PURIFICATION AND PROPERTIES OF
COWPEA MOSAIC VIRUS RNA REPLICASE

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PURIFICATION AND PROPERTIES OF COWPEA MOSAIC VIRUS RNA REPLICASE
(with a summary in Dutch)

Proefschrift

ter verkrijging van de graad
van doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op vrijdag 17 maart 1978
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen.

STELLINGEN

1. Ter bevordering van de verkeersveiligheid dienen alle bestuurders van voertuigen verplicht te worden overdag met ontstoken verlichting te rijden.

2. De gedetailleerde kennis van het Qβ replicase is tot nu toe eerder een belemmering dan een stimulans geweest voor het onderzoek over eukaryotische virus RNA replicases.

3. Het is een misvatting, dat het ontbreken van matrijs-specificiteit van eukaryotische virus RNA replicases verklaard kan worden door de afwezigheid van eiwitfactor(en) analoog aan die van het Qβ replicase.

   C. LeRoy, C. Stussi-Garaud en L. Hirth. 1977. Virology 82, 48.
   J.T. May en R.H. Symons. 1971. Virology 44, 517.
   A. Traub, B. Diskin, H. Rosenberg en E. Kalmar. 1976. J. Virol. 18, 375.
   M. Zaitlin, C.T. Duda en M.A. Petti. 1973. Virology 53, 300.

4. De bewering in de Grote Nederlandse Larousse Encyclopedie dat genamplificatie een synoniem is van genactivatie is foutief.

   Grote Nederlandse Larousse Encyclopedie. Uitg. Scheltens en Giltay. N.V. 's-Gravenhage.

5. De experimenten van Salvato en Fraenkel-Conrat betreffende de *in vitro*
    translatie van het RNA van tabaks necrose virus zijn zeer onvolledig en
    rechtvaardigen niet de conclusie dat er mogelijk drie initiatieplaatsen
    voor de translatie zijn.

> M.S. Salvato en H. Fraenkel-Conrat. 1977. Proc. Natl.
> Acad. Sci. USA 74, 2288.

6. Het weglaten van (een lijst met) woorden uit de volks- en schuttingtaal
    in het Nieuw Nederlands Handwoordenboek van 'Van Dale' - terwijl daar-
    entegen wel afzonderlijke lijsten met namen uit de Grieks-Romeinse
    oudheid en uit de bijbel zijn toegevoegd - miskent het levend taalgebruik
    en getuigt van een elitaire taalopvatting.

> Nieuw Handwoordenboek der Nederlandse Taal.
> Achtste, opnieuw bewerkte en aangevulde druk door dr. F.
> De Tollenaere en dr. A.J. Persijn. 1975. Martinus Nijhoff,
> 's-Gravenhage.

7. Het verdient aanbeveling om van overheidswege maximumprijzen vast te
    stellen voor Franse wijnen in Nederlandse restaurants.

8. De recent ontwikkelde RNA sequentie analyse methoden van Simoncsits *et
    al.*, Donis-Keller *et al.* en Gupta en Randerath zijn minder universeel
    dan gesuggereerd wordt.

> A. Simoncsits, G.G. Brownlee, R.S. Brown, J.R. Rubin en
> H. Guilley. Nature 269, 833, 1977.
> H. Donis-Keller, A.M. Maxam en W. Gilbert. Nucleic Acids
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> 1977. Nucleic Acids Res. 4, 3441, 1977.

9. Het salaris van röntgenologen dient regelmatig te worden doorgelicht.

Pim Zabel

Aan mijn ouders
Voor Petra
Mariska
Ivor

VOORWOORD

Hoewel de titelpagina slechts één auteursnaam draagt, is het tot stand komen van dit proefschrift te danken aan de inzet van een team. Daarom wil ik graag mijn oprechte dank betuigen aan:

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- allen die ik vergeten ben te vermelden.

CONTENTS

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ABBREVIATIONS AND SYMBOLS

AMV	alfalfa mosaic virus
ATP	adenosine-5'-triphosphate
BBMV	broad bean mottle virus
BMV	brome mosaic virus
BPMV	bean pod mottle virus
CCMV	cowpea chlorotic mottle virus
CMV	cucumber mosaic virus
cpm	counts per minute
CPMV	cowpea mosaic virus
CTP	cytidine-5'-triphosphate
Ci	Curie
DEAE-	diethylaminoethyl-
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpm	desintegrations per minute
DTE	dithioerythritol
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetate
g	centrifugal field (number times gravity)
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
M	molar
M.W.	molecular weight
mRNA	messenger ribonucleic acid
P_i	inorganic phosphate
PP_i	pyrophosphate
PEMV	pea enation mosaic virus
PMSF	phenyl methyl sulphonyl fluoride
poly(A)	polyriboadenylic acid
poly(C)	polyribocytidylic acid

poly(G)	polyriboguanylic acid
poly(U)	polyribouridylic acid
PVX	potatovirus X
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
S	Svedberg, the unit of sedimentation
SDS	sodium dodecyl sulphate
STNV	satellite tobacco necrosis virus
TMV	tobacco mosaic virus
TNV	tobacco necrosis virus
Tris	Tris(hydroxyl)aminomethane
tRNA	transfer ribonucleic acid
TRSV	tobacco ringspot virus
TYMV	turnip yellow mosaic virus
UTP	uridine-5'-triphosphate

