EARLY STAGES IN COWPEA CHLOROTIC MOTTLE VIRUS INFECTION

J.W.ROENHORST

Promotor: Dr. R.W. Goldbach
Hoogleraar in de Virologie
Co-promotor: Dr. Ir. B.J.M. Verduin
Universitair Hoofddocent bij de vakgroep Virologie

Johanna Wesselina Roenhorst

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Ik bedank iedereen die heeft bijgedragen aan het tot stand komen van dit proefschrift.

STELLINGEN

1. Het bestuderen van initiële interacties tussen plantevirus en plantecel wordt sterk bemoeilijkt door het feit dat slechts enkele van de bij inoculatie in overmaat aanwezige virusdeeltjes verantwoordelijk zijn voor infectie.
   Dit proefschrift.

2. De penetratie-mechanismen endocytose en fusie zijn voor virus-infecties bij planten van geen of ondergeschikt belang.
   Hsu *et al.* (1983). *In* "Current Topics in Vector Research". (K.F. Harris, Ed.), Vol. 1, pp. 189-214. Praeger Publisher s, New York.
   Dit proefschrift.

3. Het feit dat Sulzinski en Zaitlin de onderzijde, en Fannin en Shaw de bovenzijde, van het blad gebruikten voor inoculatie verklaart mogelijk waarom alleen de eerstgenoemden in staat waren primaire infectie in mesophylcellen te bewerkstelligen.
   Sulzinski and Zaitlin (1982). *Virology* 121, 12-19.
   Fannin and Shaw (1987). *Plant Science* 51, 305-310.

4. De conclusie van Gergerich en Scott dat door kevers overgebrachte virussen in staat zijn niet-verwonde cellen te infecteren is voorbarig te noemen, daar zij geen rekening hebben gehouden met de mogelijkheid dat stoombehandeling leidt tot verwonding van cellen.
   Gergerich and Scott (1988). *J. gen. Virol.* 69, 2935-2938.

5. De suggestie van Horikoshi en medewerkers dat de remmende werking van het manteleiwit op de RNA synthese van "brome mosaic virus" *in vitro* te wijten zou zijn aan interactie met de bindingsplaats van het replicase wordt onvoldoende ondersteund door hun experimentele resultaten.
   Horikoshi *et al.* (1987). *Virology* 158, 15-19.

6. Het feit dat in met luzernemozaïekvirus geïnoculeerde tabaksbladeren het eiwit met een moleculaire massa van 32,000 Dalton wordt aangetroffen in de middenlamellen van celwanden aan het infectiefront toont niet aan dat het op deze plaats ook een functie in virustransport heeft.
   Stussi- Garaud *et al.* (1987). *J. gen. Virol.* 68 1779-1784.

7. De isolatie van natuurlijke tobravirus recombinanten toont opnieuw aan dat identificatie op basis van serologie niet sluitend is.
   Robinson *et al.* (1987). *J. gen. Virol.* 68, 2551-2561.

8. Gezien het sterk homologe karakter van viroïden verdient het aanbeveling nieuwe isolaten van deze pathogenen op basis van hun nucleotidenvolgorde te identificeren.

9. Letterlijk vertaalde gebruiksaanwijzingen zijn zelden verhelderend.

10. De status welke ontleend wordt aan het dragen van ambtskleding wordt in sterke mate bepaald door de omgeving.

11. Gezien het gevaar dat onoordeelkundig gebruik van chemicaliën met zich mee brengt, verdient het aanbeveling in het onderwijsprogramma van potentiële gebruikers meer aandacht te besteden aan het veilig werken met deze stoffen.

12. Te hard rijden kost in Nederland te weinig.

Stellingen behorend bij het proefschrift:

"Early stages in cowpea clorotic mottle virus infection"

10 oktober 1989 J.W. Roenhorst

CONTENTS

CHAPTER 1

INTRODUCTION

"Viruses are entities whose genome is an element of nucleic acid either DNA or RNA, which reproduce inside living cells and use the cell's synthetic machinery to direct the synthesis of specialized particles, the virion, which contain the viral genome and transfer it to other cells", according to the definition presented by Luria and Darnell (1967). More recently, Harrison (1984) described a virus particle as "a structure for transferring nucleic acid from one cell to another", adding that "the nucleic acid may be either RNA or DNA and, in both cases particles of varying complexity are found. Observed structures reflect requirements for efficient and accurate assembly, for exit and re-entry, and for correctly localized disassembly". These definitions emphasize three characteristics of the virus particle: i) its infectivity, i.e., the ability to multiply upon penetration into a suitable host cell and to be transferred to other cells, ii) the ability to exist in a non-cellular state, and iii) the obligate parasitism at the genetic level.

Upon penetration into a susceptible host cell, the virus particle disassembles and the viral genome directs the cellular machinery to replication of viral nucleic acids and synthesis of virus specific proteins. Newly synthesized nucleic acids and coat protein molecules are assembled to new nucleocapsids, and sometimes surrounded by a lipid membrane, which are then ready for starting a next round of infection.

During the last decades, much progress has been made in the understanding of the multiplication process of eukaryotic viruses. The emerge of molecular and cell biological techniques provided tools to elucidate the structural organization of the viral genome and its strategies for replication and expression. In addition, biophysical studies provided information on the architecture and assembly of nucleocapsids. However, the knowledge of early stages in virus infection, i.e., penetration into the (host) cell and uncoating of the viral genome, has remained very scarce and fragmentary. For plant viruses even less data seem to be available than for animal viruses.

The aim of the experiments described in this thesis was to gain more insight in the early stages in plant virus infection. The two central questions to be answered were: (1) how and in what form does the plant virus enter the cytoplasm of a newly infected cell, and (2) what mechanism is responsible for uncoating of the plant viral nucleocapsid?

With regard to the ways by which plant viruses enter (host) cells several mechanisms have been proposed (for review see Shaw, 1985). Specific interactions with both the cell wall (Gaard and de Zoeten, 1979; De Zoeten, 1981; De Zoeten and Gaard, 1984) and plasma membrane (Banerjee *et al.*, 1981a,b; Durham, 1978) have been suggested to be involved in virus entry. Passage of the plasma membrane was supposed to occur by endocytosis (Cocking and Pojnar, 1969; Cocking, 1970; Takebe, 1975) or through pores or lesions formed as a result of damage of the membrane (Burgess *et al.*, 1973a,b; Kassanis *et al.*, 1977; Watts et al., 1981).

Also for uncoating of the viral nucleocapsids several mechanisms have been proposed. Based on results in different experimental systems indications were obtained for uncoating to take place prior to or during penetration into the plant cell (De Zoeten, 1981; Durham, 1978) as well as after appearance in the cytoplasm (Wilson, 1985). In the former case, both cell wall and plasma membrane have been suggested to be involved in the process of uncoating. In the latter case, cytoplasmic ribosomes were supposed to take part in a process of cotranslational disassembly of metastable virus particles.

The inconclusive results on the initial interactions between plant viruses and cells prompted us to reinvestigate these early stages of infection. The various studies described in this thesis all focussed on the cowpea chlorotic mottle virus (CCMV) - cowpea protoplast system.

First, experiments were performed in order to investigate the role of different entry mechanisms, i.e., direct penetration and endocytosis, in infection of plant protoplasts. Therefore, binding of CCMV to cowpea protoplasts was studied under various conditions in relation to virus entry and infection (Chapter 3).

With regard to the way of uncoating of the CCMV genome the possible involvement of cotranslational disassembly, as first proposed for tobacco mosaic virus (TMV) (Wilson, 1984), was tested in cell-free translation systems (Chapter 4). Referring to the observed association of virus particles and ribosomes, a further characterization of this interaction was initiated by studying binding of CCMV to ribosomal proteins in electroblot assays (Chapter 5). Finally, isolated cowpea protoplasts were used to investigate the possible role of cotranslational

disassembly in uncoating of CCMV *in vivo* (Chapter 6).

CCMV was chosen because of its relatively simple structure, the extensive knowledge of its nucleoprotein particles and the protein-nucleic acid interactions (Verduin, 1978; Krüse, 1979; Vriend, 1983). CCMV is a small spherical plant virus belonging to the bromovirus group. This virus group comprises three definite members: the type member brome mosaic virus (BMV), broad bean mottle virus (BBMV), and CCMV. The properties of these viruses have been extensively reviewed (Bancroft, 1970; Lane, 1974; Bancroft and Horne, 1977; Lane, 1979). CCMV consists of three types of nucleoprotein particles containing four species of single-stranded, positive-sense RNA molecules (RNA-1, -2, -3, and -4) with lengths of 3171, 2776, 2173, and 835 nucleotides, respectively (Allison *et al.*, 1989; Dzianott and Bujarski, 1989) (Fig. 1). RNA-1 and RNA-2 are encapsidated separately, and RNA-3 and RNA-4 are packed together in polyhedral particles about 26 nm in diameter (Lane, 1974). The coat of each particle consists of 180 identical protein subunits with M_r 20,253 arranged in a shell with icosahedral symmetry and a triangulation number of three (Caspar and Klug, 1962; Dzianott and Bujarski, 1989). On basis of their buoyant density, the three nucleoprotein particles are denoted as heavy (H; RNA-1), medium dense (M; RNA-3 and -4), and light (L; RNA-2) particles (Bancroft and Flack, 1972). RNA-1 and RNA-2 code for polypeptides with molecular masses of 109,006 Da and 92,789 respectively (Davies and Verduin, 1979; Dzianott and Bujarski, 1989). RNA-3 directs the synthesis of a 33,075 M_r product while RNA-4 is a RNA-3-derived subgenomic messenger RNA for capsid protein (Fig. 1). For successful infection of plants, all three nucleoprotein particles are required. When inoculating with extracted RNA, however, infection is obtained with a mixture of RNA-1, -2, and -3.

The function of the RNA-1, -2, and -3 products in CCMV infection is unknown. However, in analogy to BMV it can be suggested that the RNA-1 and -2 encoded polypeptides are involved in viral replication (Kiberstis *et al.*, 1981). The RNA-3 product, most probably, is involved in spread of infection throughout the plant.

The *in vitro* dissociation and association processes of CCMV as a model for *in vivo* uncoating and assembly, respectively, have been extensively reviewed and studied by Verduin (1978). *In vitro* CCMV, like all members of the bromovirus group, appears to be stable around pH 5.0, and to sediment at 88 S. For BMV an increase of pH to 7.5 at low ionic strength ($\mu < 0.2$) was found to cause "swelling" of the particles and to make the virus sensitive to RNases and proteases (Pfeiffer and Hirth, 1975). This swelling causes a drop in sedimentation coefficient to 78 S,

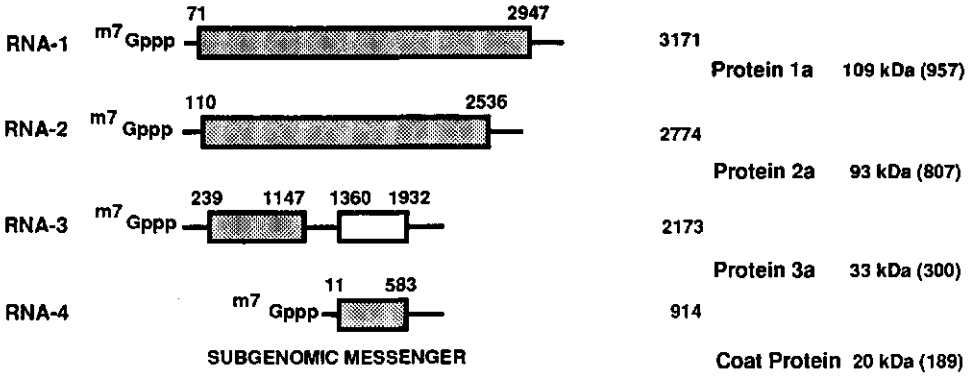

Figure 1. Schematic representation of CCMV, showing genomic organization and proteins encoded by the viral genome. Open-reading frames in the RNAs are represented as open bars, with the nucleotide positions of start and stop codons indicated; numbers at the right indicating total length. Shaded bars represent open-reading frames that are translated into viral proteins with molecular masses as listed at the right of the figure; numbers between brackets indicating the number of amino acid residues. RNA-1 and RNA-2 both function as monocistronic mRNA. RNA-3 contains two cistrons of which the second (open bar) is only translated from a subgenomic mRNA, RNA-4.

and appears to be irreversible. Reversibility can be obtained in the presence of divalent cations (i.e., Mg^{2+}), but in this case swelling at pH 7.5 does not proceed to its full extend (Chauvin *et al.*,1978). Raising the pH at increased salt concentration ($\mu > 0.5$) causes the swollen virus to dissociate into RNA-protein complexes and protein dimers (Bancroft and Hiebert, 1967). A schematic view of the swelling and dissociation is given in Figure 2.

Isolated mesophyll protoplasts of cowpea, *Vigna unguiculata* cv. California Blackeye, were used to study early stages in plant virus infection. Protoplasts can be prepared from plant tissue by a treatment with enzymes macerating the tissue and degrading the cellulose wall of plant cells (Cocking, 1960; Takebe *et al.*, 1968). Such isolated protoplasts can be inoculated with plant viruses or even with their naked genome *in vitro*, and are capable of synthesizing intact new virus particles (for reviews see Zaitlin and Beachy, 1974; Takebe, 1975; Mühlbach, 1982; Takebe, 1983; Sander and Mertes, 1984). In contrast to intact plants, relatively high numbers of homogenous cells can be infected simultaneously, while cell-to-cell spread is excluded. Therefore, this defined cell system allows the investigation of the basic molecular processes in virus replication at the cellular level.

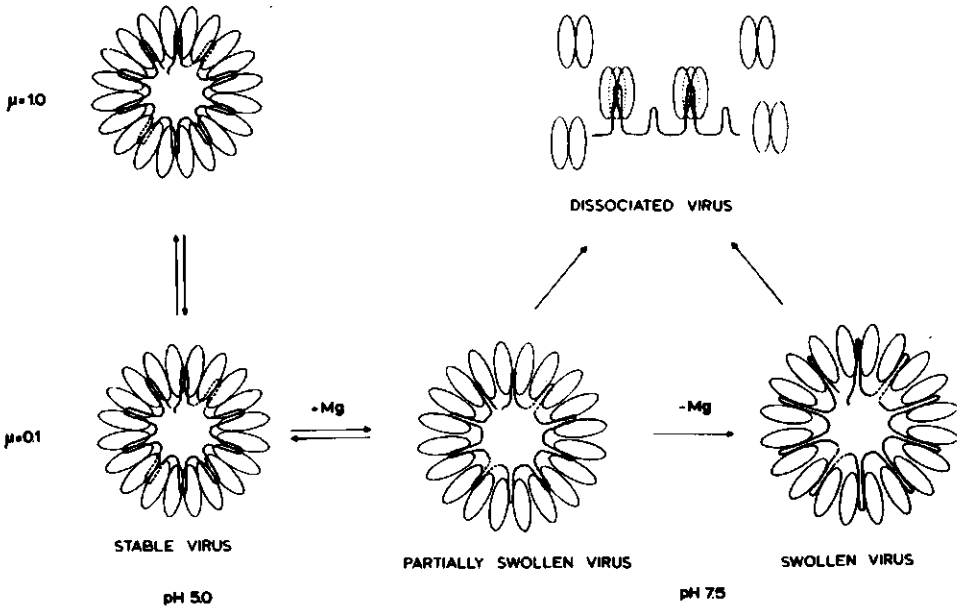

Figure 2. Schematic representation of the swelling and dissociation of the bromoviruses. In vertical position ionic strength and in horizontal position pH has been varied. At low ionic strength and pH 7.5 the influence of magnesium ions on swelling is shown. A virus particle is represented by a cross section of the icosahedral particle, the strings and ellipses representing RNA molecules and protein subunits, respectively (from Verduin, 1978).

It should be realized, however, that a protoplast system has its limitations. Protoplasts are single cells that do not exist under natural conditions. They lack a rigid cell wall and cell-to-cell connections are absent. Isolated protoplasts are cultured in media totally different from the environment in plant tissue with respect to among others nutrient composition, hormone balance and tonicity. Several authors have documented the effects of osmotic stress in protoplasts of various sources (Lazar *et al.*, 1973; Premecz *et al.*, 1978; Fleck *et al.*, 1982). In isolated protoplasts a dramatic change in gene expression was observed, including a decrease in total RNA and protein synthesis (Fleck *et al.*, 1982). On the other hand, a more than ten-fold increase of RNase level was found (Lazar *et al.*,1973). Whether such altered physiological state influences virus multiplication is not known. Nevertheless, it might be clear that experimental data obtained with isolated protoplasts should be interpreted with caution.

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CHAPTER 2

EARLY EVENTS IN VIRUS INFECTION A COMPARISON BETWEEN PLANT AND ANIMAL VIRUSES

CONTENTS

1. INTRODUCTION

Initial interactions between a virus and its host cell have been studied in much less detail than other stages of the viral infection process. Reasons for this are the complexity of the cellular membrane, the additional cell wall in case of plant cells, and the fact that only a few percent of the attaching and penetrating virus particles are actually causing infection. Initial interactions are defined as those essential activities occurring during the first contact between virus and cell until the moment that virus-directed translation or transcription starts. In these interactions three discrete processes may be distinguished:

i) attachment of virus particles to cells,
ii) passage through the cellular membrane (cell wall), and
iii) release of the viral genome from the nucleocapsids.

These distinct processes, which in fact represent a great variety of interactions, will be illustrated by a description of some of the best characterized mechanisms as shown schematically in Figure 1.

For plant viruses even less data on initial interactions are available than for animal viruses. Therefore this review will start with a brief overview of the present knowledge of mechanisms employed by animal viruses (Section 2). Plant viruses are discussed in the next section (Section 3) and where possible the available data are compared with the current models and hypotheses in animal virology.

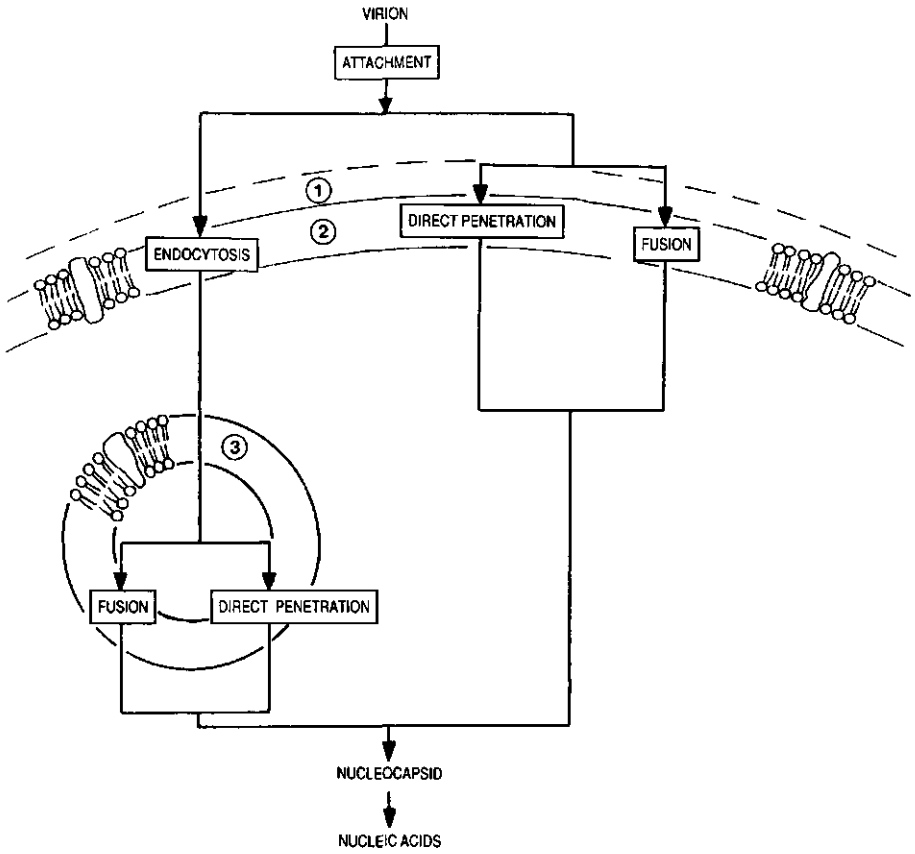

Figure 1. Schematic representation of initial interactions between a virus and its host cell, for both animal- and plant systems. "Virion" representing both enveloped and non-enveloped viruses; numbers indicating cell wall (1), plasma membrane (2), and endosomal membrane (3).

