractionation of the cells, a fraction containing virus-specific (vesicle) structures could be isolated in which the majority of the replicase activity and CPMV-specific dsRNA was located. Comparison of proteins extracted from these fractions from infected and mock-infected leaves resulted in the detection of two proteins, besides the coat proteins. These proteins, with molecular weights of about 170,000 and 72,000 could only be detected after the development of the radioactive labelling technique using ^{35}S as sulphate.
In vitro studies using the messenger-dependent lysate from rabbit reticulocytes (MDL) and wheat germ extracts show that the virion RNAs stimulate the incorporation of amino acids in acid precipitable material. The optimization and characteristics of both *in vitro* systems are described in chapter IV.
Product analysis of the synthesized polypeptides shows that the CPMV-RNAs can be reproducibly translated into well defined products (chapter V), indicating that these RNAs act well as messengers in the cell-free systems used. From the characteristics of these systems and the product analysis, it has become clear that CPMV-RNAs needed particular conditions for their *in vitro* translation (chapter IV and V). In wheat germ extracts the addition of spermidine (0.4 mM) together with a lowering of the Mg^{2+} concentration to about 3.0 mM was required for the efficient translation of CPMV-RNAs. Furthermore, increasing the K^{+} concentration to 130 mM was advantageous for the translation into high molecular weight polypeptides. In the MDL the addition of heterologous tRNA was absolutely necessary for the translation of CPMV-RNA into high molecular weight products.
Estimations of the molecular weights by polyacrylamide gel electrophoresis in SDS containing gels, of the products synthesized, showed that there is a considerable variation in the molecular weights determined. The molecular weight of BP (the largest product synthesized under direction of B-RNA) was estimated to be 180,000 to 190,000. MP_1 (the large molecular weight product synthesized under direction of M-RNA) has a molecular weight that varies from 110,000 to 125,000. The other large polypeptide synthesized under direction of M-RNA (MP_2) has an estimated molecular weight between 105,000 and 95,000.
Comparison between the polypeptides synthesized in both types of extract shows that the products are — at least electrophoretically — identical (chapter VI). Also in chapter VI it is described that comparison with virus-specific proteins found *in vivo* gives little information about either the origin of the virus-specific proteins found *in vivo* or about the nature of the *in vitro* synthesized polypeptides.
In all the *in vitro* translation experiments no coat proteins could be detected among the translation products. In chapter VII experiments are described which were undertaken to reveal the translation strategy of CPMV-RNAs. From these experiments it was concluded that it was very unlikely that the coat proteins are synthesized from

subgenomic messengers nor from internal initiation sites.
The overall conclusion is that the large polypeptides synthesized under direction of B- and M-RNA are probably precursor molecules from which the coat proteins (and other virus-specific proteins) are generated by a mechanism of posttranslational cleavage. Processing experiments in either wheat germ extracts or MDL did not, up until now, reveal any precursor-product relationship between the polypeptides synthesized. This is in contrast to other findings where in MDL the M-RNA specific products could be processed (Pelham, *Virology* 96, 463), albeit into products not recognized as being virus-specific proteins.

SAMENVATTING

Dit proefschrift handelt over de eiwitten die gesynthetiseerd worden onder invloed van het cowpea mozaiëk virus.
Het cowpea mozaiëk virus (CPMV) is een enkelstrengs RNA virus waarvan het genoom verdeeld is over twee RNA moleculen die beide nodig zijn voor een succesvolle infectie. Het RNA van CPMV heeft een structuur die erg veel lijkt op die van de virussen uit de dierlijke picorna virus groep en dat van de nepovirussen. Met name de aanwezigheid van een (klein) eiwit aan het 5'-eind en een polyA staart aan het 3'-eind van de CPMV-RNAs duidt hierop. In de inleiding wordt beschreven hoe plantenvirussen met een enkelstrengs (+) RNA genoom ingedeeld kunnen worden in virussen van drie verschillende soorten, op basis van hun 'translatie strategie'.
1. virussen die via zg. subgenomische messengers er voor zorgen dat al de vertaalbare RNA moleculen monocistronische messengers zijn.
2. virussen waarvan het RNA genoom als polycistronische messenger vertaald wordt.
3. virussen waarbij het RNA genoom vertaald wordt in een groot eiwit, waarna de andere (virus-specifieke) eiwitten ontstaan nadat er een proteolytische splitsing van het grote eiwit heeft plaats gevonden.
De indeling van CPMV bij een van deze virus soorten wordt ook hier beschreven. In eerste instantie is gekeken naar de eiwitten die na virus infectie opgespoord kunnen worden in cowpea planten. Hiertoe was het noodzakelijk om een goede radioactieve labellings techniek te ontwikkelen voor eiwitten en bladeren.
In hoofdstuk III wordt beschreven dat de ontwikkeling van deze techniek resulteerde in een labellingsmethode waarbij hele planten — op watercultures — gevoed werden met ^{35}S in de vorm van sulfaat.
Celfractionering van *Vigna* bladeren leidde tot de isolatie van een fraktie, waarin de meerderheid van de replicase activiteit en van het CPMV-specifieke dsRNA kon worden aangetroffen. Polyacrylamide gel electroforese van eiwitten uit deze frakties, uit gezond en met CPMV geïnfecteerde bladeren, toonde aan dat er twee eiwitten konden worden aangetoond buiten de CPMV mantel eiwitten. Deze eiwitten, met een moleculair gewicht van ongeveer 170,000 en 72,000, konden allen worden aangetoond nadat de labellings techniek ontwikkeld was.
Vertaling van de CPMV-RNAs in zowel een celvrij systeem van konijnen reticulocyten als in tarwe kiemen extrakten toonden aan dat de CPMV-RNAs in staat waren om de inbouw van amino zuren in zuur-precipitabel materiaal te stimuleren. De optimalisering van het 'messenger-dependent reticulocyte lysate' (MDL) en het tarwe kiemen systeem wordt beschreven in hoofdstuk IV. Verder is daarin ook beschreven wat de karakteristieken van beide systemen zijn. Uit deze karakteristieken en uit de analyse van de gesynthetiseerde producten (hoofdstuk IV en V) werd duidelijk dat de CPMV-RNAs bijzondere omstandigheden nodig hebben om tot een efficiënte vertaling te komen in de beide systemen. In tarwe kiemen extrakten bleek toevoeging van spermidine (0.4 mM) tegelijk met een verlaging van de magnesium concentratie (naar ongeveer 3 mM) noodzakelijk voor een efficiente vertaling. Verder bleek dat een verhoging van de kalium concentratie naar 130 mM zeer voordelig werkte op de synthese van hoog moleculaire producten. In MDL bleek de toevoeging

van heteroloog tRNA absoluut noodzakelijk om te komen tot een vertaling van de CPMV-RNAs in hoog moleculaire producten.
De schattingen van de molecuul gewichten van de producten die gesynthetiseerd werden onder invloed van de beide CPMV-RNAs liepen nogal uiteen in de verschillende experimenten. BP, het grote polypeptide dat gesynthetiseerd werd onder invloed van B-RNA gaf een molecuul gewicht dat varieerde van 190,000 tot 180,000. Het grootste polypeptide van M-RNA (MP_1) gaf een variërend molecuul gewicht te zien van 110,000 tot 125,000. Het iets kleinere M-RNA specifieke polypeptide MP_2 werd geschat op een molecuul gewicht dat ligt tussen de 105,000 en 95,000.
Een vergelijking tussen de produkten die in de verschillende soorten celvrije systemen gesynthetiseerd werden toonde aan dat er — ten minste wat de electroforetische mobiliteit betreft — geen verschil bestaat tussen de producten die gemaakt werden in het ene of in het andere celvrije systeem (hoofdstuk VI). Verder wordt in hoofdstuk VI beschreven dat de vergelijking tussen de polypeptiden die *in vitro* gesynthetiseerd werden en de eiwitten die *in vivo* aangetroffen werden, weinig informatie op leverde over de oorsprong van de virus-specifieke eiwitten uit geïnfecteerde cellen, of over de aard van de *in vitro* gesynthetiseerde producten.
In alle *in vitro* translatie experimenten konden de mantel eiwitten niet aangetroffen worden tussen de translatieproducten. In hoofdstuk VI worden experimenten beschreven die uitgevoerd werden om de translatie strategie van de CPMV-RNAs op te helderen. De conclusie van deze experimenten was dat het zeer onwaarschijnlijk is dat de mantel eiwitten vertaald worden van zg. subgenomische messengers. Ook vertaling vanaf een interne startplaats wordt onwaarschijnlijk geacht.
De uiteindelijke conclusie luidt dan ook dat de beide CPMV-RNAs vertaald worden in grote eiwitten waaruit de mantel eiwitten (en andere virus-specifieke eiwitten) ontstaan door proteolytische splitsing van de precursor moleculen. Door dit translatie mechanisme wordt CPMV dan ook ingedeeld bij de virussen genoemd onder 3.
In dit proefschrift worden ook zg processing experimenten beschreven, die echter geen precursor-product relatie konden aantonen voor *in vitro* gesynthetiseerde eiwitten. Dit is niet in overeenstemming met andere experimenten waarin de M-RNA producten specifiek gesplitst konden worden (Pelham, *Virology* 96, 463), echter in producten die niet herkend kunnen worden als virus-specifieke eiwitten.

CURRICULUM VITAE

Eugène Johannes Stuik werd op 6 november 1945 te Amsterdam geboren.
Na het behalen van het gymnasium β diploma werd in 1965 aangevangen met de studie scheikunde aan de Universiteit van Amsterdam. In december 1972 werd het doctoraal diploma behaald met als hoofdvak biochemie en als bijvakken plantenfysiologie en radiochemie.
Van januari 1971 tot mei 1974 was hij werkzaam als wetenschappelijk ambtenaar op het Coronel laboratorium van de Universiteit van Amsterdam, alwaar onderzoek verricht werd naar de toxicologie van zware metalen — lood in het bijzonder — in het menselijk lichaam, onder leiding van prof. dr. R.L. Zielhuis.
Vanaf mei 1974 was hij verbonden aan de vakgroep moleculaire biologie van de landbouw hogeschool te Wageningen, daartoe in staat gesteld door de stichting Z.W.O. met financiële hulp van S.O.N.