2. ANIMAL VIRUSES

For a considerable number of animal viruses initial stages of infection have been investigated and extensively reviewed (Lonberg-Holm and Philipson, 1974; Bukrinskaya, 1982; Dimmock, 1982; Marsh, 1987). Although a great variety of early interactions seems to exist, most animal viruses, when infecting a cell, follow one of the routes indicated in the scheme in Figure 1. The next paragraphs will consider successive stages of these routes, focussed on some of the most extensively studied viruses.

2.1. Attachment

The first step of infection involves binding of the virus to the cell surface. Although this attachment does not automatically guarantee successful entry, it is a necessary step (Mims, 1986). Attachment, which leads to infection in most cases, is the result of a specific interaction between cellular receptors and viral attachment proteins. Cells lacking the proper receptor are infected either very inefficiently or not at all.

The specificity of binding varies for different viruses. Some viruses bind to a wide range of cell types, whereas others are highly restricted. This may depend on either the distribution of a certain receptor over different cell types or the ability of the virus to bind different receptors.

2.1.1. Cellular Receptors

Host-cell receptors for viruses can be defined as those structures on the cell surface, which bind virus as a prerequisite for infection. Any normal constituent of the plasma membrane is a potential virus receptor. These components include phospholipids, glycolipids and integral membrane proteins or glycoproteins, which serve normal cellular functions (Co *et al.*, 1986).

Relatively few viral receptors have been identified thus far. The number of receptors is usually very low: 10^4 to 10^5 molecules per cell (Lonberg-Holm and Philipson, 1981). It is often difficult to distinguish non-specific from specific binding. Possible host-cell receptors for viruses that have been reported in literature are listed in Table 1. It should be noted that some receptors are better characterized than others and not all reported receptors are widely accepted.

Table 1. Host-cell receptors for viruses.

Virus	Host-cell receptor
adenovirus	class I HLA histocompatibility molecule
Epstein-Barr virus	C3d receptor CR2 of B lymphocytes
hepatitis B virus	hepatocyte receptor for polymerized serum albumin (PSA) via PSA hepatocyte receptor for polymeric IgA
human cytomegalovirus	class I HLA histocompatibility molecule via β_2-microglobulin
human immunodeficiency virus	CD4 (T4) molecule of T lymphocyte
human T-cell leukemia virus	class I HLA histocompatibility molecule interleukin 2 receptor
influenza viruses	sialoglycoproteins and sialoglycolipids (gangliosides)
lactate dehydrogenase virus	class II Ia histocompatibility molecule of macrophage
murine leukemia virus	lymphoma cell surface IgM T-cell receptor
rabies virus	acetylcholine receptor sialoglycoproteins
reovirus 3	β-adrenergic receptor sialoglycoproteins
Semliki Forest virus	class I HLA and H-2 histocompatibility molecules
vaccinia virus	epidermal growth factor receptor
vesicular stomatitis virus	phosphatidylserine

according to Lentz (1988)

The nature, number and distribution of host-cell receptors are important factors in determining the host range or tissue tropism of a virus (Fields and Greene, 1982; Mims *et al.*, 1986). Viruses infecting lymphocytes bind to molecules present on the surface of these cells such as complement receptors, immunoglobulins and T-cell receptors. On the other hand, different cells may be infected by the same virus when they express the appropriate receptor. The T4-receptor is found on the surface of brain cells as well as on T-lymphocytes, explaining the dual neurotropic and lymphotropic character of human immunodeficiency virus (Maddon *et al.*, 1986). In some cases, virus particles coated with subneutralizing concentrations of antibody can bind to and be internalized by cells with surface Fc receptors (Gollins and Porterfield, 1984).

For some host-cell receptors the structure of the receptor site has been identified. One of the best known is the sialic acid of oligosaccharides, present on many cell-surface glycoproteins and glycolipids, as the influenza virus hemagglutinin's receptor (Wiley and Skehel, 1987).

2.1.2. Viral Attachment Proteins

Viral attachment proteins are those proteins located on the surface of the virus particle that are involved in attachment to host-cell receptors. These proteins are either virus specific integral membrane glycoproteins (spikes) of enveloped viruses or capsid proteins of non-enveloped viruses.

Among the best characterized viral attachment proteins are those of influenza virus (Wilson, 1986; Wiley and Skehel, 1987), poliomyelitis virus (Hogle *et al.*, 1985) and human rhinovirus 14 (Rossmann *et al.*, 1985). X-ray crystallographic analyses provided the three-dimensional structure of the surface proteins of these viruses, which enabled us to study their interaction with the host in more detail.

The hemagglutinin, which is the attachment protein of influenza virus, is composed of a globular head and a fibrous tail (Wilson, 1986; Wiley and Skehel, 1987). The globular head has many exposed loops extending to the surface furthest from the viral membrane. The sialic acid binding site is located at the center of these protruding loops, consisting of a highly conserved pocket. The amino acid chains forming the surface of the pocket are positioned in such a way that they can make direct contact with the cellular receptor. The binding site is inaccessible to neutralization by antibodies, which explains its high degree of conservation. In contrast, the protruding loops at the surface show a high degree of antigenic variation. Hence, extensive changes at the surface enables the virus to escape immune recognition, while the essential receptor-recognition site remains preserved.

For poliovirus and human rhinovirus, both non-enveloped viruses, the receptor binding site is located in a cleft in the protomer surface (Hogle *et al.*, 1985; Rossmann *et al.*, 1985). One side of the cleft is formed by the VP1-pentamer; the other side by VP2 and VP3 subunits. In case of rhinovirus, mutations leading to resistance to monoclonal antibodies occurred in amino acids which protrude from the protomer surface (Rueckert *et al.*, 1986). The floor of the cleft, which most probably is the receptor recognition site, appears to be protected from attack by antibodies, which are too big for penetration.

Thus, as far as three-dimensional structures are available, for both enveloped and non-enveloped viruses viral attachment proteins seem to contain highly conserved receptor recognition sites inaccessible to neutralization by antibodies, while protruding surface structures accommodate extensive mutations enabling the virus to escape immune recognition.

2.2. Penetration

Entry of animal viruses into their host cells occurs either directly through the plasma membrane, or, after endocytosis of the virus particles, through the membrane of an endocytotic vesicle or endosome (Figure 1.).

Most viruses enter via the latter, indirect, way. Following attachment, both receptors and attached virus particles are internalized by absorptive or receptor mediated endocytosis. Some viruses, e.g., influenza virus and Semliki Forest virus, enter the cell via coated pits which, upon internalization of the vesicles, become part of the endosomal compartment whose contents become acidified (pH 5). This acidification is an important factor in the further unknown process of entry of virus particles or their genomes into the cytoplasm. In case of influenza virus, low pH triggers the hemagglutinin to change conformation, which in addition to a proteolytic cleavage activates its ability to induce fusion between the viral and endosomal membrane (Landsberger and Sehgal, 1986; White *et al.*, 1986; Wiley and Skehel, 1987). This activation includes the exposure of a previously buried hydrophobic peptide (denoted fusion peptide), which, by inserting into the target membrane, brings the viral and host-cell membrane physically close enough to fuse. It is suggested that the viral fusion peptide, by withdrawing lipid, causes a destabilization of the target membrane, resulting in intermixing of the phospholipids of the two bilayers (Landsberger and Sehgal,1986).

The involvement of hydrophobic peptides in virus-entry seems a rather wide-spread phenomenon in animal virology. Viruses penetrating directly through the plasma membrane, such as paramyxoviruses, may obtain fusion with the target membrane in a way quite similar to influenza virus (Hsu *et al.*, 1981). In this case, however, activation of the fusion peptide is not dependent on exposure to pH 5, but occurs after attachment to the cellular receptor at neutral pH.

Also non-enveloped viruses may use "hydrophobic domains" of nucleocapsid proteins to mediate penetration into the host cell. For example, at low pH, adenovirus exposes a hydrophobic domain of its penton base. This domain, by interaction with the lipid bilayer, initiates disruption of the endosomal membrane, thereby allowing virus particles to enter into the cytosol (Seth *et al.*, 1984;

Blumenthal, 1986). Also poliovirus capsid proteins expose hydrophobic domains at pH 5 (Olsnes *et al.*,1986). However, the mechanism by which these domains mediate translocation is not understood yet.

2.3. Disassembly (Uncoating)

Once penetrated into the cell, the viral genome has to be released from the particle or nucleocapsid. In case of alphavirus nucleocapsids, which appear in the cytoplasm after fusion of the viral and endosomal membrane, a transfer of capsid protein is observed from the nucleocapsids to the large subunits of cellular ribosomes (Wengler and Wengler, 1984). This transfer is supposed to be part of the reactions leading to the release of the viral genomic RNA into the cytoplasm. The exact mechanism how capsid proteins are released from nucleocapsids is not known yet. Later in infection, however, a transfer in the opposite direction, i.e., from ribosomes to preassembled nucleocapsids, occurs (Söderlund and Ulmanen, 1977; Wengler *et al.*, 1984). These observations lead to the hypothesis that the disassembly and assembly of alphavirus nucleocapsids is regulated by a process which could be named "receptor-mediated nucleocapsid disassembly". According to this hypothesis acceptors exist for capsid protein molecules in uninfected cells, which early in infection bind these proteins and thereby initiate disassembly of these complexes, while later on these acceptors have to be saturated with newly synthesized protein before efficient assembly of nucleocapsids can occur (Wengler, 1987). The existence of such a receptor, as regulator of nucleocapsid disassembly and assembly, may be a feature used by other viruses as well.

For the non-enveloped picornaviruses, however, different mechanisms seem to be involved. As these viral nucleocapsids already undergo conformational changes prior to penetration into the cytoplasm, disassembly may be initiated at en earlier stage (Olsnes *et al.*, 1986; Neubauer, 1987). The exposure of hydrophobic domains, supposed to mediate translocation across the endosomal membrane, probably also represents the initial event leading to disassembly of the particles. Whether these altered virus particles or just the naked genomes enter the cytoplasm is not elucidated yet. In spite of this uncertainty it is clear that the mechanism of disassembly of these non-enveloped viruses, most probably, is different from the "receptor-mediated" disassembly of alphavirus nucleocapsids.

3. PLANT VIRUSES

Compared to animal virus infections, the information about initial stages in plant virus infections is scarce and far more fragmentary. This is probably caused by the fact that studies on plant cells are hampered by the presence of a cell wall.

The majority of our present knowledge about early events in plant virus infections has been obtained from studies on mechanically inoculated leaves and isolated leaf cell protoplasts. Both experimental systems have their own advantages and disadvantages: the former in providing a system with intact plant cells in tissue, the latter in providing a defined cell system where synchronous infections can be obtained while cell-to-cell spread is excluded. Therefore, studies on both systems will be briefly discussed (for more detailed reviews see: Zaitlin and Beachy, 1974; Takebe, 1975; De Zoeten, 1981; Watts *et al.*, 1981; Mühlbach, 1982; Takebe, 1983; Sander and Mertes, 1984; Takebe, 1984; Shaw, 1985; Zaitlin and Hull, 1987).

In the following paragraphs, no discrimination is made between enveloped and non-enveloped viruses, as the viral envelope might only have a function in replication in the arthropod vector. Indeed, some plant rhabdoviruses (Hsu *et al.*, 1983; Gaedigk *et al.*, 1986) have been shown to replicate in insect cells.

3.1. Attachment

In contrast to animal systems, where specific interactions at the cell surface often provide a selection mechanism for compatibility, plant systems do not seem to discriminate at this level (Atabekov, 1975). Numerous non-host plants show subliminal infections on virus inoculation, indicating that replication occurs at least in initially infected cells (Sulzinski and Zaitlin, 1982). Furthermore, various plant viruses have been shown to replicate in isolated protoplasts of non-host plants (Furusawa and Okuno, 1978). In view of these observations the involvement of specific interactions at the cell surface in plant virus infections seems questionable.

Non-specific attachment, however, was observed. After infiltration of tobacco rattle virus (TRV) particles into leaf panels of host and non-host plants, virus particles were found to be bound to cell walls bordering intercellular spaces (Gaard and De Zoeten, 1979), and for both plants shortening of these bound particles has been observed. Also Kurtz-Fritsch and Hirth (1972), when studying uncoating of turnip yellow mosaic virus (TYMV) and brome mosaic virus (BMV) on inoculation of chinese cabbage and barley leaves, respectively, reported on

both reversible and irreversible binding. Only the latter was supposed to be involved in infection. As in both studies no further data were available on the localization and the fate of those virus particles causing infection, the role of extracellular attachment remains unclear.

By using compressed-air guns for mechanical virus transmission, Laidlaw (1987) found a close correlation between leaf susceptibility and surface area of extruded cytoplasm, and therefore concluded that virus particles bind to or are absorbed by the plasma membrane covering these cytoplasmic extrusions. The extent of infection in this system was shown to be dependent on static attraction as well as a close contact between virus particles and plasma membrane. These observations agreed well with those obtained by studies on binding of virus particles to isolated leaf cell protoplasts (Motoyoshi, 1973; Wyatt and Shaw, 1975; Zhuravlev *et al.*, 1975; Watts *et al.*, 1981). In none of these systems, however, the relation between attachment and infection has been reported.

Thus, neither studies on mechanically inoculated leaves, nor studies on isolated protoplasts, revealed adequate and conclusive information on attachment to the cell surface in relation to infection of plant cells. For understanding of this process, more knowledge of virus transmission under natural conditions seems indispensable.

3.2. Penetration

Unlike animal viruses, most of which are self-supporting in penetration, entry of plant viruses, at least in experimental systems, often is dependent on external damaging of the (host) cell. Although plant virus capsid proteins are found to interact with isolated plasma membranes (Kiho and Shimomura, 1976) and artificial membranes (Banerjee *et al.*, 1981a,b; Datema *et al.*, 1987) in most cases no infection is obtained on inoculations with virus only.

For mechanical virus inoculation of leaves, abrasive substances, such as carborundum and celite, are used. Rubbing leaf surfaces with one of these substances is supposed to produce local, transient wounds through which virus particles may penetrate.

In case of isolated protoplasts, viral entry sites are obtained by either treatment with polymers as poly-L-ornithine (PLO) and polyethylene glycol (PEG) (Sander and Mertes, 1984), or electric shock (Nishiguchi *et al.*, 1986; Watts *et al.*, 1987). Both treatments are supposed to induce transient perturbations of the plasma membrane during which penetration of virus particles may occur.

With regard to the mechanism of penetration, the information obtained by these experimental systems is very inconclusive. During mechanical inoculation of leaves cytoplasmic extrusions towards the leaf surface may bind and internalize virus particles in a similar way as observed for isolated protoplasts (Watts *et al.*, 1981; Laidlaw, 1987). However, it is unknown if either direct penetration or endocytosis of bound virus particles (Figure 1) leads to infection, as the formation of both lesions and vesicles seems to be stimulated during inoculation (Grout *et al.*, 1973). Therefore, the fate of virus particles, present in the cytoplasm as well as in the endocytotic vesicles, has to be determined. Furthermore, for a better understanding of the penetration process, in these experimental systems as well as under natural conditions, more information on the role of the vector is required.

3.3. Disassembly (Uncoating)

Disassembly of several plant viruses has been studied on inoculation of both host and non-host plants (Machida and Kiho, 1970; Kurtz-Fritsch and Hirth, 1972; Gaard and De Zoeten, 1979; Matthews and Witz, 1985). In these studies, initiation of disassembly was found to occur very soon after inoculation. However, with regard to the site and mechanism of this process, rather different results were obtained.

For TRV, a shortening of particles has been observed to occur on the cell wall (Gaard and de Zoeten, 1979). Indications exist that this extracellular dissociation is needed before infection can occur. However, in this study the possibility that intact virions enter the cells and cause infection could not be excluded.

Evidence for an intracellular site of disassembly was obtained for several viruses, e.g., tobacco mosaic virus (TMV) (Machida and Kiho, 1970; Kiho, 1972). Soon after inoculation of tobacco leaves parental virus particles were observed to be associated with cellular ribosomes. Moreover, primarily in cell-free translation systems (Wilson, 1984a,b), but later in leaf cells as well (Shaw *et al.*, 1986), these virus-ribosome complexes were shown to direct protein synthesis. Therefore, it was proposed that disassembly might occur as a consequence of translation of the viral genome, i.e., "cotranslational disassembly".

A similar disassembly mechanism has been proposed for some isometric and bacilliform virus particles, because *in vitro* studies revealed their ability to direct protein synthesis (Brisco *et al.*, 1985,1986). However, the highly stable particles of TYMV formed an exception as their encapsidated RNA appeared not to be available for translation *in vitro*. In addition, the appearance of empty protein

shells following mechanical inoculation of leaves (Kurtz- Fritsch and Hirth, 1972; Matthews and Witz, 1985) suggested that, in this case, the genome is released from almost intact protein shells. The nucleoprotein particles probably lose a pentamer or hexamer of protein when the RNA is released.

Uncoating by either direct genome release or cotranslational disassembly is unlikely to occur on intact virus particles. For example, alkali-treatment of TMV particles was found to markedly stimulate cotranslational disassembly *in vitro* (Wilson, 1984a). The pH 8 treatment was suggested to alter the 5'-terminal nucleoprotein structure in such a way that the 5'-end of the RNA becomes available for initiation of translation. Also "pre-swelling" of isometric and bacilliform virus particles, which stimulated viral protein synthesis, was supposed to increase the accessibility of the RNA (Brisco *et al.*, 1986).

Destabilization *in vivo* has been subjected to many speculations, as in most studies the destabilization observed could not be related to infection. Interactions with both cell wall (De Zoeten, 1981) and plasma membrane (Durham, 1978; Banerjee *et al.*, 1981a,b) have been proposed. In the latter case, the local concentration of especially calcium ions was supposed to be involved as well (Durham, 1977,1978). In addition, the association of capsid protein with intracellular membranes in TYMV-infected cells (Hatta and Matthews, 1976) might suggest the involvement of these membranes.

Other mechanisms of intracellular destabilization may include the involvement of "capsid protein - binding sites", which, like the large ribosomal subunit in case of alphavirus infections, withdraw capsid protein molecules from the incoming virus particles. The presence of such receptor sites in plant cells is supported by observations on "cross-protected" (Sherwood and Fulton, 1982; Sherwood, 1987) and transgenic plants expressing the viral capsid protein (Powell Abel *et al.*, 1986; Loesch-Fries *et al.*, 1987; Nelson et al., 1987; Tumer et al., 1987; Van Dun *et al.*, 1987). In both cases, the presence of serologically related or homologous capsid protein, was found to protect against (super) infection with virus, most probably by preventing the uncoating of incoming virus particles. Indeed, recent findings of Register and Beachy (1988) on isolated protoplasts of these transgenic plants favour the hypothesis that protection is due to a blockage of sites where virus uncoating is initiated. However, the existence of such intracellular receptor sites for capsid proteins still has to be proven.

4. CONCLUDING REMARKS

In initial stages of both animal and plant virus infections three distinct processes can be recognized: i) attachment, ii) penetration, and iii) disassembly (uncoating) (Figure 1), which finally will result in translation and replication of the viral genome. The mechanisms by which this result is achieved, however, may be completely different, depending on the type of virus, host cell, experimental system, etcetera. On the other hand, apparently different interactions may rely on similar basic principles.

When comparing initial interactions in animal and plant virus infections, most striking differences are observed with regard to attachment. In animal systems, this process involves highly specific interactions between viral attachment proteins and cellular receptors, which give rise to a biological relevant reaction, i.e., penetration of the virus. In plant systems, attachment is non-specific and only results in penetration when externally induced wounds are present. This difference may explain why in animal systems discrimination between host and non-host cells is performed at the cell surface, while in plant systems discrimination occurs at a later stage.

In contrast, the process of penetration is much more similar, as a transient disturbance of the membrane is required in both systems. In animal systems viral proteins do account for disturbance and subsequent virus passage of either plasma or endosomal membrane; in plant systems membrane disturbance and virus penetration is, most probably, dependent on external wounding. How far, in the latter case, penetration under natural conditions is dependent on external wounding is still unknown. However, the observation that aphids, and possibly other arthropod vectors as well, penetrate plasma membranes during "probing" activities (Tjallingii, 1985; Lopez-Abella *et al.*, 1988), suggest that natural transmission also involves external damaging of the plama membrane.

Finally, disassembly, especially when assumed to occur intracellularly, may form an important point of contact in comparing animal and plant virus infections (Wilson, 1985). At the moment, however, for both systems the available information on this process is still fragmentary and incomplete, and hence a grateful subject for speculations.

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CHAPTER 3

BINDING OF COWPEA CHLOROTIC MOTTLE VIRUS TO COWPEA PROTOPLASTS AND RELATION OF BINDING TO VIRUS ENTRY AND INFECTION

J.W. Roenhorst, J.W.M. van Lent, and B.J.M. Verduin

ABSTRACT

Cowpea chlorotic mottle virus (CCMV) and cowpea protoplasts were used to study initial interactions between virus and protoplast. Protoplasts and virus were incubated under varying conditions of temperature, pH, ionic strength and the presence of added compounds. Both the amount of ^{35}S-labelled virus bound to protoplasts and the percentage of infected cells were determined. At 0 and 25° the amount of virus associated with protoplasts increased with the amount of virus added. With inoculum of 25×10^6 virus particles per protoplast, 4×10^3 and 14×10^3 particles per protoplast were bound at 0 and 25°, respectively. In the presence of polyethylene glycol, 85×10^3 associated particles per protoplast were bound at both temperatures and ca. 50% of the protoplasts became infected. No infection occurred in the absence of PEG. Variation of pH or ionic strength in the absence of PEG caused little to no change in binding and no infection. In the presence of PEG, increase of pH resulted in lower binding, but infectivity was not affected. Increasing ionic strength, however, increased both binding and infectivity. The presence of unlabelled CCMV, tobacco mosaic virus coat protein, bovine serum albumin, and polycations during inoculation in the absence of PEG decreased the amount of bound CCMV. In contrast, CCMV coat protein, which

has a positively charged N-terminal arm, increased binding. In the presence of PEG the effects were similar, although larger amounts of virus were bound. The percentage of infection was reduced by all additives to 5-25 %. Addition of ammonium chloride, which inhibits endocytotic virus uptake in animal cells, during inoculation as well as in culture media, did not reduce infectivity. These data do not support a specific receptor-mediated endocytotic uptake of virus but favor a nonspecific mechanism of entry, possibly through membrane lesions. Observations in the electron microscope support the latter mechanism.

INTRODUCTION

The mechanism by which non-enveloped plant viruses enter their host cells is still disputed (for review see Shaw, 1985) and has recently attracted more attention because some viruses are used as vectors to introduce foreign genes into cells. Specific interactions with both the cell wall (De Zoeten, 1981; De Zoeten and Gaard, 1984) and plasma membrane (Durham, 1978; Banerjee *et al.*, 1981a,b) have been suggested as modes of entry with concurrent uncoating of the viral genome. Passage of the plasmalemma may occur by (receptor-mediated) endocytosis (Cocking and Pojnar, 1969; Cocking, 1970; Takebe, 1975) or through pores or lesions (Burgess *et al.*, 1973a,b; Kassanis *et al.*, 1977; Watts *et al.*, 1981). Recently a process of cotranslational disassembly of metastable virus particles has been proposed (Wilson, 1985) in which ribosomes induce the uncoating.

The inconclusive results on the initial interactions between virus and cells prompted us to reinvestigate these interactions. Cowpea chlorotic mottle virus (CCMV) and isolated mesophyll protoplasts of cowpea were used as a model system to study the initial interactions between CCMV and its host cell in relation to infection. Protoplasts and virus were incubated under varying conditions of temperature, pH, ionic strength, and the presence of added compounds. Both the amount of ^{35}S-labelled virus bound to protoplasts and the percentage of infected cells were determined. Besides the effects of addition of PEG, a mediator of infection, ammonium chloride, an inhibitor of the acidification of endocytotic vesicles, and several other compounds were tested. The binding and infectivity experiments were supplemented with electron microscopic examination of embedded and sectioned protoplasts immunogold labelled with antiserum against CCMV.

MATERIALS AND METHODS

Isolation and storage of virus

CCMV and brome mosaic virus (BMV) were propagated in *Vigna unguiculata* cv. California Blackeye and *Hordeum vulgare* cv. Moore, respectively. Viruses were isolated and purified as described by Verduin (1978) and stored in virus buffer (CCMV, 0.1 M sodium acetate buffer, pH 5.0, containing 1 mM EDTA and 1 mM sodium azide; BMV, CCMV-virus buffer including 10 mM $MgCl_2$) at 4°.

^{35}S-Labelling of virus

Cowpea and barley seeds were incubated for 48 hr at 25° in the dark in moistened vermiculite. The germinated seeds were transferred to Hoagland's mineral salt solution, deficient in sulphate, and placed in a growth cabinet. The growing conditions of this hydroponic culture were illumination with fluorescent tubes (25 kWatt/m^2 at the height of the primary leaves) at 25° and a relative humidity >75% for 12 hr and 30 min, followed by darkness for 11 hr and 30 min at 22° (Huxley and Summerfield, 1976). After 10 and 5 days, cowpea and barley leaves, respectively, were inoculated with CCMV and BMV at a concentration of 1 mg/ml in inoculation buffer: 0.01 M sodium phosphate, pH 6.0, containing 5 mM $MgCl_2$. Twenty four hours after inoculation leaves were removed from the plant and incised at 2-mm distances. This material was floated on 50 ml of Hoagland's solution containing 50-150 μCi ^{35}S-sulphate (ca. 300 mCi/mM). Three days thereafter the leaves were harvested and the virus was isolated as described (Verduin, 1978). Purity of the labelled virus (sp act 1000-2000 cpm/μg) was checked on polyacrylamide gels. All radioactivity migrated with the coat protein band.

Preparation of protoplasts

Cowpea mesophyll protoplasts were isolated as described by Van Beek *et al.* (1985) with minor modifications of the washing solution [2.5 mM 2(N-morpholino) ethane sulfonic acid-KOH (MES, Sigma), pH 5.6, 0.6 M mannitol] and enzyme [0.8% cellulase and 0.05% Macerozym (Yakult Pharmacological Industries) in washing solution] solutions. Only protoplast suspensions containing at least 80% viable protoplasts after isolation were used in further experiments.

Inoculation of protoplasts

Inoculation was done essentially as described by Van Beek *et al.* (1985). A pellet of 2 x 10^6 protoplasts was resuspended in 25 µl virus buffer containing 100 µg CCMV or BMV. Immediately thereafter 0.2 ml of 3 mM $CaCl_2$ with or without 40% (w/v) polyethylene glycol (PEG, M_r 6000) was added, mixed, and diluted with 1.8 ml washing solution. After incubation for 30 min at 25° the protoplasts were sedimented and washed three times in washing solution at room temperature.

Incubation of protoplasts at 0° was done on ice. Washing, before as well as after inoculation, was performed at 4° with ice-cold washing solution.

When pH and ionic strength had to be varied, the isolated protoplasts were washed once in the appropriate solution, prior to inoculation. Washing solutions of different pH were made without MES. Ionic strength was varied with sodium chloride and osmolarity was kept constant by changing the mannitol concentration.

To test the effect of ammonium chloride, protoplasts were incubated prior to inoculation for 30 min at 25° in washing solution containing the appropriate amount of ammonium chloride. This concentration was maintained during all further steps, including 16 hr incubation in culture medium.

Addition of unlabelled virus, (coat) protein and polycations was done as follows: the protoplast pellet was resuspended in 25 µl of virus buffer containing 100 µg ^{35}S-labelled CCMV or BMV followed by the addition of 0.2 ml of washing solution containing either 400 µg CCMV, CCMV coat protein (empty protein shells, Verduin, 1974), tobacco mosaic virus (TMV) coat protein (Fraenkel-Conrat, 1957) or bovine serum albumin (BSA), or 3.2 µg polycation [poly-L-ornithine (PLO, M_r 100,000-200,000) or poly-L-lysine (PLL, M_r 30,000)]. From here, the standard inoculation procedure was followed with the exception that only 1.6 ml washing solution was used. The dilution effect introduced in the competition experiment was tested with the addition of 0.2 ml washing solution.

The viability of the protoplasts, as determined by fluorescein diacetate staining, (Widholm, 1972) did not change with the altered inoculation conditions.

Culture of inoculated protoplasts

After the third washing, 2 x 10^6 protoplasts were resuspended in 2.5 ml of culture medium (Aoki and Takebe, 1969), whereby the concentration of mannitol was raised to 0.6 M, 6-benzyladenine was omitted, 2.5 mM MES was added and

10 µg/ml gentamicin (Sigma) was used as antibiotic. Protoplasts were kept at 25° under continuous illumination with fluorescent tubes (25 kWatt/m^2).

Quantitation of virus bound to protoplasts

Protoplasts were inoculated with ^{35}S-labelled virus (sp. act. ca. 1500 cpm/µg) following the standard procedure. After the third washing, when at least 95% of the unbound virus was removed, the protoplasts were disrupted with 0.5 ml 10% (v/v) ethanol in 1 M sulphuric acid, incubated for 15 min at room temperature and bleached in 0.5 ml of commercial bleach solution (3% hypochlorite). Scintillation fluid (14 ml) (Hydroluma, Lumac), was added and the radioactivity was determined by liquid scintillation counting. All values were corrected for virus precipitation, adsorption to the tubes and the presence of unbound virus.

Fluorescent-antibody staining of infected protoplasts

After incubation for ca. 16 hr in culture medium, protoplasts were prepared for immunofluorescence microscopy according to Van Beek *et al.* (1985). For each sample ca. 1000 non-autofluorescent protoplasts were counted. With CCMV maximum infectivity varied between 40 and 80% depending on the quality of the protoplasts.

Infectivity test

Protoplasts, ca. 18 hr after start of inoculation procedure, were homogenized in inoculation buffer and tested on half-leaves of *Chenopodium hybridum*. Both CCMV and BMV appeared to multiply in cowpea protoplasts as the number of lesions increased with time of incubation from 0 to 48 hr after inoculation. Thus infectivity was not caused by inoculum virus bound to the protoplasts.

Electron microscopy of protoplasts

Embedding, sectioning and immunogold labelling of antigen in protoplasts was done as described (Van Lent and Verduin, 1986).

RESULTS

When increasing amounts of CCMV were added to isolated cowpea protoplasts at both 0 and 25° increasing numbers of virus particles were bound (Fig. 1A). In interpreting the binding no discrimination was made between attached and internalized virus particles. At 25° three times more virus was bound than at 0°,

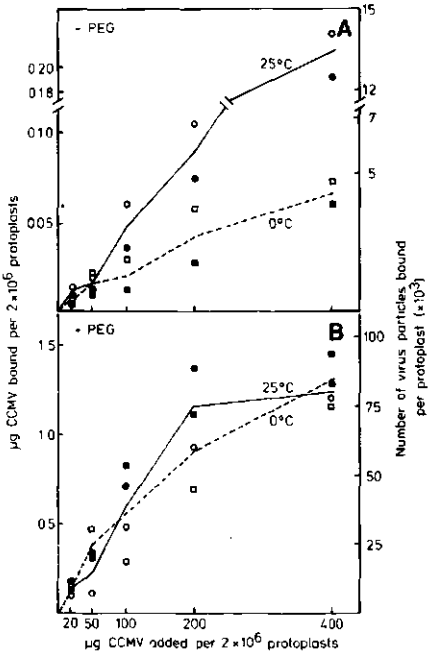

Figure 1. Binding of CCMV to cowpea protoplasts. The effect of the amount of CCMV added during inoculation was tested in the absence (A) and in the presence (B) of PEG. A pellet containing 2 x 10^6 protoplasts was inoculated with 20-400 µg ^{35}S-labelled CCMV (sp. act. 1000-2000 cpm/µg) with and without PEG. After inoculation the protoplasts were incubated for 30 min at either 25° (—) or 0° (---). After washing the protoplasts were disrupted and bleached and radioactivity was determined by liquid scintillation counting. Solid and broken lines connect the average values from two independent experiments: ○, ● at 25° and □, ■ at 0°.

probably as a consequence of inhibition of endocytotic uptake at low temperature. This difference was not observed when PEG was present during inoculation (Fig. 1B). At the same time the total number of particles bound increased approximately 10-fold to 85 x 10^3 particles per protoplast, and at this level saturation appeared to be reached.

Infection of protoplasts only occurred after inoculation in the presence of PEG (results not shown). The relation between percentage of infection and virus concentration is shown in Fig. 2. Maximum level of infectivity was reached when ca. 25 x 10^3 virus particles were bound per protoplast. Although at least three times less binding was sufficient for 20% infection, an average binding of 9 x 10^3 particles per protoplast did not result in infection when PEG was omitted during inoculation. Changing the sequence of addition within the inoculation procedure, i.e., adding PEG before virus or adding PEG and virus at the same time, decreased binding as well as infection in both cases. Binding was lowered from ca. 30 x 10^3 to ca. 6 x 10^3 and ca. 5 x 10^3 particles per protoplast while infectivity dropped from 81% to 6 and 22%, respectively (results not shown).

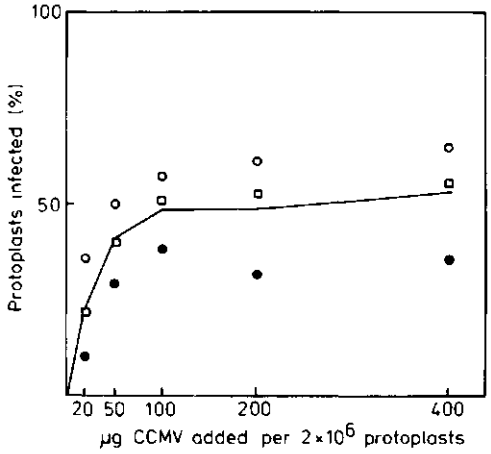

Figure 2. The Effect of the amount of CCMV added during inoculation on infection of cowpea protoplasts by CCMV. A pellet containing 2 x 10^6 protoplasts was inoculated with 20-400 µg CCMV in the presence of PEG and incubated for 30 min at 25°. The protoplasts were subsequently washed and cultured for ca. 18 hr, after which the percentage of infection was determined by fluorescent antibody staining. The solid line connects average values from three independent experiments, indicated as ○, ● and □.

To further analyze the nature of the binding and its relation to protoplast infection we varied the pH and ionic strength during inoculation. Variation of pH between 3.5 and 7.5 only slightly influenced CCMV binding in the absence of PEG (Fig. 3A), while a twofold decrease of binding was found after inoculation in its presence (Fig. 3B). Performing the same experiments with another bromovirus, BMV, having a higher isoelectric point (6.8) than CCMV (3.5), binding increased by a factor of 1000 in the absence (Fig. 4A) and a factor of 50 in the presence of PEG (Fig. 4B). The increased binding may be explained by the rather aspecific binding between the positively charged BMV and the negatively charged plant cells. With BMV, variation of pH indicated an optimum in the amount of binding around pH 5, irrespective of the absence or presence of PEG. We have no explanation for these results. BMV was able to infect cowpea protoplasts in the presence of PEG and the maximum infectivity was about 10%.

Variation of the ionic strength between 0 and 0.35 M NaCl decreased the binding of CCMV in the absence of PEG (Fig. 5A), indicating its electrostatic nature, and increased it approximately twofold in the presence of PEG (Fig. 5B). Although inoculation of protoplasts with CCMV in the absence of PEG did not result in infection, the effects of pH and ionic strength on infectivity were still determined after inoculation with and without PEG. The percentage of infected

Figure 3

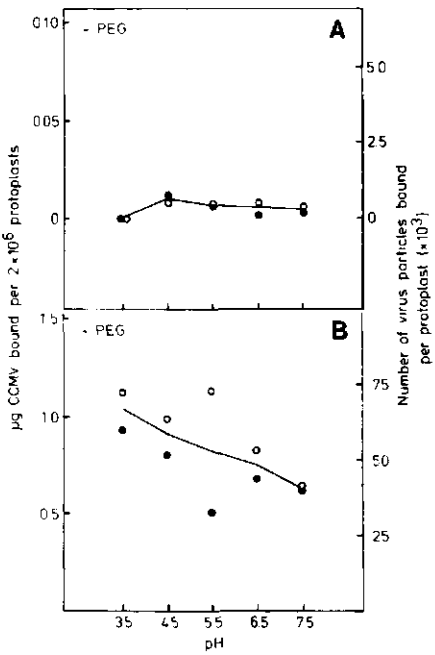

Figure 4

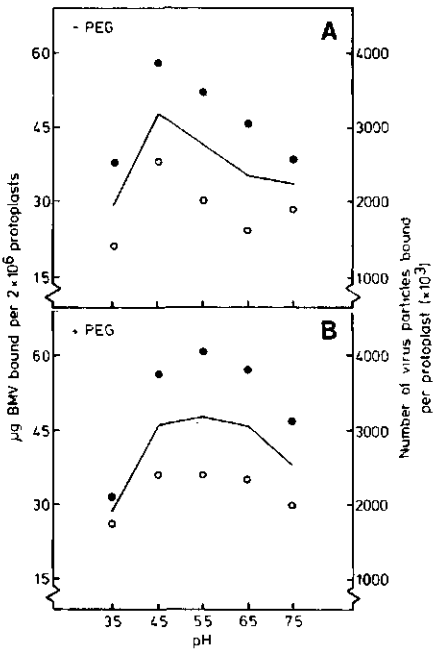

Figure 3. Effect of pH on binding of CCMV to cowpea protoplasts. Pellets containing 2×10^6 protoplasts were inoculated with 100 µg ^{35}S-labelled CCMV (sp. act. 1000-2000 cpm/µg) in the absence (A) and in the presence (B) of PEG and incubated for 30 min at 25°. Prior to inoculation the protoplasts were washed once in washing solution of appropriate pH. The same solution was used during inoculation and further washing. After washing the protoplasts were disrupted and bleached and radioactivity was determined by liquid scintillation counting. Solid lines connect the average values from two independent experiments, indicated by O and ●.

Figure 4. Effect of pH on binding of BMV to cowpea protoplasts in the absence (A) and in the presence (B) of PEG. Conditions were as described for CCMV in Fig. 3.

protoplasts with PEG and different pH values remained constant at 60% with different pH values and increased by ca. 15% with increasing NaCl concentration (data not shown). In the absence of PEG protoplasts were not infected.

Although variation in pH and ionic strength did not suggest specific binding sites we nevertheless tested CCMV binding in the presence of several added compounds to screen for competition (Table 1). In the absence of PEG the addition of washing solution itself, i.e., inoculation under more diluted conditions,

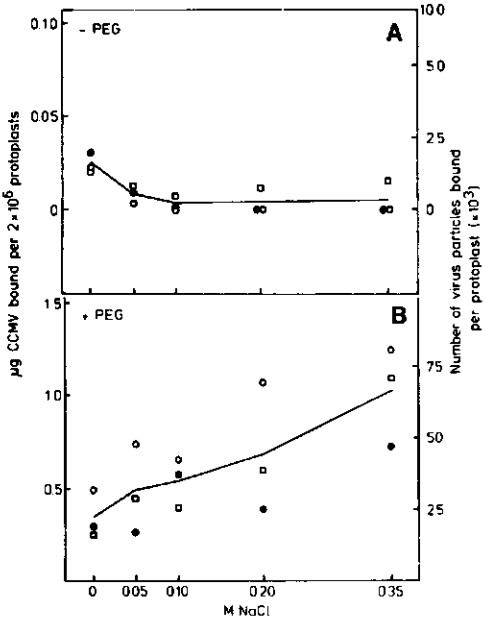

Figure 5. Effect of sodium chloride concentration (ionic strength) on binding of CCMV to cowpea protoplasts. Isolated protoplasts were sedimented in solutions of the appropriate ionic strength. Resuspended pellets containing 2×10^6 protoplasts were inoculated with 100 µg ^{35}S-labelled CCMV (sp. act. 1000-2000 cpm/µg) in the absence (A) and in the presence (B) of PEG and incubated for 30 min at 25°. Solutions with the same ionic strength were used during inoculation and further washing. After washing the protoplasts were disrupted and bleached and radioactivity was determined by liquid scintillation counting. Solid lines connect the average values from two independent experiments, indicated by ○ and ●.

decreased binding about 25%. The presence of extra unlabelled virus did not change this decrease, nor did TMV coat protein, BSA, PLL and PLO. Only CCMV coat protein, having a positively charged N-terminal arm, increased binding, emphasizing the electrostatic nature of the binding. In all cases infectivity was zero.

In the presence of PEG, the tendencies were roughly similar, although higher binding numbers were obtained. The percentage of infected protoplasts was decreased by all additions. The addition of washing solution caused a decrease from 71 to 46%, possibly due to a decrease in inoculum concentration. However, this decrease was only partially compensated by the addition of extra unlabelled CCMV. Addition of CCMV coat protein, as empty protein capsids, reduced infectivity of the control by about 50%. A more drastic decrease to about 7% infected protoplasts was caused by TMV coat protein, BSA and both polycations. Therefore, the competition experiments did not support specific binding.

Finally the role of endocytotic uptake of virus with respect to infection was tested by the addition of ammonium chloride. The presence of this compound did

Table 1.

EFFECT OF VARIOUS COMPOUNDS PRESENT DURING INOCULATION OF COWPEA PROTOPLASTS IN THE ABSENCE AND PRESENCE OF PEG ON BINDING AND INFECTIVITY OF CCMV[a]

	Number of virus particles bound per protoplast (x 10^6)		percentage of infected protoplasts	
Additive	**-PEG**	**+PEG**	**-PEG**	**+PEG**
none	3.1	36.1	0	71
washing medium	2.3	25.6	0	46
unlabelled CCMV	2.5	17.0	0	58
CCMV coat protein	173.9	646.4	0	24
TMV coat protein	2.8	18.3	0	7
BSA	2.1	13.5	0	8
PLO	3.8	25.9	0	7
PLL	2.6	23.9	0	6

[a] A resuspended pellet containing 2 x 10^6 protoplasts was inoculated with 100 µg ^{35}S-labelled CCMV in the presence of 400 µg of the compound, in both the absence and the presence of PEG. After inoculation protoplasts were incubated for 30 min at 25° and washed three times in washing solution. Binding was determined by liquid scintillation counting after disruption and bleaching of the protoplasts. Percentage of infection was determined by fluorescent antibody staining ca. 18 hr after inoculation.

not result in decreased infectivity (Table 2), suggesting that endocytosis was not important for infection.

A possible role of endocytosis in protoplast infection was at first suggested by electron microscopic data. Freshly isolated protoplasts showed endocytotic vesicles (Fig. 6A) and the number of vesicles increased in the presence of PEG, used during inoculation (Fig. 6B). However, in time, fusion of vesicles and concurrent aggregation of virus particles in crystalline-like arrays occurred (Fig. 6C), rather than release of virus from the vesicles into the cytoplasm, to start virus replication. In many protoplasts virus-containing vesicles were observed without significant numbers of gold particles in the cytoplasm. In contrast, infected protoplasts (Fig. 6F) gave high numbers of gold particles in the cytoplasm. Only a few of these protoplasts also showed virus-containing vesicles. In other words virus infection is more strongly correlated with gold particles in the cytoplasm

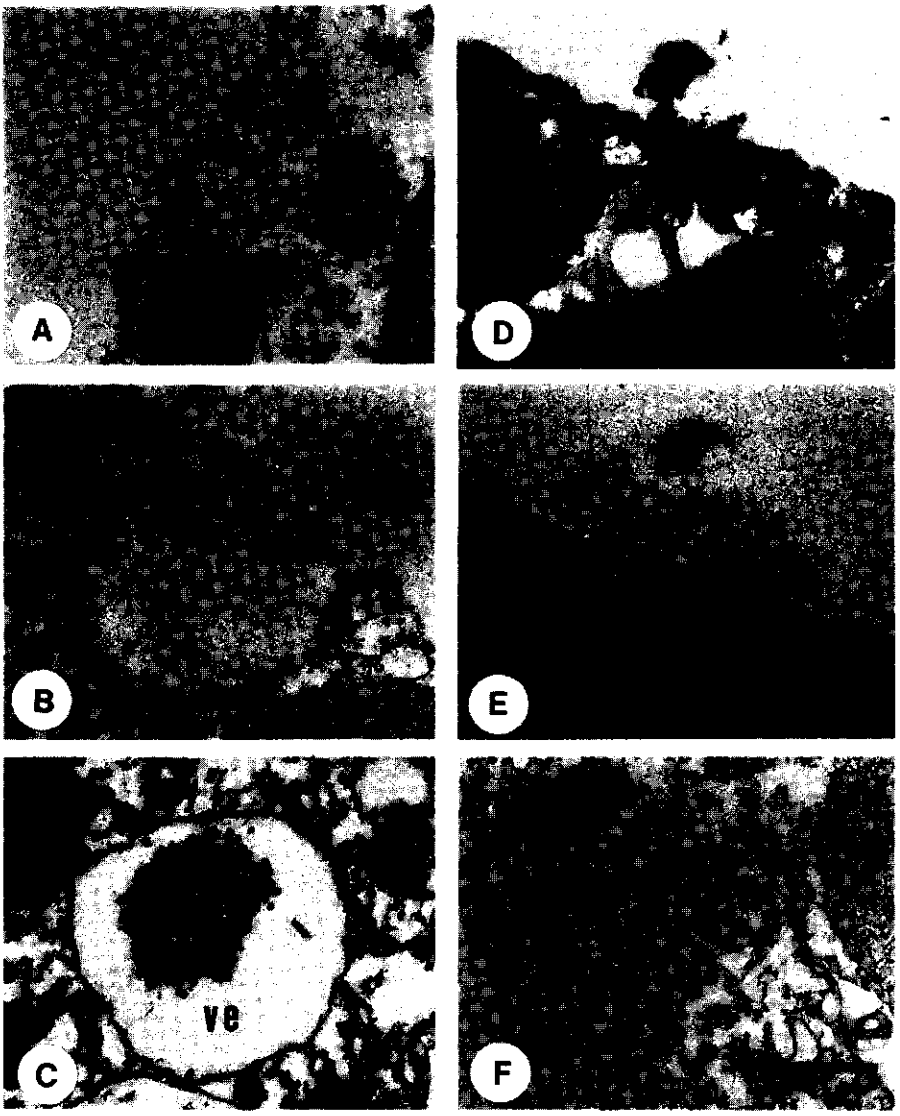

Figure 6. Entry and multipliction of CCMV in cowpea protoplasts. Sections of methacrylate-embedded protoplasts at 0 (A,B,D,E) and 6 hr (C,F) after inoculation with CCMV were incubated with antibodies raised against CCMV (B,C,E,F) and 16 nm protein A-gold. At t=0 invagination of the plasma membrane (A) containing labelled virus particles (B) was seen next to virus particle aggregates either penetrating (D) or at the surface of the protoplast (E). At t=6 hr invaginated inoculum-virus was found in crystalline-like arrays (C), while only inoculum-virus which had entered the cytoplasm caused synthesis of new coat protein in the cytoplasm (F). Ve, vesicles; Cy, cytoplasm. Bar represents 300 nm.

Table 2.

EFFECT OF AMMONIUM CHLORIDE CONCENTRATION ON VIABILITY OF COWPEA PROTOPLASTS AND THEIR INFECTION BY CCMV[a]

Ammonium chloride concentration (mM)	Percentage of viable protoplasts		Percentage of CCMV-infected viable protoplasts
	Mock-inoculated	CCMV-inoculated	
0	62/72 [b),c)]	82/81	88/95[b),c)]
2	57/70	78/78	95/90
10	63/71	81/80	91/89
50	57/67	80/83	91/89
100	—/69	—/83	—/—[d)]

[a] A resuspended pellet containing 2 x 10^6 protoplasts was preincubated in washing solution supplemented with an appropriate amount of ammonium chloride. Subsequently these protoplasts were sedimented and inoculated in the same solution with 100 µg virus in the presence of PEG and incubated for 30 min at 25°. The ammonium chloride concentration was maintained during all further steps, including 16 hr incubation in culture medium. Percentage of infection was determined by fluorescent antibody staining ca. 18 hr after inoculation.

[b] Determined 18 hr after inoculation.

[c] Two independent experiments.

[d] —, not determined; viability of freshly isolated protoplasts was 83/90%.

than virus-containing vesicles. Gold particles in the cytoplasm do not correlate with virus-containing vesicles. This result also weakened the possibility of a role of endocytosis in the infection of protoplasts. Evidence for other possible ways of entry was also obtained by electron microscopy. Aggregates of virus particles were frequently found at damaged areas of the protoplast membrane (Fig. 6D) or bound to the surface (Fig. 6E). All data suggest a mechanism of entry whereby individual virus particles or aggregates of particles penetrate into the cytoplasm through a lesion in the plasma membrane.

DISCUSSION

The data presented revealed that initial contact between CCMV and cowpea protoplasts resulted in rather aspecific binding, mainly based on electrostatic interactions between virus particles and plasma membrane. Recent studies on interactions of plant viruses and viral coat proteins with mixed model membranes revealed ionic interaction, mainly between negatively charged vesicles and the positively charged N-terminal arm of viral coat proteins (Datema *et al.*, 1987). On addition of PEG, both precipitation of virus (Hebert, 1963) and stimulated endocytosis caused higher binding numbers.

An alternative to endocytotic virus uptake would be the entry through membrane lesions. These areas of damaged membranes caused an increase in solute leakage rate and occurred under conditions of cell expansion (Willing and Leopold, 1983). These conditions are induced during inoculation of protoplasts with PEG, where protoplasts, dehydrated for a short period in 40% PEG, are rehydrated after dilution to 4% PEG. The phenomena of shrinking and swelling and solute leakage have been observed with light microscopy and conductivity measurements, respectively (W. van Dis, personal communication).

Endocytotic virus uptake, however, did not appear to contribute to infection significantly, as infectivity was influenced by neither a decrease of the inoculation temperature to 0^{o} (results not shown) nor increasing concentrations ammonium chloride (Table 2), both known to inhibit this process (Motoyoshi *et al.*, 1974). Also electron microscopic data (Fig. 6; Van Lent and Verduin, 1986), showing the presence of virus-containing vesicles in uninfected protoplasts, were in agreement with these results. Therefore, we suggest that mainly virus particles which penetrated through membrane lesions contributed to the infection of protoplasts. This conclusion agrees quite well with the observation that increased injury of protoplasts resulted in higher infectivity numbers (Motoyoshi *et al.*, 1974, Okuno and Furusawa, 1978). Also the introduction of nucleic acid and virus particles into protoplasts by electroporation is likely to occur through membrane lesions (Watts *et al.*, 1987).

The results indicated PEG to be responsible for the introduction of membrane lesions, as infection failed to occur when it was omitted during inoculation. The question arose how PEG induced these membrane lesions. Studies on artificial membranes (Arnold *et al.*, 1983) as well as studies on hen erythrocytes (Ahkong *et al.*, 1975) and isolated carrot protoplasts (Boss, 1983) demonstrated that changes in polarity and hydration caused by PEG could contribute to alterations in the

membrane surface. These structural rearrangements were shown to stimulate both membrane fusion and endocytosis, and to facilitate incorporation of external components into the membrane (Grout *et al.*, 1973). Moreover, PEG is known to stimulate virus aggregation (Hebert, 1963), especially in the presence of sodium chloride. As infection of cowpea protoplasts with CCMV was stimulated on addition of sodium chloride, aggregation near the plasma membrane also may improve infection conditions.

The role of aggregation, however, could be disputed, as on inoculation with aggregates, obtained by preincubation of CCMV and PEG before inoculation, infectivity was lowered from 80 to 20%. A direct and close contact between PEG and the protoplast membrane seemed to be necessary to induce membrane lesions and allow subsequent penetration and infection by virus particles. The presence of proteins or polycations seemed to protect the protoplasts and prevent subsequently added virus from entering. The possible relationship between this contact and protoplast damage was also demonstrated by the observation that 48 hr after inoculation with virus about 70% of the protoplasts were still viable, versus 40% in case of mock-inoculation (results not shown).

These data support the mechanism for entry of virus by initial physical association, as also shown by Watts and King (1984) in tobacco protoplasts, and subsequent internalization through membrane lesions. This mechanism found for protoplasts may be applicable to the cytoplasmic extrusions found by Laidlaw (1987) after inoculation of tobacco leaves with several plant viruses. His observations lead to the conclusion that virus particles bind to, or are absorbed by, exposed cytoplasm, or more probably the covering plasmalemma, and are withdrawn into the cell when the extrusions are retracted. The proposed internalization of whole particles, possibly destabilized by membrane or cell wall components, may be followed by a process of cotranslational disassembly shown for TMV *in vivo* (Shaw *et al.*, 1986) and CCMV *in vitro* (Brisco *et al.*, 1986; unpublished results).

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CHAPTER 4

VIRUS-RIBOSOME COMPLEXES FROM CELL-FREE TRANSLATION SYSTEMS SUPPLEMENTED WITH COWPEA CHLOROTIC MOTTLE VIRUS PARTICLES

J.W. Roenhorst, B.J.M. Verduin, and R.W. Goldbach

ABSTRACT

When particles of cowpea chlorotic mottle virus (CCMV) were added to cell-free extracts from wheat germ, the encapsidated viral genome was translated into polypeptides similar to the translation products specified by unencapsidated viral RNA (as shown before by M.J. Brisco, R. Hull, and T.M.A. Wilson, 1986, *Virology* 148, 210-217). The rate of protein synthesis observed upon addition of virus particles was much slower than that of extracted RNA and the quantity of protein formed was only 10% of that of extracted RNA. Using sucrose and cesium-chloride gradient analysis, virus-ribosome complexes, containing up to four ribosomes per virus particle, were isolated from translation mixtures supplemented with CCMV particles. These complexes, with densities intermediate of those of virus (1.36 g/cm^3) and ribosomes (1.58 g/cm^3), were analyzed and quantified in the electron microscope. Less than 5% of the particles was found in association with ribosomes. To verify whether these complexes were involved in the process of cotranslational disassembly, tobacco mosaic virus was analyzed with the same techniques and methods. The results found for TMV were similar to those found for CCMV except that virus-ribosome complexes with up to 20 ribosomes per virus particle were observed. The implications of the process

of virion-directed translation for the structure of the particle as well as the role of this process *in vivo* are discussed.

INTRODUCTION

The mechanism of cotranslational disassembly has been demonstrated for tobacco mosaic virus (TMV) both *in vitro* (Wilson, 1984a,b, 1985; Wilson & Watkins, 1985, 1986; Turner *et al.*, 1987) and *in vivo* (Wilson & Shaw, 1985; Shaw *et al.*, 1986). The disassembly process of destabilized rod-shaped particles, stripping coat protein molecules from the 5'-end of the RNA, was easily observed by electron microscopy and the existence of translationally active complexes of virus particles and ribosomes ("striposomes") has been shown. Also spherical viruses like brome mosaic virus (BMV), cowpea chlorotic mottle virus (CCMV), and southern bean mosaic virus (SBMV) have shown virion-directed translation *in vitro* (Brisco *et al.*, 1985; 1986a,b), although the mechanism of uncoating for those viruses is more complex. For SBMV some putative translation complexes have been shown when particles treated with EGTA at pH 8.0 were supplemented to wheat germ extracts. In all cases some form of swelling either before or during incubation was required for virus-specific protein synthesis.

In this paper we describe the isolation and characterization of CCMV-ribosome complexes formed in cell-free protein synthesizing systems. In both rabbit reticulocyte lysates and wheat germ extracts viral specific proteins were synthesized upon addition of intact CCMV particles. Specific virus-ribosome complexes could be isolated from these mixtures by sucrose and CsCl gradients, which were then further analyzed in the electron microscope. The results obtained suggest that uncoating of spherical viruses may also occur by cotranslational disassembly.

MATERIALS AND METHODS

Isolation, purification and storage of virus and RNA

CCMV was propagated in *Vigna unguiculata* cv. California Blackeye. The virus was isolated and purified as described by Verduin (1978a,b) and stored in 0.1 M sodium acetate buffer, pH 5.0, containing 1 mM EDTA and 1 mM sodium azide at 4°. ^{35}S-Labelled virus was obtained as previously described (Roenhorst *et al.*, 1988). TMV was purified according to procedure 5 as described in Noordam

(1973). RNA was extracted from purified virus as described by Verduin (1978a,b) and RNA was stored in water at -20°.

Treatment of virus particles for *in vitro* translation

To obtain CCMV particles in different stages of swelling purified virus preparations (10 mg/ml) were dialyzed for 3 hr at 4° against 1 mM sodium acetate buffer, pH 5.0 (nonswollen), 50 mM Tris-HCl buffer, pH 7.5, containing either 10 mM $MgCl_2$ (partially swollen) or 10 mM EDTA (totally swollen). Destabilized TMV was obtained by dialysis for 3 hr at room temperature against 50 mM Tris-HCl buffer, pH 8.1.

Treatment of CCMV with micrococcal nuclease

Partially, totally and nonswollen virus particles (1 mg/ml) as well as unencapsidated RNA (0.2 mg/ml) were treated at 25° for 30 min with micrococcal nuclease (Boehringer) at up to 1000 U/ml in the presence of 1 mM $CaCl_2$. Reactions were stopped with 2 mM EGTA buffer, pH 7.5. To remove EDTA totally swollen virus was dialyzed for 2 hr at 4° against 50 mM Tris- HCl buffer, pH 7.5 prior to nuclease treatment.

In vitro translation and product analysis

In vitro translation experiments were done with cell-free extracts from wheat germ (General Mills, Vallejo, California), and prepared as described (Davies and Kaesberg, 1974; Davies, 1979; Davies and Verduin, 1979), or with rabbit reticulocyte lysates (Green Hectares). Both cell-free systems were stored in small aliquots in liquid nitrogen. Standard incubations (5 µl) in rabbit reticulocyte lysate contained either 0.01 mg/ml extracted RNA or 0.05 mg/ml virus. The endogenous levels of ca. 40 mM K^+ and 1.8 mM Mg^{2+} were supplemented with 60 mM potassium acetate and variation in K^+ and Mg^{2+} concentration was accomplished by the addition of 20-40 mM potassium acetate and 0-2 mM magnesium acetate. Standard incubations (5 µl) in wheat germ extract contained either 0.1 mg/ml extracted RNA or 0.5 mg/ml virus for CCMV and 2.0 mg/ml virus for TMV. Incubations with CCMV were done at 60 mM K^+ and 2.5 mM $MgCl^{2+}$ (endogenous levels), while for TMV extra potassium acetate (40 mM) and magnesium acetate (0.4 mM) were added. Per sample ca. 5 µCi L-[^{35}S]methionine (New England Nuclear Corp., 1084 Ci/mmol) was added. After 1.5 hr incubation at 28°, sample buffer according to Laemmli (1970) was added and the samples were incubated for 3 min in boiling water before loading on 16-cm-long 15%

(w/v) polyacrylamide gels (Laemmli, 1970). Denatured proteins were separated at a constant current of 35 mA during ca. 3.5 hr and ^{35}S-labelled polypeptides were visualized by fluorography on Kodak XAR5 X-ray film.

Sucrose density gradient analysis of *in vitro* translation mixtures

For rate zonal centrifugation analysis incubations with wheat germ extract were scaled up to a volume of 450 µl, with 100 µCi [^{35}S]methionine per sample. After 0 (control) or 30 min incubation at 28°, the samples were mixed with an equal volume of a solution containing 25 mM cycloheximide (Sigma) and 25 mM $MgCl_2$ (to inhibit further translation). These mixtures were diluted to 7 ml in 50 mM triethanolamine-HCl buffer, pH 7.5, containing 20 mM KCl and 10 mM magnesium acetate (TEKM-buffer). Virus particles, ribosomes, and translation complexes were sedimented in a Beckman Type 50Ti rotor for 4 hr at 40,000 rpm and 4° through a 2-ml-20% (w/v) sucrose cushion. The pellets were resuspended in ca. 200 µl TEKM-buffer on a rotary shaker overnight at 4°. The resuspended material was then layered on a 15-50% (w/v) linear sucrose gradient made up in TEKM-buffer and centrifuged (Beckman SW41 rotor, 38,000 rpm, 1.25 hr, 4°). The gradients were fractionated with an ISCO model 185 density gradient fractionator and the contents were monitored at both 254 and 280 nm with a LKB Uvicord III densitometer. Fractions of 0.25 ml were collected and mixed with 7 ml Hydrocount (Baker) and counted in a Packard Tri-Carb Model 2450 liquid scintillation spectrometer or used for further analysis in CsCl gradients.

Fixation and CsCl density gradient analysis of nucleoprotein complexes

Sucrose gradient fractions containing virus, ribosomes, and translation complexes were pooled and concentrated by centrifugation in a Beckman Type 50Ti rotor for 4 hr at 40,000 rpm and 4°. The pellets were resuspended overnight in ca. 100 µl TEKM-buffer on a rotary shaker at 4°. Complexes were fixed in a final concentration of 5% (w/v) formaldehyde for 1 hr on ice. This fixed material was layered on top of 5 ml of CsCl (density 1.48 g/ml), dissolved in TEKM-buffer containing 1% (w/v) formaldehyde, and centrifuged overnight to equilibrium in a Beckman SW55Ti rotor at 48,000 rpm and 4°. The gradients were fractionated with an ISCO Model 185 density gradient fractionator using Maxidense to replace the contents of the tubes. Fractions of 0.175 ml were collected and counted in a liquid scintillation spectrometer as described above, or used for electron microscopy.

Electron microscopy of "virus-ribosome" complexes

Twenty-five microliters of CsCl fractions containing the complexes were put on grids and washed with TEKM-buffer, containing 1% (w/v) formaldehyde. After a second fixation in 1% (w/v) glutaraldehyde in TEKM-buffer the nucleoprotein material was negatively stained with 2% (w/v) uranyl acetate in double-distilled water and viewed in a Philips CM12 electron microscope. TMV-ribosome complexes were further analyzed by determining both rod length and number of associated ribosomes for at least 100 complexes in each fraction.

RESULTS

In vitro translation of cowpea chlorotic mottle virus particles

When CCMV particles were added to an *in vitro* translation system, prepared from either rabbit reticulocytes or wheat germ (results not shown), translation products (Figs. 1A and B, lanes 6-17) were similar to those formed with unencapsidated RNA (Figs. 1A and B, lanes 2-5). The major polypeptides had molecular masses of 105, 100, 35, and 23 kDa, as calculated from the protein markers with known molecular masses. When encapsidated RNA was added the overall rate of [^{35}S]methionine incorporation was lower and the time of appearance of the largest protein product was considerably later than with unencapsidated RNA (results not shown). Maximum levels of incorporation were about 10 times less compared to unencapsidated RNA, when the amount of RNA was the same in both cases.

Preswelling of virus particles, by dialysis against pH 7.5 prior to the addition to the translation system, hardly influenced the results. The swollen virus became more sensitive to the calcium dependent micrococcal nuclease (Fig. 2). Nuclease treatment of totally swollen virus particles resulted, similar to treatment of unencapsidated RNA, in total abolishment of translation (Fig. 2, lanes 16 and 4). Since EDTA, present in the buffer of totally swollen virus, would inactivate the nuclease (Fig. 2, lane 13), it was removed by dialysis prior to enzyme addition. Only part of the RNA of partially swollen virus was inactivated by micrococcal nuclease (Fig. 2, lane 10), while nonswollen virus appeared virtually unaffected by this treatment (Fig. 2, lane 7). These results demonstrate that translation products were made from encapsidated RNA and not from RNA adsorbed to the outside of the virus particle. It was therefore concluded that the pH and ionic

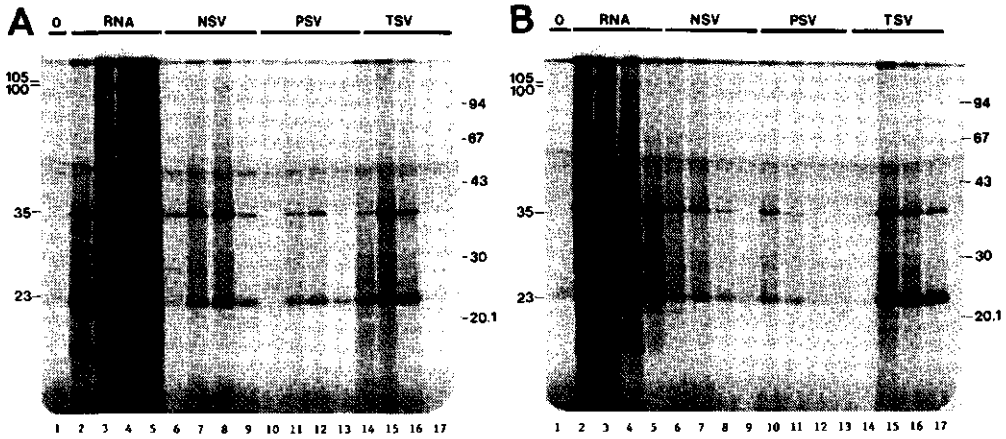

Figure 1. The effect of K^+ concentration (A) and Mg^{2+} concentration (B) on *in vitro* translation products from rabbit reticulocytes supplemented with encapsidated or unencapsidated CCMV-RNA. Incubations were programmed with extracted RNA (lanes RNA, 2-5), intact nonswollen virus (lanes NSV, 6-9), partially swollen virus (lanes PSV, 10-13), or totally swollen virus (lanes TSV, 14-17). No template was added to the mixture loaded in lane 1 (marked 0). Translation reaction mixtures contained either 10 µg/ml RNA or 50 µg/ml virus. Variation of K^+ concentration (A) were 60 (lanes 2, 6, 10, 14), 100 (lanes 1, 3, 7, 11, 15), 140 (lanes 4, 8, 12, 16), or 180 mM (lanes 5, 9, 13, 17) at a constant Mg^{2+} concentration of 2.2 mM. Variations of Mg^{2+} concentration (B) were 1.7 (lanes 2, 6, 10, 14), 2.2 (lanes 1, 3, 7, 11, 15), 2.7 (lanes 4, 8, 12, 16), or 3.2 mM (lanes 5, 9, 13, 17) at a constant K^+ concentration of 100 mM. All concentrations include endogenous ions. Tracks were loaded with 1.5 µl of incubation mixture diluted 1:1 with 2x concentrated sample buffer (Laemmli, 1970).

strength of the translation systems were sufficient to destabilize CCMV and enable ribosomes to translate the encapsidated viral genome.

The concentrations of both K^+ and Mg^{2+} in the translation mixture were varied, since these ions influenced both translational activity (Davies and Kaesberg, 1974) and the stability of the nucleoprotein particles (Adolph, 1975a,b). Within the range tested, an increase in K^+ concentration resulted in higher levels of translation of unencapsidated RNA compared to encapsidated. With encapsidated RNA, however, a maximum was reached at 100-140 mM, probably since virus particles are more stable above 100 mM. A similar effect was found with increasing Mg^{2+} concentrations. In general, an increase in Mg^{2+} concentration, within the range tested, resulted in rather similar levels of translation for unencapsidated RNA, and decreased translation of encapsidated RNA. Totally swollen virus was an

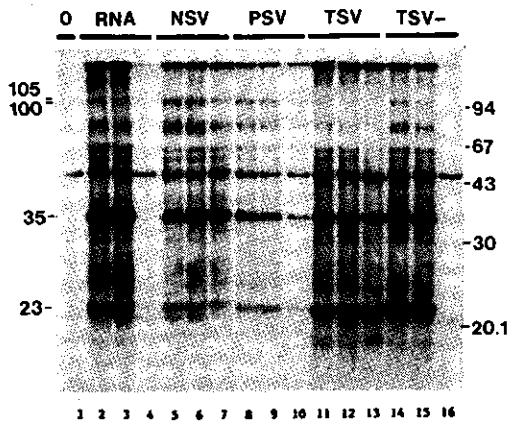

Figure 2. The effect of micrococcal nuclease treatment on encapsidated and unencapsidated CCMV-RNA with respect to the *in vitro* translation in rabbit reticulocyte lysates. Incubations were programmed with 2 μg/ml RNA (lanes RNA, 2-4) or 60 μg/ml virus [lanes NSV, 5-7; PSV, 8-10; TSV, 11-13 (see Fig. 1); TSV- (=TSV dialyzed against 50 mM Tris-HCl pH 7.5), 14-16]. The mixtures were either not treated (2, 5, 8, 11, 14) or incubated for 30 min at 25° in the absence (lanes 3, 6, 9, 12, 15) or in the presence (lanes 4, 7, 10, 13, 16) of 1000 U/ml micrococcal nuclease. The K^+ and Mg^{2+} concentrations were 100 and 1.7 mM, respectively. No template was added to the incubation mixture loaded in lane 1 (marked 0).

exception because such preparations contained EDTA that lowered the effective concentration of Mg^{2+}. With the so found optimum conditions for translation of encapsidated RNA in rabbit reticulocyte lysates as well as wheat germ extracts, an attempt was made to isolate virus-ribosome complexes and to analyse the fate of the translated virus particle.

Sucrose gradient and CsCl analysis of *in vitro* translation mixtures

Wheat germ extracts supplemented with CCMV particles and [^{35}S]methionine were incubated for 30 min at 28° in the presence of cycloheximide, an inhibitor of peptide chain elongation, added at the beginning (control) or at the end of the incubation time and prior to analysis on sucrose density gradients (Fig. 3A). For comparison, unencapsidated CCMV-RNA was incubated and analyzed similarly. After fractionation of the gradients and counting the radioactivity of the fractions (Fig 3B), faster sedimenting structures were observed with both encapsidated and unencapsidated RNA. Complexes formed of virus and ribosomes sedimented faster than complexes of RNA and ribosomes. To test the method with a virus

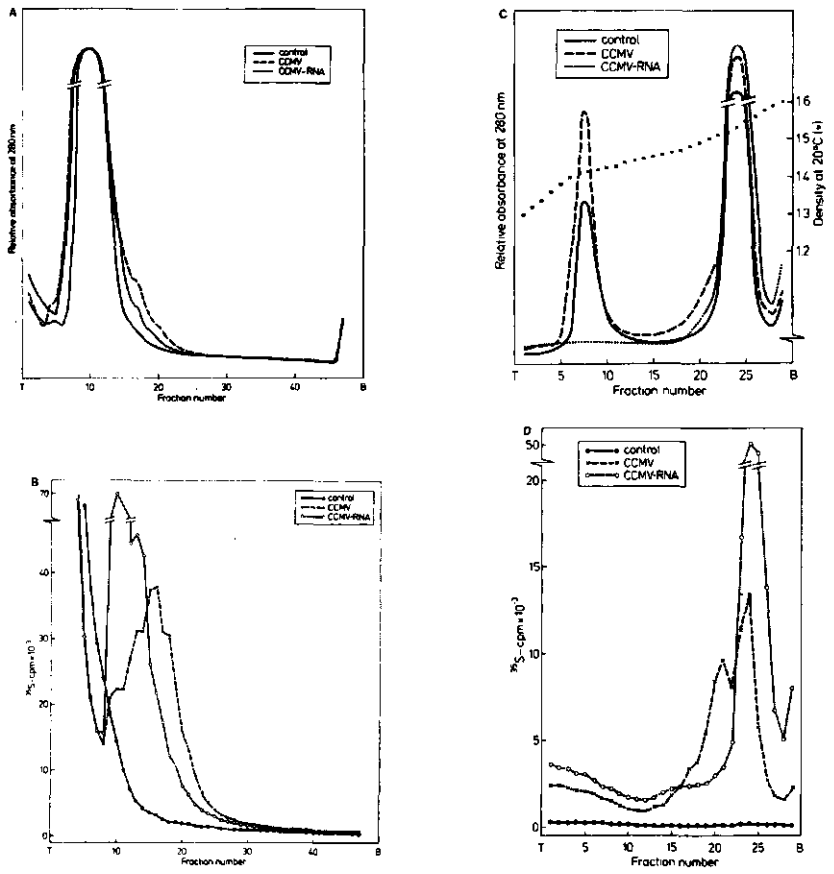

Figure 3. Sucrose gradient and CsCl gradient analysis of [^{35}S]methionine containing wheat germ extracts (450 µl) supplemented with 0.1 mg/ml extracted CCMV-RNA or 0.5 mg/ml nonswollen CCMV particles (A,C), and incubated for 30 min at 28° with 12.5 mM cycloheximide added at either t = 0 (virions (●—●)) or t= 30 min (virions (X--X), RNA (O...O)). The incubation mixtures were diluted in TEKM-buffer and ribosomes, virus particles, and translation complexes were sedimented through a 20% (w/v) sucrose cushion. After resuspension, this material was centrifuged in linear 15-50% (w/v) sucrose gradients (A, B). The gradients were monitored for absorption at 280 nm (A) and fractionated. Fractions were counted for [^{35}S]methionine (B) or used for further analysis on CsCl gradients (C, D). Fractions 7 to 25 of sucrose gradient (A) were concentrated by centrifugation. After resuspension and formaldehyde fixation the material was centrifuged to equilibrium in a CsCl solution (initial density 1.48 g/cm^3). The gradients were monitored at 280 nm or fractionated and radioactivity counted. Panels (A) and (B) represent sucrose gradients and panels (C) and (D) CsCl gradients. Panels (A) and (C) show the relative absorbance at 280 nm and (B) and (D) the distribution of the radioactivity in cpm. Sedimentation is from left to right.

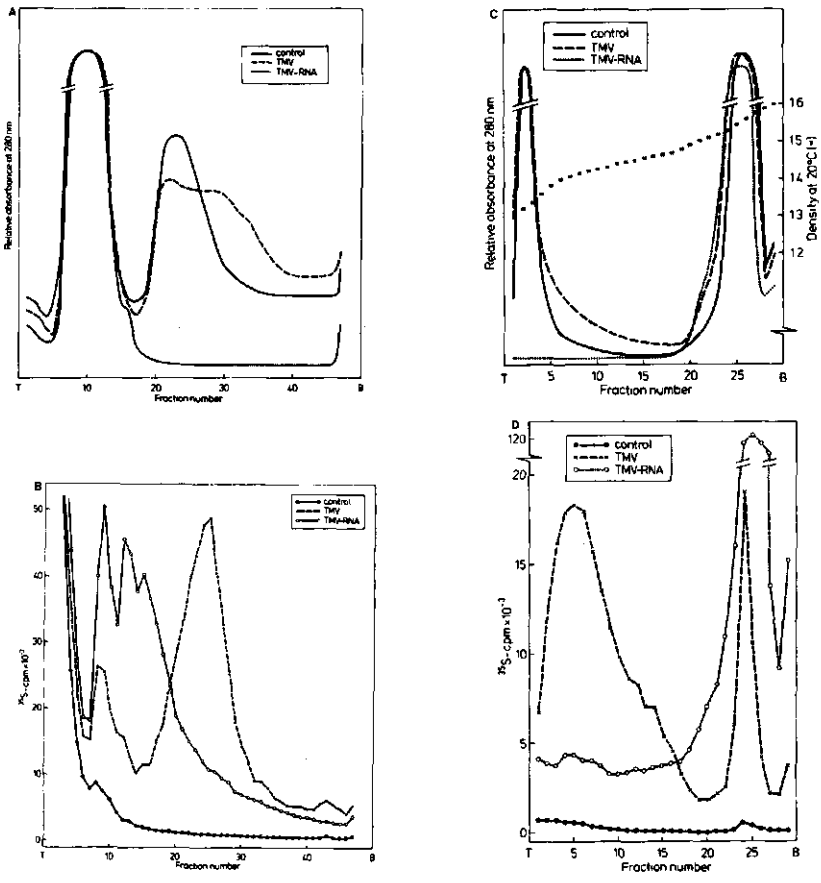

Figure. 4. Sucrose gradient and CsCl gradient analysis of [^{35}S]methionine containing wheat germ extracts (450 µl) supplemented with 0.1 mg/ml extracted TMV-RNA or 2 mg/ml TMV particles (dialyzed at pH 8.1), incubated, and treated as described for CCMV in Fig. 3. For CsCl gradients fractions 7 to 35 of sucrose gradient (A) were concentrated by centrifugation, resuspended, fixed, and centrifuged to equilibrium in CsCl as described for CCMV in Fig. 3. The gradients were fractionated and radioactivity was determined. Virions with cycloheximide added at t = 0 (●—●) or t = 30 min (X---X); RNA with cycloheximide added at t = 30 min (O...O).

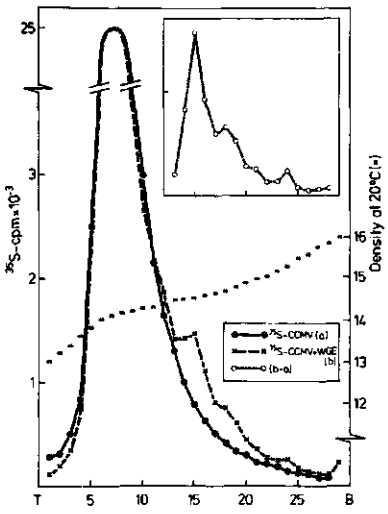

Figure 5. CsCl gradients of 150 µg ^{35}S-labelled CCMV (sp. act. 500 cpm/µg, ●—●, a) and of the same amount of labelled virus incubated with wheat germ extract for 30 min at 28° (X---X, b) and further processed as described in Fig. 3. The insert shows the difference between b and a representing the labelled virus-ribosome complexes. Densities in 0.1 g/cm^3. Fractions of 0.175 ml were collected.

which does not cosediment with ribosomes, the same analysis was done with destabilized TMV-particles and TMV-RNA (Figs. 4A and B). Also with TMV, ribosomes and virus particles formed faster sedimenting complexes than ribosomes and RNA. In all cases wheat germ extracts with virus particles mixed with cycloheximide at t = 0 did not form any radioactive complexes, indicating that active protein synthesis is a prerequisite for this complex formation.

To improve the separation between virus, ribosomes, and virus-ribosome complexes, we used CsCl gradients in which TMV (ρ = 1.32 g/cm^3) and CCMV (ρ = 1.36 g/cm^3) banded just below the meniscus, while ribosomes (ρ = 1.58 g/cm^3) formed a band near the bottom of the gradient in the tube. Fractions 7 to 25 of CCMV (Fig. 3A) and 7 to 35 of TMV (Fig. 4A) were concentrated by centrifugation, fixed, and subsequently centrifuged to equilibrium in CsCl gradients.

Figures 3C and 4C show the absorbance at 280 nm of the contents of the tubes loaded with CCMV and TMV fractions, respectively. Figures 3D and 4D show the distribution of the radioactivity of the same contents. The absorbance profiles indicate a good separation between virus and ribosomes and both the RNA- and the virus-extract mixtures show a small increase in absorbance in fractions less dense than ribosomes. This position in the gradient is expected for virus-ribosome complexes, which are less dense than ribosomes alone. Only a small percentage of

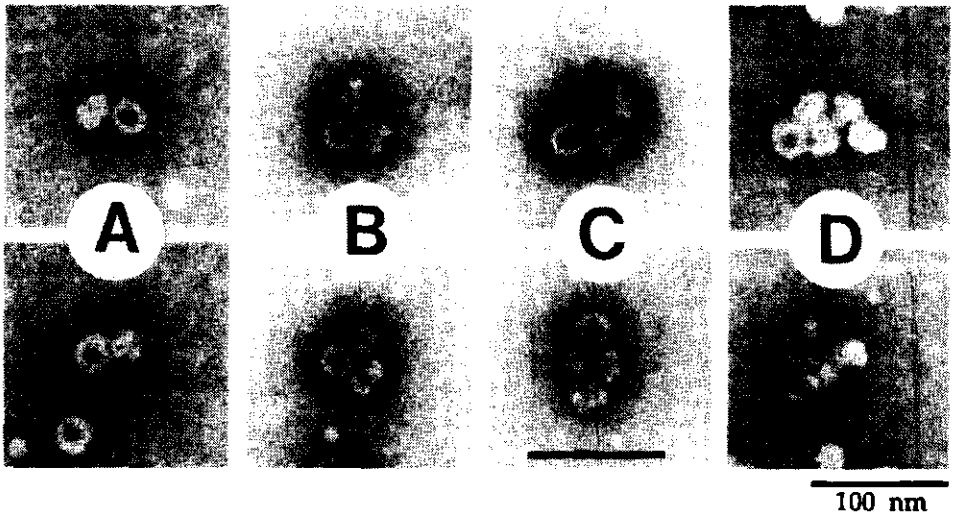

Figure 6. Electron micrographs of virus-ribosome complexes from wheat germ extract, containing nonswollen particles of CCMV, incubated, and processed as described in Fig. 3. Samples for electron microscopy were taken from fractions of a CsCl gradient (Fig. 3C). After fixation in 1% (w/v) glutaraldehyde in TEKM-buffer, complexes were negatively stained with 2% (w/v) uranyl acetate in water and viewed in a Philips CM12 electron microscope. Complexes of the most abundant type are shown for the fraction numbers 15 (A), 17 (B), 18 (C), and 19 (D). Bar represents 100 nm.

the virus particles appeared to be involved in complex formation. In the radioactivity distribution profiles only active complexes producing [^{35}S]-methionine-labelled peptides are seen. With CCMV most activity other than with ribosomes is located slightly above the ribosome position, while for TMV most activity is located below the virus position. This can be understood if we realize that for virus-ribosome complexes with one to four ribosomes per virus particle, RNA percentages of 7 to 22 for TMV and 36 to 46 for CCMV, respectively, can be calculated. All CCMV-ribosome complexes are therefore much denser. The results shown in Figs. 3 and 4 indicate that with CCMV only few particles were involved in virion-directed translation.

This was proven in a separate experiment where incubation of wheat germ extract with ^{35}S-labelled CCMV particles in stead of [^{35}S]methionine resulted in complexes between virus and ribosomes banding at higher buoyant densities than virus. Less than 5% of the labelled virus particles was found in these complexes (Fig. 5). Untreated but labelled virus did not band at these densities.

Electron microscopy

To further characterize the virus-ribosome complexes, fractions were taken from the CsCl gradients between the virus and the ribosome fractions and studied in the electron microscope. Associations between virus particles and ribosomes were observed (Fig. 6). In the case of TMV the appearance of virus was obvious (results not shown). For CCMV, virus could be distinguished from ribosomes by their regular spherical shape and the extra uranyl acetate stain in the central hole of the particle (Fig. 6). Irregular particles with two lobes, as seen in control preparations of ribosomes, were identified as ribosomes. For TMV 1 to 20 ribosomes were found in association with one virus particle. With the CCMV particles up to 4 ribosomes were observed near one CCMV particle (Fig. 6D). The fate of the translated virus particle with more than 4 ribosomes remained unclear. Further analysis of complexes between TMV particles and ribosomes revealed a positive correlation between the number of ribosomes per virus particle and fractions with increasing density and a negative correlation with the average length of the remaining virus particles (results not shown). Using these data a number of ca. 140 nucleotides bound per ribosome could be calculated.

DISCUSSION

The results presented in this paper show that CCMV, like TMV and SBMV particles, allows *in vitro* translation of at least partially encapsidated RNA. In contrast to TMV and SBMV, which both need to be destabilized prior to translation of the RNA (Wilson, 1984a, Brisco *et al.*, 1985, 1986a,b), translation was observed with CCMV when virtually untreated (nonswollen) particles were added to the rabbit reticulocyte lysate or wheat germ extract. Indeed CCMV particles will readily undergo swelling under relatively mild conditions. Both RNA-protein and protein-protein interactions are destabilized as soon as the pH exceeds 7 (Adolph, 1975a,b). Hence addition of the virus to a cell-free translation system (pH 7.5), per se, is probably sufficient to induce immediate swelling of the particles. Indeed, preswelling of virus particles, prior to addition to the translation mixture, did not lead to enhanced protein synthesis (Figs. 1 and 2), although an increased accessability of the RNA could be expected based on the increased sensitivity to nuclease treatment (Fig. 3).

Swelling is known to be affected by Mg^{2+} and ionic strength. When the K^+ concentration of the translation system exceeded 140 mM, protein synthesis of encapsidated RNA was inhibited. No inhibition was found with extracted RNA

(Fig. 1A). This indicates that the concentration of K^+ ions influences the accessibility of the RNA and hence the stability of the virus particles. With increasing Mg^{2+} concentrations, protein synthesis of both unencapsidated RNA and virus became inhibited (Fig. 1B). Therefore the effects of Mg^{2+} ions on the stability of the capsids remain unclear. With regard to both K^+ and Mg^{2+} concentration, no major differences in translation were observed between the four CCMV-RNAs, neither encapsidated nor unencapsidated. Therefore a different stability of the individual CCMV particles (light, medium dense, heavy) is not likely to regulate the expression of the different parts of the genome *in vivo*.

The isolation of CCMV-ribosome complexes *in vitro* supports the hypothesis that isometric particles undergo, as was found for the rod-shaped TMV particles (Wilson, 1984a), a process of cotranslational disassembly, during which the RNA remains continuously protected from degradation by either coat protein or ribosomes. Protection by ribosomes is easily demonstrated for TMV, where a very close packaging of ribosomes at the edge of a TMV particle can be observed. Moreover, a correlation seems to exist between particle length, i.e., length of RNA strands already freed from protein, and the number of associated ribosomes, (results not shown). For CCMV, a maximum of only four ribosomes per virus particle has been found (Fig. 6). Using the data from TMV this would suggest that ca. 20% (560 nucleotides) of the RNA content of the particle can bind to ribosomes, while a sperical-shaped virus particle can still be seen in the electron microscope. A further release of the RNA from the virus particle probably desintegrates the particles since no complexes have been found with more than four ribosomes. This agrees with the findings of earlier assembly work, that demonstrated the need of both RNA-3 and RNA-4 (824 nucleotides) to have efficient assembly and formation of stable virus particles (Verduin, 1978b). At low ionic strength these virus particles may disintegrate into a linear structure whereby coat protein dimers remain attached to the RNA which is not yet bound by ribosomes. In the virus-ribosome complex we have not seen any small cytoplasmic ribonucleoprotein particles called prosomes (Schmid *et al.*, 1984; Kremp *et al.*, 1986). These particles have been found to regulate *in vitro* translation of both TMV- and CPMV-RNA (Horsch *et al.*, 1985), although it has not been clearly demonstrated if they function as a particle *in vivo* or are an isolation artifact. Their role in virus infections and possibly determining host range has yet to be determined.

It should be noticed that for both CCMV and TMV, most of the virus particles were found unaffected during *in vitro* translation (Figs. 3A and 4A). Based on the

experiment with ^{35}S-labelled CCMV (Fig. 5), less than 5% of the virus particles were estimated to be involved in cotranslational disassembly. At the moment this low efficiency cannot be explained. Neither is it known whether a relation exists to the low efficiency of infection generally observed for plant viruses *in vivo* (Furumoto and Mickey, 1967).

Experiments are in progress to elucidate the role of cotranslational disassembly during early stages of infection *in vivo* and the possible relation with other nucleoprotein particles, like informosomes (Dorokhov *et al.*, 1984).

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CHAPTER 5

INTERACTION BETWEEN COWPEA CHLOROTIC MOTTLE VIRUS AND WHEAT GERM RIBOSOMAL PROTEINS

ABSTRACT

To further characterize the interaction between CCMV and wheat germ ribosomes binding of virus particles to electroblots of denatured ribosomal proteins was studied. A set of approximately 20 ribosomal proteins was found to be involved, 10-15 proteins belonging to the large ribosomal subunit, the remaining being part of the small subunit. Several other viruses tested, all bound to the same set of ribosomal proteins. The observed binding to these denatured ribosomal proteins give tentative indications of a possible functional role of this interaction *in vivo*.

Cell-free translation systems have been demonstrated to be of great value to reconstruct initial stages of plant virus infection (Wilson, 1984a,b; Brisco *et al.*, 1985, 1986). Since Wilson (1984a,b) first observed ribosome-directed uncoating of tobacco mosaic virus (TMV) particles in cell-free translation systems, these experimental systems have been applied to several other viruses (Brisco *et al.*, 1985, 1986). The value of these systems, however, became clear when the proposed cotranslational disassembly of TMV *in vitro* (Wilson 1984a,b) was confirmed by experiments *in vivo* (Shaw *et al.*, 1986). Besides TMV, several spherical and bacilliform plant viruses have been found to undergo cotranslational disassembly *in vitro* (Brisco *et al.*, 1985, 1986).

Also for cowpea chlorotic mottle virus (CCMV), member of the bromovirus group, the formation of translationally active virus-ribosome complexes has been demonstrated (chapter 4). With regard to the mechanism of cotranslational

disassembly and the interaction between individual viral and ribosomal proteins, however, little is known. In case of animal alphaviruses, it is proposed that cytoplasmic ribosomes may be involved in destabilization of the viral nucleocapsid by functioning as cellular binding site for viral capsid protein (Wengler, 1987).

To further characterize the interaction between CCMV and wheat germ ribosomes, binding of virus particles to electroblots of denatured ribosomal proteins was investigated. A set of approximately 20 ribosomal proteins was found to be involved. To elucidate whether the observed binding might represent a more common and possibly functional interaction, binding of CCMV was compared with other plant viruses.

Viruses were isolated and purified as described for CCMV, brome mosaic virus (BMV; Verduin, 1978), southern bean mosaic virus (SBMV; Van Lent and Verduin, 1985), blackeye cowpea mosaic virus (BLCMV; Dijkstra *et al.*, 1987) and TMV (Noordam, 1973; procedure 5).

Ribosomes and ribosomal subunits were isolated from wheat germ extract, prepared according to Davies and Verduin (1979), using linear sucrose density gradients. Non-dissociated 80 S ribosomes were isolated from 10-50% (w/v) sucrose gradients, made up in TEKM-buffer (50 mM triethanolamine-HCl, pH 7.5, 20 mM KCl, and 10 mM magnesium acetate) and centrifuged for 16 hr 20,000 rpm in a Beckman SW 28 rotor at 4^{o}. Ribosomal subunits were obtained from 80 S ribosome suspensions by addition of EDTA (f.c. 20 mM) and subsequent centrifugation into 15-30% (w/v) sucrose gradients made up in TEKE-buffer (50 mM triethanolamine-HCl, pH 7.5, 20 mM KCl, and 1 mM EDTA) for 5 hr 39,000 rpm in a Beckman SW 41 rotor at 4^{o}. Ribosomal suspensions were concentrated by centrifugation, non-dissociated ribosomes for 4 hr, and subunits for 10 hr at 45,000 rpm in a Beckman 50 Ti rotor at 4^{o}.

To investigate the interaction between CCMV and ribosomes electroblots of wheat germ ribosomal proteins, separated in denaturing polyacrylamide gels, were incubated with CCMV and binding of both virus particles and coat protein subunits was visualized by immunological staining (for details see legend of Figure 1). Binding of virus particles occurred to 10 to 15 ribosomal proteins (Fig. 1, lane 3), that comprise approximately one third of the proteins visualized by silverstaining (lane 2). These virus-binding ribosomal proteins mainly had molecular masses between 15 and 35 kDa. Most prominent binding was found for proteins of ca. 18, 25, 28, 31 and 33 kDa. The remaining proteins showing binding to a lesser extend. No binding was observed with marker proteins (lane 4).

To gain more insight in the position of these virus-binding proteins within the ribosome, additional binding experiments were performed with purified 40 and 60 S ribosomal subunits (Fig.2). Most if not all prominent bands between 15 and 35 kDa, present in the lane loaded with proteins of non-dissociated ribosomes (CCMV, lane d), were also present in the lane loaded with proteins of 60 S subunits (CCMV, lane c), indicating that all these proteins were part of the large ribosomal subunit. However, due to the higher percentage of acrylamide in the separating gel, 17.5% (w/v) compared to 15% (w/v) for Figure 1, the diffuse 18 kDa band (Fig. 1, lane 3) appeared to be comprised of another 7 proteins, which did not seem to belong to the 60 S subunit. Hence, it may be deduced that these proteins are part of the small ribosomal subunit. Unfortunately, this could not be analyzed as a result of repeatedly low recoveries of 40 S subunits from the gradients (CCMV, lane 2). The reason for this low recovery remained unclear.

To investigate whether these CCMV-binding proteins might be more commonly involved in plant virus binding, corresponding ribosomal protein blots were incubated with several other viruses (Fig. 2; viruses as indicated at the bottom). All viruses were found to bind to almost the same set of ribosomal proteins found for CCMV, although overall intensities of bands varied from virus to virus. This variation might be due to different specific activities of the antisera used. Again proteins with molecular masses between 15 and 35 kDa showed most prominent binding and were located on the large ribosomal subunit. For all viruses, with the exception of TMV, binding was not effected by increasing NaCl concentration (100-400 mM), neither by increasing heparin concentration (5 - 5 x 10^3 U/ml), the latter preventing aspecific interactions with the positively charged domains of the viral coat protein molecules (Dietzgen and Francki, 1987). These results indicate that for these viruses, electrostatic interactions were not or only of minor importance for binding to ribosomal protein blots. On the other hand, the fact that binding of TMV occurred only in the complete absence of heparin, suggests that, in this case, electrostatic interactions are essential.

These preliminary results indicate that in electroblot assays CCMV and several other plant viruses are able to bind to at least 20 different ribosomal proteins, of which approximately 10 to 15, with molecular masses mainly between 15 and 35 kDa, were found to be part of the large ribosomal subunit, while the remaining 5 to 10 smaller proteins, most probably, belong to the small subunit. The experiments presented do not yet provide information on the position and arrangement of the proteins in the native ribosome. It should be noted that it cannot be excluded that the observed binding to denatured ribosomal proteins is

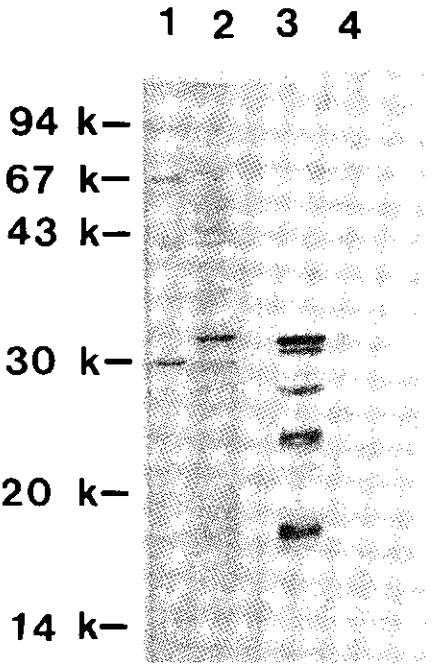

Figure 1. Binding of CCMV to electroblots of electrophoretically separated plant ribosomal proteins. Wheat germ ribosomal proteins were separated in 16-cm-long 15% (w/v) denaturing polyacrylamide gels (Laemmli, 1970) and electrophoretically transferred to nitrocellulose sheets (Schleicher and Shuell, 0.45 µm, type BA-85) (Towbin, 1979), and incubated overnight with 100 µg CCMV in phosphate buffer (10 mM NaH_2PO_4, pH 7.5, 100 mM NaCl, 10 mM magnesium acetate, 0.05% (v/v). Tween-20, 0.5% (w/v) gelatin, and heparin 10 U/ml). Binding of virus was visualized by immunological staining, i.e., by subsequent incubations (1 hr 37°) with anti-viral (1 µg/ml) and goat-anti-rabbit (0.25 µg/ml) gamma-globulins, the latter conjugated with alkaline phosphatase (BRL), diluted in the same buffer, followed by incubation (4 hr room temperature) in Nitro Blue Tetrazolium (330 µg/ml, BRL) and 5-Bromo, 4-Chloro, 3-Indolylphosphate (165 µg/ml, BRL) in 100 mM Tris-HCl buffer, pH 9.5, containing 100 mM NaCl, and 50 mM $MgCl_2$. Staining was terminated by incubation in 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM EDTA. Blots were washed thoroughly between different steps. Lane 1 and 2 show protein markers (indicated at the left) and total protein content of 10 µg non-dissociated wheat germ ribosomes, respectively, as visualized by silverstaining. Lane 3 and 4 show electroblots of ribosomal proteins and markers, respectively, incubated with CCMV and stained as described.

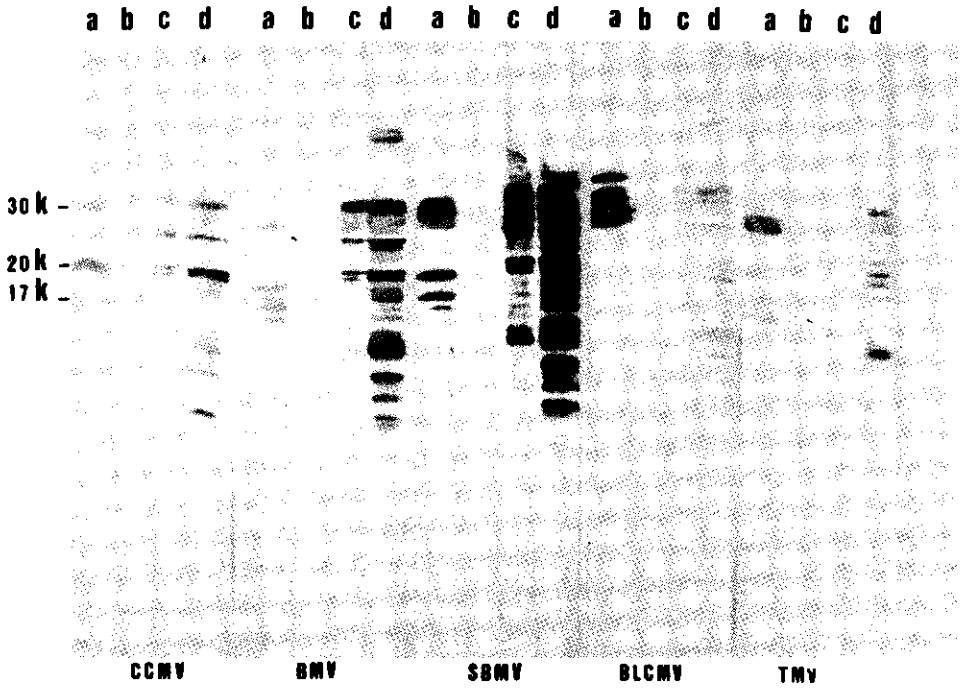

Figure 2. Binding of several plant viruses to electroblots of electrophoretically separated plant ribosomal proteins. Wheat germ ribosomal proteins were separated in 16-cm-long 17.5% (w/v) denaturing polyacrylamide gels, blotted, and incubated with virus as described in the legend of Figure 1. Incubations with TMV were performed in the absence of heparin. Lanes of different panels were loaded with markers (mixture of TMV, CCMV, and SMBV, 0.5 µg each, indicating positions of 17, 20, and 30 kDa, respectively (lane a), 5 µg 40 S (lane b), 5 µg 60 S (lane c), and 10 µg 80 S (lane d) ribosomal proteins. Viruses as indicated at the bottom.

(partially) based on interactions with hydrophobic domains, which are non-exposed in the native ribosome. However, when the observed binding reflects a functional phenomenon, it might be expected that these proteins form a, for the present, hypothetic viral binding site and are clustered on the surface of the ribosome. At the moment no structural evidence is available for such a localization of virus-binding proteins, as in contrast to the procaryotic ribosome, hardly anything is known about the position of the various ribosomal proteins in the native (plant) eucaryotic ribosome (Brimacombe *et al.*, 1978; Spirin, 1986). On the other hand, incubation of ^{35}S-labelled CCMV particles with functional ribosomes in wheat germ extracts revealed that also under more physiological

conditions approximately 1% of the labelled coat protein binds in almost equal amounts to both ribosomal subunits (unpublished results). In addition, it is worthwhile to note that the observed accumulation of viral coat protein in the nucleolus of CCMV-infected cowpea protoplasts suggests an association of virus particles or coat protein subunits with ribosomal structures *in vivo* as well (Van Lent, 1988).

With regard to the possible functional role of the observed interactions between virus particles and ribosomal proteins, can be speculated only. For animal alphaviruses, binding of viral capsid protein molecules to the large ribosomal subunit, at the onset of infection, was suggested to fulfil a role in destabilization of the viral nucleocapsid (Wengler, 1987). For the present, it remains unclear whether these interactions reflect a more common phenomenon that also may occur during plant virus infection. The results presented in this chapter provide evidence that a subset of approximately 20 ribosomal proteins, located on both the large and small ribosomal subunit, is able to bind CCMV and several other plant viruses in electroblot assays, and hence provide valuable starting points for elucidation of a possible functional role.

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CHAPTER 6

COTRANSLATIONAL DISASSEMBLY OF COWPEA CHLOROTIC MOTTLE VIRUS: DOES IT OCCUR IN VIVO?

ABSTRACT

Cowpea protoplasts were used to study the possible occurrence of cotranslational disassembly at the onset of cowpea chlorotic mottle virus (CCMV) infection. After lysis of protoplasts, inoculated with CCMV and incubated for 30 min at 25°, virus-ribosome complexes could be isolated from the cell extract by zonal and density gradient centrifugation. Control experiments indicated, however, that complexes were also formed during or after lysis of the cells. To distinguish those complexes involved in cotranslational disassembly from the, most probably, extracellularly formed complexes, incubations were performed in the presence of [^{35}S]methionine. Although considerable amounts of radioactivity were found associated to the ribosomal fractions of inoculated cells, no translational activity could be ascribed to the CCMV-ribosome complexes. Similar results were obtained for tobacco mosaic virus (TMV), which was included in the experiments as control. As for TMV, translationally active virus-ribosome complexes had been isolated successfully from extracts of epidermis strips from inoculated tobacco leaves (Shaw *et al.*, 1986), the inability to demonstrate the presence of such complexes in cowpea protoplasts may indicate that this is due to features of the experimental system. On the other hand, the observation that CCMV-ribosome complexes were formed with very high affinity, even when initiation of translation was inhibited, provides evidence that association of virus particles and ribosomes precedes the process of cotranslational disassembly. The possibility that translationally active complexes might have been masked by a bulk of complexes that had not initiated translation yet will be discussed.

INTRODUCTION

To date, the hypothesis that plant viruses enter their host cells via lesions seems to gain more and more experimental support (for reviews see: De Zoeten, 1981; Watts *et al.*, 1981; Shaw, 1985; Zaitlin and Hull, 1987). However, less agreement exists about the question whether uncoating of the virus particles will occur prior to, during, or after penetration into the plant cell.

Recently, evidence has been obtained that during mechanical inoculation, at least in the experimental systems used, apparently intact virus particles become internalized into the cell (Laidlaw, 1987; Chapter 3). Hence, uncoating by a mechanism of cotranslational disassembly, as observed for some positive strand RNA viruses *in vitro* (Wilson, 1984; Brisco *et al.*, 1986; Chapter 4), was proposed to occur *in vivo* as well. For tobacco mosaic virus (TMV), this hypothesis was supported by the observation of translationally active virus-ribosome complexes in epidermal cells of tobacco leaves soon after inoculation (Shaw *et al.*, 1986; Plaskitt *et al.*, 1987). However, whether this observation stood for a coincidental event, restricted to this model system, or represented a more general mechanism of uncoating during plant virus infections, still remained unknown.

A different virus and experimental system, i.e., cowpea chlorotic mottle virus (CCMV) and isolated cowpea protoplasts, was used to search for functional virus-ribosome complexes. For this spherical virus, virion-directed translation has been demonstrated in cell-free protein synthesizing systems (Brisco *et al.*, 1986; Chapter 4). To study the possible occurrence *in vivo*, a method essentially the same as that used for isolation of CCMV-ribosome complexes from cell-free translation mixtures was employed for their isolation from lysates of inoculated protoplasts.

The results obtained indicate that CCMV-ribosome complexes are formed with high affinity. Although the formation of such complexes is likely to precede the process of cotranslational disassembly, their involvement in the latter could not be established. Possible reasons are discussed.

MATERIALS AND METHODS

Isolation, purification and storage of virus

CCMV was propagated in *Vigna unguiculata* cv. California Blackeye. The virus was isolated and purified as described by Verduin (1978a,b) and stored in 0.1 M

sodium acetate buffer, pH 5.0, containing 1 mM EDTA and 1 mM sodium azide, at 4°. TMV was purified according to procedure 5 as described by Noordam (1973).

Preparation of protoplasts

Cowpea mesophyll protoplasts were isolated as described by Van Beek *et al.* (1985), with minor modification of the washing solution [2.5 mM 2 (N-morpholino)-ethane sulfonic acid-KOH (MES, Sigma), pH 5.6, 0.6 M mannitol] and enzyme solution [0.8% cellulase and 0.05% Macerozyme (Yacult Pharmalogical Industries) in washing solution]. Only protoplast suspensions containing at least 80 % viable cells were used for inoculation experiments.

Inoculation of protoplasts

Inoculation was done essentially as described by Van Beek *et al.* (1985). A pellet of 12×10^6 protoplasts was mixed with 25 µl washing solution containing 600 µg CCMV or 2 mg TMV, immediately thereafter 0.5 ml of a solution containing 40% (w/v) polyethylene glycol (PEG, M_r 6000) and 3 mM $CaCl_2$ was added, homogenized, and diluted with 4.5 ml washing solution. After incubation for 30 min (different times will be indicated) at 25°, the protoplasts were divided into two aliquots: 2×10^6 protoplasts to determine the percentage of infected cells and 10×10^6 protoplasts to isolate virus-ribosome complexes. After sedimentation, both aliquots were washed three times in washing solution, including 10 mM cycloheximide (Sigma) when complexes had to be isolated.

For labeling of complexes 200 µCi L-[^{35}S]methionine (NEN, 1084 Ci/mmol) was added both to 20 ml enzyme solution containing 4-6 leaf halves 2 hr before isolation of the protoplasts and to the 25 µl inoculum. After incubation, washing was done in the presence of both 10 mM cycloheximide and 10 mM unlabeled L-methionine.

Determination of the percentage of infected cells

The aliquot containing 2×10^6 protoplasts was resuspended in 2.5 ml of culture medium as described by Aoki and Takebe (1969), in which the concentration of mannitol was raised to 0.6 M, 2.5 mM MES was added, 6-benzyladenine was omitted, and 10 µg/ml gentamycin (Sigma) was used as antibiotic. Protoplasts were kept at 25° under continuous illumination with fluorescent tubes (25 kW/m^2). After ca. 16 hr, protoplasts were prepared for immunofluorescence microscopy according to Van Beek *et al.* (1985). For each sample ca. 500 non-autofluorescent protoplasts were counted. Infectivity varied between 60 and 80%.

Protoplast lysis, fixation, and isolation of virus- and ribosome-containing fractions

The aliquot of 10 x 10^6 protoplasts (pellet) was cooled on ice for ca. 2 min before addition of 5 ml ice-cold lysis buffer, containing 50 mM triethanolamine-HCl, pH 7.5, 20 mM KCl, 10 mM magnesium acetate, 5 mM EGTA, and 10 mM cycloheximide. The protoplasts were resuspended and kept on ice for 15 min to accomplish cell-lysis. Then 5% (w/v) formaldehyde was added for fixation. Following another 15 min incubation on ice the lysates were centrifuged for 10 min in a Sorvall SS34 rotor at 10,000 rpm and 4°. The clear supernatants (ca. 7 ml) were layered on 10-50% (w/v) linear sucrose gradients made up in 28 ml 50 mM triethanolamine, pH 7.5, 20 mM KCl, 10 mM magnesium acetate (TEKM-buffer) containing 1% (w/v) formaldehyde, and centrifuged for 15 hr in a Beckman SW28 rotor at 18,000 rpm and 4°. The gradients were fractionated with an ISCO model 185 density gradient fractionator and the contents were monitored at both 254 and 280 nm with a LKB Uvicord III densitometer. The virus- and ribosome-containing fractions, i.e., fractions containing the material with sedimentation coefficients between 40 and 200 S, were collected and used for further analysis in CsCl gradients. When protoplasts were incubated in the presence of [^{35}S]methionine the lysis buffer was supplemented with 10 mM unlabelled L-methionine.

CsCl density analysis of nucleoprotein complexes

The collected virus- and ribosome-containing fractions were pooled and concentrated by centrifugation in a Beckman Type 50-2Ti rotor for 4 hr at 40,000 rpm and 4°. The pellets were resuspended overnight in ca. 100 µl TEKM-buffer containing 1% (w/v) formaldehyde, on a rotary shaker at 4°. The suspensions were mixed with 5 ml CsCl (density 1.48 g/cm^3), dissolved in the same buffer and centrifugation analysis was done as described previously (Chapter 4).

Electron microscopy

Twenty five microliters of CsCl-fractions were put on grids and washed with TEKM-buffer, containing 1% (w/v) formaldehyde. After a second fixation in 1% (w/v) glutaraldehyde in TEKM-buffer, the nucleoprotein material was negatively stained with 2% (w/v) uranyl acetate in double distilled water and viewed in a Philips CM12 electron microscope.

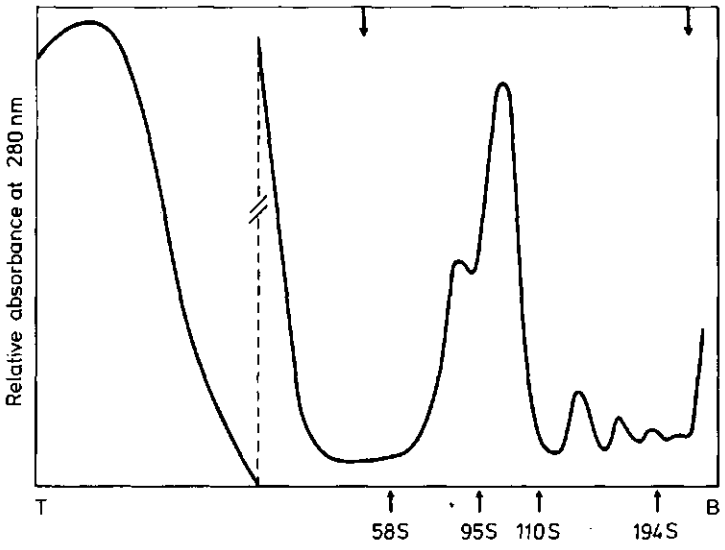
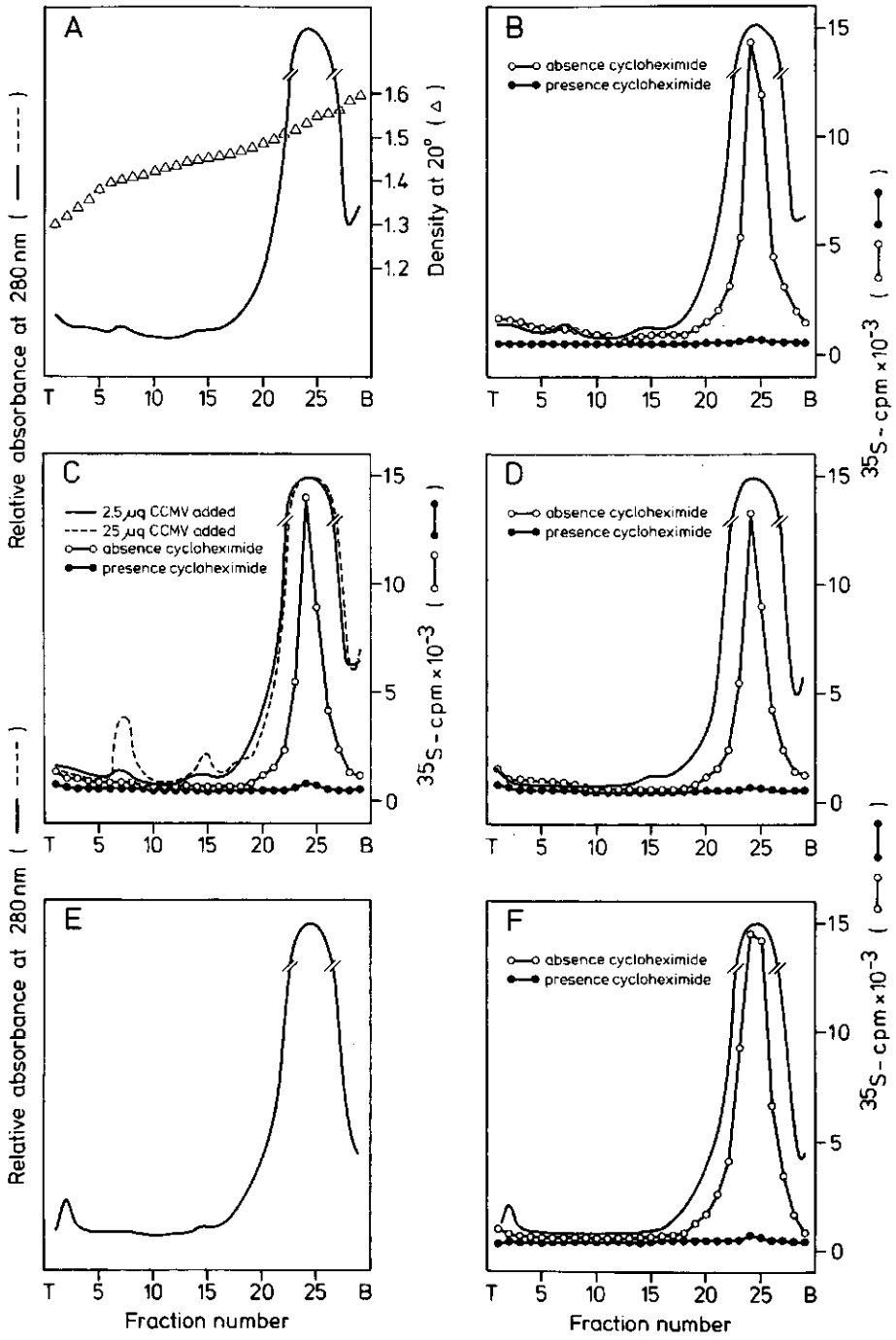


Figure 1. Sucrose gradient analysis of a lysate of 10 x 10^6 cowpea protoplasts inoculated with cowpea chlorotic mottle virus (CCMV). After inoculation, 0 or 30 min incubation at 25°, and washing, protoplasts were lysed in lysis buffer, containing 10 mM cycloheximide. The lysates were fixed in 5% (w/v) formaldehyde and after low speed centrifugation the clear supernatants were centrifuged in 10-50% (w/v) linear sucrose gradients made up in TEKM-buffer, containing 1% (w/v) formaldehyde. The gradients were monitored for absorption at 280 nm and fractionated. Fractions containing material with sedimentation coefficients between ca. 40 and 200 S (indicated by downward arrows) were used for further analysis on CsCl gradients. Sedimentation is from left to right. The positions of markers (cowpea mosaic virus -T, -M, and -B components, and tobacco mosaic virus: 58, 95, 110, and 194 S, respectively), run in sister tubes, are indicated by upward arrows.

RESULTS

A method essentially similar to that used for the isolation of virus-ribosome complexes from cell-free translation systems supplemented with CCMV (Chapter 4) was employed to study the possible occurrence of such complexes in CCMV-infected cowpea protoplasts. Inoculation of protoplasts, of which 60-80% became infected, was followed by cell-lysis and low speed centrifugation. The supernatant was subjected to sucrose gradient centrifugation and the absorbance profile at 280 nm is shown in Figure 1.


A
B
C
D
E
F
absence cycloheximide
presence cycloheximide
2.5 µg CCMV added
25 µg CCMV added
Relative absorbance at 280 nm (—— ----)
Density at 20° (△)
35S - cpm x 10-3 (o—o ●—●)
Fraction number
T 5 10 15 20 25 B
1.2 1.3 1.4 1.5 1.6
0 5 10 15

Free protein and nucleic acid molecules as well as small nucleoprotein complexes sediment close to the top of the gradient, clearly separated from virus, ribosomes, and complexes sedimenting faster than 80 S. Further analysis of these virus- and ribosome-containing fractions (between arrows in Fig. 1) on CsCl density gradients (Fig. 2) revealed a good separation between free virus particles (fraction 7, ρ = 1.36 g/cm3), ribosomes (fraction 24, ρ = 1.58 g/cm3), and the virus-ribosome complexes banding in between (see also Chapter 4). At the position of virus ribosome complexes (fractions 15 to 19) there was no difference between inoculated protoplasts lysed at time zero (Fig. 2A) or after 30 min incubation at 25° (Fig. 2B). However, when fractions of both gradients were examined in the electron microscope, virus-ribosome complexes containing up to three ribosomes per virus particle were found (Fig. 3). Moreover, these complexes were also present when non-inoculated protoplasts were lysed in the presence of virus (Fig. 2C (—)). Even an increase of absorbance around fractions 15 and 19 was found when the amount of virus present during lysis was increased (Fig. 2C (---)). No differences in morphology could be observed between the complexes found with CCMV-inoculated and non-inoculated protoplasts, i.e., no differences with regard to the number of ribosomes bound per virus particle and their position. Virus-ribosome complexes were also found when fixation was omitted, and therefore could not be considered as fixation artifacts. Hence, it had to be concluded that the formation of CCMV-ribosome complexes was not restricted to virus particles infecting intact protoplasts and that a substantial amount of these complexes formed after lysis of the cells.

In order to elucidate whether at least part of the complexes observed was formed inside the protoplasts as a result of a specific virion-directed translation process, inoculation was performed in the presence of [^{35}S]methionine. In this case, translationally active virus-ribosome complexes could be distinguished from inactive complexes on basis of incorporated radioactivity.

Figure 2. Cesium chloride density gradient analysis of sucrose gradient fractions (40-200 S) of lysates of protoplasts, inoculated with 500 µg CCMV (A,B), non-inoculated lysed in the presence of either 2.5 or 25 µg CCMV (C), mock-inoculated (D), or inoculated with 2 mg TMV (E,F). Protoplasts were lysed either immediately after inoculation (A,E) or after 30 min incubation at 25° (B,C,D,F). Incubation with 200 µCi [^{35}S]methionine was performed in the presence (control, ●-●) or absence (O-O) of 10 mM cycloheximide. Initial density of the gradients was 1.48 g/cm^3. Sedimentation is to the right. Density profiles of all tubes as indicated in diagram A (Δ).

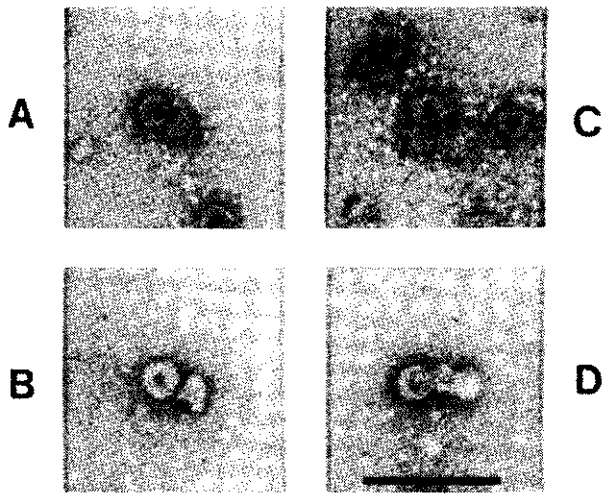


Figure 3. Electron micrographs of virus-ribosome complexes from lysates of CCMV-inoculated cowpea protoplasts, incubated and processed as described in Figures 1 and 2. Samples for electron microscopy were taken from fractions of a CsCl gradient (Fig. 2B). After fixation in 1% (w/v) glutaraldehyde in TEKM-buffer, complexes were negatively stained with 2% (w/v) uranyl acetate in water and viewed in a Philips CM12 electron microscope. Complexes of the most abundant type (50-75%) are shown for fraction numbers 15 (A,B) and 19 (C,D). Bar represents 100 nm.

In the presence of cycloheximide, an inhibitor of protein synthesis, almost no radioactivity was found (Figs. 2B, C, and D). In the absence of cycloheximide, however, non-inoculated (Fig. 2C), mock-inoculated (Fig. 2D), and protoplasts inoculated with CCMV (Fig. 2B) showed an extensive labeling in the ribosomal fractions (fractions 21-26). In contrast, virus-ribosome complexes, present in the lysates of CCMV-inoculated protoplasts (fractions 15-19) did not show a significant incorporation of [^{35}S]methionine. Increasing the incubation time up to 4 hr did not change these results (results not shown).

Also for TMV, which was analyzed with the same techniques and methods (Figs. 2E, and F), no significant amounts of incorporated radioactivity were found except for the ribosome-containing fractions (Fig. 2F). These results indicate that, if cotranslational disassembly occurs in isolated protoplasts, the amount of virus involved in the formation of functional virus-ribosome complexes remains below the level of detection. In case of CCMV, most if not all complexes observed representing "inactive" complexes formed after lysis of the cell.

In a final experiment, therefore, aurintricarboxylic acid (10 mM), an inhibitor of initiation of protein synthesis (Marcus *et al.*, 1970), was added to the lysis buffer as an attempt to prevent the formation of virus-ribosome complexes during or after lysis of the cells, i.e., to restrict the formation of complexes to the protoplast interior. However, also this inhibitor was unable to prevent the association of CCMV particles and ribosomes (results not shown). Hence, it had to be concluded that CCMV and ribosomes form complexes with very high affinity even before translation has been initiated and that this phenomenon may have masked the detection of functional virus-ribosome complexes *in vivo*.

DISCUSSION

The results presented in this paper demonstrate that CCMV particles and ribosomes from cowpea protoplasts form complexes with high affinity. Analysis of protoplast lysates revealed that approximately 10% of the CCMV particles present had become associated with ribosomes. However, whether the presence of these complexes reflected the occurrence of cotranslational disassembly remained questionable, as complexes were found not only in lysates of CCMV-inoculated protoplasts, but also in lysates of mock- and non-inoculated protoplasts, lysed in the presence of this virus.

To distinguish those complexes involved in cotranslational disassembly from those formed during or after lysis of the protoplasts, incubations were performed in the presence of [^{35}S]methionine. Although considerable amounts of radioactivity were found associated to the ribosomal fractions of inoculated cells, no translational activity could be ascribed to the virus-ribosome complexes. Also for TMV, for which translationally active virus-ribosome complexes had been isolated from extracts of epidermis strips from inoculated tobacco leaves (Shaw *et al.*, 1986), no functional complexes were detected in the cowpea protoplast system. Hence, our inability to demonstrate the translational activity of CCMV-ribosome complexes might be due to features of the experimental system.

Isolated mesophyll protoplasts may differ in various aspects from the intact cells in epidermis strips. For isolation, protoplasts are devoid of cell walls, which may be essential for destabilization of virus particles (Gaard and De Zoeten, 1979). In addition, inoculation with polyethylene glycol (PEG) causes a severe osmotic shock, which has been shown to dramatically change the cellular metabolism (Lazar *et al.*, 1973; Premecz *et al.*, 1978; Fleck *et al.*, 1982). On the other hand, PEG is known to aggregate virus particles (Hebert, 1963), which may be

disadvantageous for the interaction of individual particles with cytoplasmic ribosomes. Moreover, the fact that a substantial part of the virus particles becomes trapped in endocytotic vesicles as a result of the PEG-treatment (Chapter 3), may reduce the number of potential candidates for cotranslational disassembly considerably. Finally, it cannot be excluded that intracellular conditions, which may be different for epidermal and mesophyll cells, may effect the probability of uncoating of the virus particles.

In spite of the fact that the experiments presented do not demonstrate the formation of translationally active virus-ribosome complexes *in vivo*, they indicate that complexes are formed with very high affinity, even when initiation of translation is inhibited. These results are in agreement with the previously observed binding of CCMV to ribosomal protein blots (Chapter 5). Although, there is no evidence for a functional role of this interaction yet, the ability of CCMV-ribosome complexes to perform translational activity in cell-free translation systems (Chapter 4), suggests that association of virus particles and ribosomes precedes the process of cotranslational disassembly. Why only such a small fraction of the virus particles establishes such interaction (*in vitro* less than 5%) remains unsolved. The same holds true for the question whether all complexes will eventually perform cotranslational disassembly.

In conclusion, our results on cotranslational disassembly of CCMV in isolated cowpea protoplasts indicate that CCMV-ribosome complexes are formed with very high affinity. Although, translational activity of this complexes could not be demonstrated, evidence has been obtained that the association of virus particles and ribosomes precedes the process of cotranslational disassembly. Hence, the presence of translationally active complexes might have been masked by a bulk of complexes that had not initiated translation yet. For demonstration of cotranslational disassembly of CCMV *in vivo*, therefore, more sensitive and/or specific tools, e.g., specific antibodies reacting with the N-terminus of non-structural viral proteins, to detect translationally active virus-ribosome complexes, might be required.

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CHAPTER 7

GENERAL DISCUSSION

This thesis describes a study of early stages of plant virus infection. These stages have remained relatively unknown despite the fact that our insights in later stages have greatly increased during the past ten years. Especially the fact that often only a few percent of the inoculated particles is actually involved in infection and the failure to detect these particles among the bulk of non-infecting hampered such investigations.

In Chapter 2 the present state of knowledge of early stages of animal as well as plant virus infections is briefly reviewed. From this chapter it is clear that for plant viruses, the understanding of the process of penetration and uncoating was even more fragmentary than for animal viruses. The inconclusive results on initial interactions between plant virus and cell, especially the lack of information on the relation between events occurring on inoculation and virus entry and infection, have given rise to a reinvestigation of these interactions.

Chapter 3 describes the binding of cowpea chlorotic mottle virus (CCMV) to isolated cowpea protoplasts in relation to virus entry and infection. The results support a mechanism of virus entry by initial physical association of virus particles with the plasma membrane and subsequent internalization through membrane lesions. Virus taken up by vesicles, formed by invagination of the plasma membrane (endocytosis), did not seem to be involved in infection. This mechanism of entry via membrane lesions may be supported by the results obtained by Laidlaw (1987). Also in this case, it was proposed that, after inoculation of tobacco leaf epidermal cells, virus particles, bound to the plasma membrane of cytoplasmic extrusions, were carried passively into the cell during their retraction from the leaf surface. The properties of these cytoplasmic extrusions may be identical to those of isolated protoplasts. Furthermore, studies on the probing and feeding behaviour of aphids with regard to the relation between stylet paths made during superficial probing, and the ability to transmit nonpersistent viruses, revealed that a direct contact with the cytoplasm is

required for transmission of virus (Lopez-Abella *et al.*, 1988). Hence, also under natural conditions, penetration of plant virus particles into a cell seems to be dependent on external damaging of the protoplast membrane. These results indicate that uncoating of the viral genome, most probably, occurs intracellularly.

In Chapter 4 "cotranslational disassembly", as first proposed for the uncoating of tobacco mosaic virus (TMV) (Wilson, 1984), was investigated as a possible mechanism of uncoating for CCMV. Addition of CCMV particles to cell-free translation systems revealed that the "encapsidated" viral genome was translated into viral specific proteins, while, moreover, translationally active virus-ribosome complexes could be isolated from the mixture. These results indicated that also spherical virus particles may, at least *in vitro*, become uncoated by cotranslational disassembly.

In Chapter 5 the interactions between CCMV and ribosomes were analyzed in more detail by studying binding of virus to wheat germ ribosomal proteins in electroblot assays. The results obtained indicated that at least twenty proteins, derived from the 40 as well as the 60 S ribosomal subunit, are able to bind CCMV or its coat protein subunits.

Chapter 6 reports on the possible involvement of cotranslational disassembly in uncoating of CCMV *in vivo*. Indeed, from lysates of inoculated cowpea protoplasts CCMV-ribosome complexes could be isolated. However, their involvement in cotranslational disassembly could not be demonstrated. Neither for TMV, for which uncoating by cotranslational disassembly in epidermal cells of inoculated tobacco leaves was demonstrated (Shaw *et al.*, 1986), translationally active complexes were detected. Therefore, it was concluded that the cowpea protoplast system probably was unsuitable for studying these initial interactions with viruses. In addition to the interfering treatments of the protoplasts during isolation as well as inoculation, as discussed in Chapter 6, it should be realized that in intact leaf tissue, mesophyll cells have other functions than epidermal cells. This implicates that intracellular, cell-type dependent, conditions may effect the probability of uncoating of the virus particles. For TMV, for instance, indications exist that uncoating is just a single event occurring in the initially infected (epidermal) cell, while spread of infection from cell to cell is obtained by transport of naked RNA (Atabekov and Dorokhov, 1984).

In spite of the fact that the experiments with cowpea protoplasts did not demonstrate the occurrence of cotranslational disassembly of CCMV *in vivo*, they revealed that part of the virus particles became associated with cytoplasmic ribosomes, even when the initiation of translation was inhibited. These results

suggested that binding of virus particles to ribosomes precedes cotranslational disassembly. In analogy to the interactions observed with cytoplasmic ribosomes and alphavirus nucleocapsids in animal cells (Wengler and Wengler, 1984), it is particularly attractive to propose that also for plant viruses ribosomes fulfil a role in destabilization of the nucleocapsid prior to initiation of cotranslational disassembly.

Besides the observed interactions between CCMV and ribosomal proteins, as described in Chapter 5, further evidence for an intracellular "uncoating" site was obtained in recent investigations on the mechanism of resistance as observed in protoplasts of transgenic tobacco plants (Register and Beachy, 1988). It was found that constitutive expression of the coat protein of TMV resulted in resistance to infection by virions, while little or no resistance was observed upon inoculation with either naked TMV-RNA or pH 8-treated, i.e., destabilized, virions. Therefore, it was suggested that the expression of the TMV coat protein gene in transgenic cells prevented the virus from uncoating. In addition a model was proposed in which protection may be due to blockage of intracellular sites where virus uncoating takes place. If these sites would have a specific affinity for TMV coat protein, the coat protein synthesized in the transgenic cell could physically block these sites, preventing the incoming TMV particles from uncoating.

In relation to this hypothesis, the observed binding of CCMV and some other plant viruses to ribosomal proteins, suggest that ribosomes are likely candidates for functioning as such intracellular "uncoating" site. On the other hand, the fact that almost the same set of ribosomal proteins was found to be involved in binding of different viruses, while the resistance observed in transgenic plants appeared to be virus specific, indicates that additional mechanisms of resistance have to be proposed for explaining these results.

With regard to the initial interactions between CCMV and its host cell, the results so far are summarized in the following model (Fig. 1). It should be realized that this model is only applicable to a very small part of the virus particles as the majority of particles is not involved in penetration (Chapter 3) and uncoating (Chapter 4) at all. In addition, the experimental evidence has been obtained in completely different systems.

Following mechanical damage of the (cell wall and) plasma membrane intact virus particles enter the cytoplasm of a host cell (A, Chapter 3), where they associate with ribosomal subunits (B, Chapter 6). This interaction affects the virus particles in such a way that the 5'-end of the viral RNA becomes accessible for initiation of translation (C). The first ribosome starts translation and by

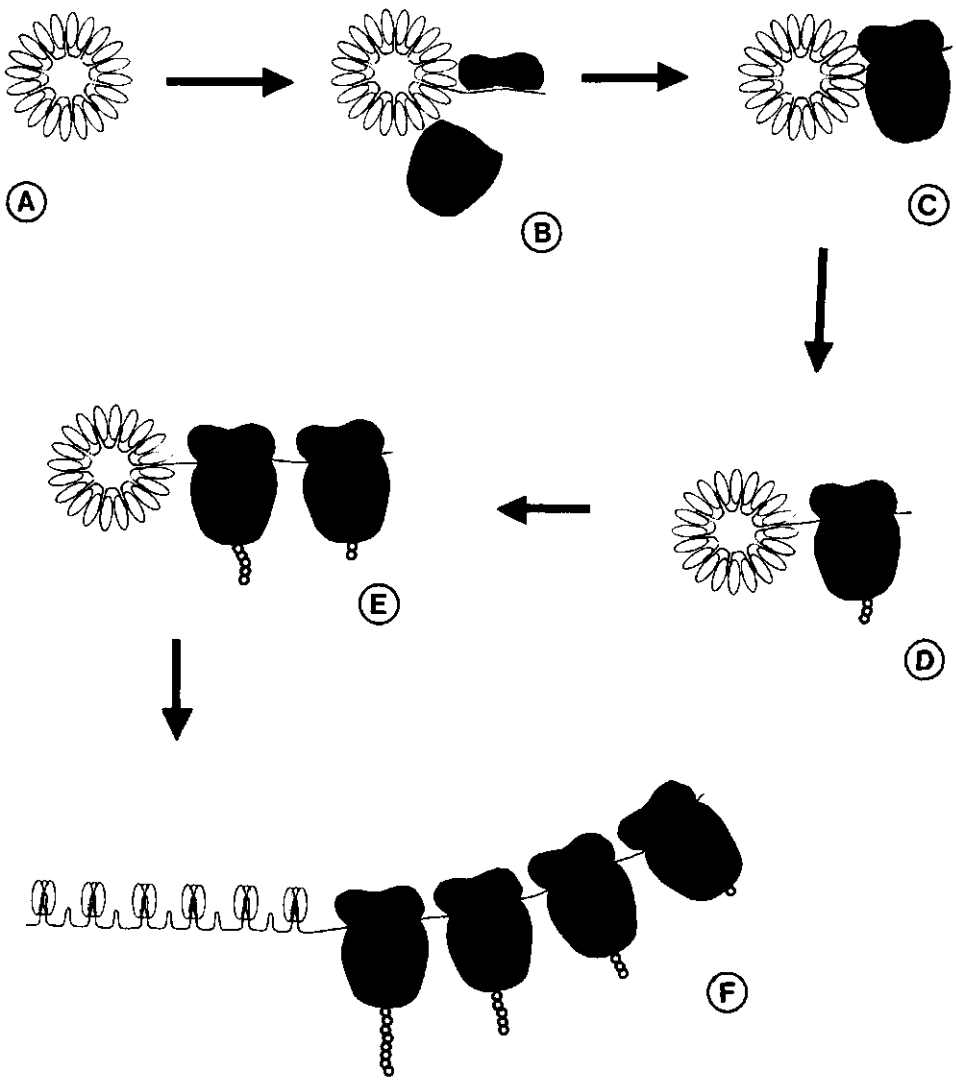

Figure 1. Model for initial interactions between CCMV and cytoplasmic ribosomes. For description see text. A virus particle is represented by a cross section of the icosahedral particle, the strings and ellipses representing RNA molecules and protein subunits, respectively. Ribosomes are represented by the black structures, showing both the small and large ribosomal subunits. The newly synthesized polypeptides are represented by chains of small circles.

proceeding, the RNA is pulled out of the protein shell (D, Chapter 4), thereby allowing more ribosomes to initiate translation (E, Chapter 4). After approximately 20% of the RNA has been pulled out of the protein shell, virus particles loose their stability and disintegrate into linear structures, in which the coat protein remains attached to the viral RNA (F, Chapter 4), the coat protein being removed by the first ribosome during proceeding of translation.

It is clear that for some steps there is more evidence than for others. Especially the second and third step (B and C) are highly hypothetical. Although several ribosomal proteins have been found able to bind the viral coat protein (Chapter 5), their relation to the initiation of translation is not understood. Evidence exists that the initiation of translation is performed by only 40 S ribosomal subunits (Kozak, 1980). Thus, for gaining more evidence for this model, it will be necessary to investigate whether 40 S ribosomal subunits are able to withdraw the 5'-end of the viral RNA from the nucleocapsid prior to the formation of functional 80 S ribosomes. Moreover, the occurrence of cotranslational disassembly of CCMV *in vivo* has to be firmly established.

The results in this thesis also illustrate that studies on initial interactions between virus and plant cell have to cope with a number of practical difficulties. Especially the experiments performed with isolated protoplasts, as described in Chapters 3 and 6, demonstrate that those virus particles and events really involved in infection of the cell, are masked by a bulk of non-infecting virus particles.

In spite of this, indications have been obtained that cytoplasmic ribosomes are involved in uncoating of the viral genome at the onset of CCMV infection. Therefore, based on the results obtained, it seems worthwhile to further characterize the interactions between CCMV and ribosomes, especially with regard to the ability of 40 S ribosomal subunits to get access to the 5'-end of viral RNA molecules. Moreover, a better understanding of the initial interactions between a plant virus and its host cell may provide new insights on the mechanisms by which viruses are able to get access to the cellular machinery and, in addition, to take over its direction at the cost of even whole plants.

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CHAPTER 8

SAMENVATTING

Virussen zijn infectieuze eenheden, bestaande uit nucleïnezuren welke omgeven zijn door een eiwitmantel en eventueel een membraan. Voor wat betreft hun vermenigvuldiging zijn virussen afhankelijk van een levende gastheercel, waarbij ze over het vermogen moeten beschikken deze binnen te dringen, zich erin te vermenigvuldigen en zich vervolgens naar andere cellen te verspreiden.

Gedurende de laatste decennia is onze kennis over virussen en hun vermogen tot infectie enorm toegenomen. De opkomst van de moleculair- en celbiologische technieken heeft bijgedragen tot een gedetailleerde kennis van zowel de organisatie van het virale genoom, als de mechanismen die aan de expressie en replicatie ervan ten grondslag liggen. Daarnaast hebben biofysische studies informatie verschaft over de structuur en assemblage van het virale deeltje. De kennis over de initiële stadia van het infectieproces, het binnendringen in de gastheercel en de ontmanteling van het virale genoom bleef echter zeer schaars. Het feit dat in veel gevallen slechts enkele virusdeeltjes verantwoordelijk zijn voor infectie heeft vooral bestudering van de initiële interacties tussen virus en gastheercel aanzienlijk bemoeilijkt.

Dit proefschrift beschrijft het onderzoek verricht aan deze initiële stadia van virusinfecties bij planten. Twee vragen stonden hierbij centraal: (1) hoe en in welke vorm dringt een plantevirus een te infecteren cel binnen en (2) welk mechanisme is verantwoordelijk voor de ontmanteling van het virale genoom? Als model voor dit onderzoek is gekozen voor cowpea chlorotic mottle virus (CCMV) en geïsoleerde cowpea (*Vigna unguiculata*) mesophyl protoplasten. Een aantal eigenschappen van dit modelsysteem zijn weergegeven in de inleiding (Hoofdstuk 1).

Met betrekking tot de initiële interacties tussen virus en gastheercel is voor zowel dier- als plantevirussen een beknopt literatuuroverzicht gegeven van de tot nu toe bekende mechanismen (Hoofdstuk 2). Zowel op het niveau van de binding aan het celoppervlak, het binnendringen in de gastheercel, als ook op het niveau

van de ontmanteling van het virale genoom, zijn verschillen en overeenkomsten tussen dier- en plantevirussen belicht. De meest opmerkelijke verschillen werden aangetroffen bij de binding en binnendringing van de gastheercel. Is voor de meeste diervirussen binding aan specifieke componenten van de plasmamembraan (receptoren) noodzakelijk voor penetratie, voor plantevirussen lijkt alleen een beschadiging van de plasmamembraan voldoende.

Om na te gaan in hoeverre ook voor plantevirussen (specifieke) interacties met membraancomponenten van belang zijn voor infectie van de cel, is de binding van CCMV aan cowpea protoplasten bestudeerd in relatie tot binnendringing en infectie (Hoofdstuk 3). Uit de resultaten van dit onderzoek bleek dat de binding van CCMV aan geïsoleerde protoplasten gebaseerd is op (aspecifieke) elektrostatische interacties, terwijl penetratie afhankelijk is van mechanische beschadiging van de plasmamembraan. Tevens zijn aanwijzingen verkregen dat virusdeeltjes, welke middels endocytose worden opgenomen, niet betrokken zijn bij infectie van de cel. Uit deze gegevens werd geconcludeerd dat alleen die virusdeeltjes, die via membraanbeschadigingen direct in het cytoplasma van de gastheercel terecht komen, verantwoordelijk zijn voor infectie. Ten gevolge hiervan moet worden verondersteld dat ontmanteling van het virale genoom intracellulair plaatsvindt.

Aangezien voor het tabaksmozaïekvirus (TMV) inmiddels sterke aanwijzingen waren verkregen dat cytoplasmatische ribosomen een rol spelen bij ontmanteling van het genoom middels "cotranslational disassembly", werd nagegaan in hoeverre dit mechanisme ook van toepassing zou kunnen zijn op CCMV. "Cotranslational disassembly" veronderstelt dat ontmanteling plaatsvindt gelijk- tijdig met translatie van het virale genoom, waarbij in het geval van TMV het virusdeeltje vooraf zodanig behandeld moet worden dat het 5'-uiteinde van het RNA beschikbaar is voor initiatie van translatie.

Door in eerste instantie gebruik te maken van celvrije translatiesystemen werden aanwijzingen verkregen dat ook voor CCMV "cotranslational disassembly" een rol zou kunnen spelen bij de ontmanteling (Hoofdstuk 4). Na toevoeging van intacte virusdeeltjes aan dergelijke systemen werd synthese van virus specifieke eiwitten waargenomen. Bovendien kon de aanwezigheid van translationeel actieve virus-ribosoom complexen worden aangetoond.

De interactie tussen CCMV en ribosomen werd vervolgens nader geanalyseerd op eiwitniveau (Hoofdstuk 5). Hiertoe werden electroblots van ribosomale eiwitten, gescheiden onder denaturerende omstandigheden, geïncubeerd met virus, en werd het gebonden virus met behulp van immunologische methoden

zichtbaar gemaakt. Ongeveer twintig eiwitten, behorend tot zowel de grote als kleine ribosomale subeenheden, bleken virus en/of viraal mantel-eiwit te binden. Dezelfde ribosomale eiwitten bleken eveneens betrokken te zijn bij binding van een aantal andere plantevirussen. Hoewel de beschreven experimenten hierover onvoldoende uitsluitsel geven, zou de waargenomen binding van virus aan deze ribosomale eiwitten op een functionele rol kunnen duiden.

Naast deze analyse op eiwitniveau is tevens gekeken naar de rol van "cotranslational disassembly" in de ontmanteling van CCMV *in vivo* (Hoofdstuk 6). Op verschillende tijdstippen na inoculatie werden met CCMV geïnoculeerde cowpea protoplasten gelyseerd en vervolgens geanalyseerd op de aanwezigheid van virus-ribosoom complexen. Inderdaad werden virus-ribosoom complexen aangetroffen, echter in tegenstelling tot *in vitro*, kon hun translationele activiteit *in vivo* niet worden aangetoond. Hiervoor wordt een aantal mogelijke verklaringen gegeven. Anderzijds benadrukten ook deze resultaten nogmaals de sterke affiniteit tussen CCMV en ribosomen, en werden aanwijzingen verkregen dat de vorming van virus-ribosoom complexen vooraf zou kunnen gaan aan een eventuele initiatie van "cotranslational disassembly".

Tenslotte zijn de in de diverse experimentele systemen verkregen gegevens samengevat in een model (Hoofdstuk 7, Figuur 1). Dit model beoogt een beeld te geven van die gebeurtenissen die van toepassing zijn op de wellicht minder dan 0,01% van de virusdeeltjes die verantwoordelijk zijn voor infectie van de cel. Met name daar waar het de interactie tussen virusdeeltje en ribosoom betreft, voorafgaand aan de initiatie van "cotranslational disassembly", bestaan nog veel vraagtekens. Een verdere ontrafeling van deze complexe interacties, alsmede het aantonen van "cotranslational disassembly" van CCMV *in vivo*, kunnen bijdragen tot een beter begrip van de initiële stadia van virusinfecties bij planten.

CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 25 januari 1959 in Doetinchem. In 1977 legde zij het eindexamen atheneum B af aan het Ulenhof college in dezelfde plaats, waarna begonnen werd met de studie Biologie aan de Rijksuniversiteit te Utrecht. Het doctoraalexamen werd behaald met lof in februari 1985, met als hoofdvak Moleculaire Celbiologie en als bijvakken Biochemie en Experimentele Cardiologie. Van maart 1985 tot maart 1988 was zij werkzaam als wetenschappelijk assistent bij de vakgroep Virologie van de Landbouwuniversiteit te Wageningen. Het hier verrichtte onderzoek heeft geleid tot dit proefschrift. Vanaf februari 1989 is zij werkzaam als hoofd van afdeling Virologie bij de Plantenziektenkundige Dienst te Wageningen.