1. INTRODUCTION

1.1 SCOPE OF THE INVESTIGATIONS

The view of replication of eukaryote viruses containing a single-stranded RNA
genome of the plus type has its origin primarily in the area explored by the
exciting research with bacteriophage Qβ. This is not surprising, since the
replication mechanism of this virus, which was discovered only about 15 years
ago (9), has been revealed in detail (14). Particularly the Qβ replicase, the
RNA-dependent RNA polymerase synthesized in the host upon infection and respon-
sible for the replication of the viral genome, has been studied extensively and
has proved to be an alluring and fruitful enzyme to study *in vitro* (14). At this
moment it is probably one of the best known nucleic acid synthesizing enzymes.
It was shown to be the first enzyme able to faithfully replicate infectious
progeny viral RNA *in vitro* (2, 6-8), and in addition, its rather complex sub-
unit structure has been resolved. The enzyme was shown to be composed of four
nonidentical subunits encoded by two distinct genomes. One polypeptide chain is
coded by the viral genome and the three others are supplied by the host cell
(3, 5).

In contrast to these sophisticated data, the current status of knowledge about
eukaryote RNA replicases and the way they propagate the viral genome is still in
its infancy, although there has been considerable work done in this area for
about 15 years. Up to the present time, eukaryote RNA replicases have not been
purified to homogeneity. The plant virus replicases thus far isolated are far
from being pure and have not yet been identified structurally. It is also un-
known whether host (protein) factors are involved in viral RNA replication.
Whereas with Qβ replicase very detailed studies have been performed concerning
the role the individual subunits are playing in the different steps of the rep-
lication, questions arising for eukaryote replicases still refer to how to pu-
rify them. Since a detailed understanding of eukaryote virus RNA replication
will depend upon the availability of an *in vitro* replicase system, the endeav-
our is highly meaningful.

One of the major difficulties limiting progress in the purification of eukaryote virus RNA replicases stems primarily from the fact that most replicases are bound to cellular membranes and template RNA and rapidly loose their stability upon solubilization and further purification. Another disadvantage concerns the relatively low specific activity of replicase and low amount per gram of cells in comparison to the Qβ-infected *E. coli* cells. From these considerations it is evident that eukaryote virus RNA replication provides a large field of research that still needs to be explored. Purification of the replicases is therefore one of the major aims to be achieved.

The aim of our work has been to purify Cowpea Mosaic Virus (CPMV) replicase from infected *Vigna* leaves. With this goal in mind, we focused our attention, in first instance, on the detection of an RNA-dependent RNA polymerase activity, which might be specific for CPMV-infected leaves. Such an enzyme activity was found to be present in the 31,000 xg fraction and was designated as CPMV replicase.

In chapter 3, we describe the isolation and time course of appearance of this membrane-bound replicase in addition to some of its properties and the nature of the RNA products synthesized *in vitro*. In attempting to purify the replicase further, we had to release the replicase from the membranes. Following several approaches which had been applied for the solubilization of other eukaryote virus RNA replicases we at first tried to use (non)ionic detergents and/or high salt concentrations. Then we were faced with the problem of lability of the solubilized enzyme hampering subsequent purification steps. However, from a close examination of other methods known to release proteins from membranes, we learned that a stable enzyme could be obtained by avoiding the use of detergents.

In chapter 4, it is shown that the replicase can easily be detached from the membranes by washing with a Mg^{2+}-deficient buffer. Using this method, we have at our disposal a highly stable enzyme, which can be further purified and freed of endogenous template RNA by DEAE-BioGel column chromatography.

In chapter 5 we describe the assay conditions favorable for replicase activity and the template activity of a variety of synthetic, viral and nonviral RNAs. The data show that the synthetic homopolymers poly(A), poly(U), poly(G) and poly(C) cannot be used efficiently as templates to direct the synthesis of a complementary chain, in contrast to various natural RNA templates. Furthermore, preliminary studies have been carried out on the interaction of replicase and ^{32}P-CPMV RNA and on the characterization of the *in vitro* synthesized RNA products.

In chapter 6 we describe our attempts to achieve additional purification of the DEAE-purified enzyme. Glycerol gradient centrifugation was found to be a very efficient and gentle purification step. Finally, an analysis of the replicase by polyacrylamide gelelectrophoresis has been carried out.

1.2 ACCOUNT

A part of the results presented in this thesis has already been published (10-13). Studies on related subjects have not been included in this thesis but are published elsewhere (1, 4).

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2. LITERATURE REVIEW OF VIRUS RNA REPLICASES

In the early sixties the replication of viruses containing DNA was understood in broad outline, owing to the pioneering investigations on the structure and replication of bacterial and T_{even}-bacteriophage DNA and to the isolation of, and *in vitro* work on DNA polymerase (50, 250). At that time the knowledge about the replication of animal and plant RNA viruses showed a gap. In addition, the replication of eukaryote RNA viruses appeared to be a field of research difficult to explore, primarily because of the technical difficulties encountered in the biochemical analyses. Therefore, the discovery by Loeb and Zinder in 1960 (144) of the RNA bacteriophage f2 in sewage, soon followed by the discovery of several other RNA phages (Qβ, MS2, R17, M12) was met with great interest and initiated a new field of investigations. Biochemical analysis of bacteria infected with RNA phages proved to be much easier than that of the eukaryote counterpart. Furthermore, genetic studies and *in vivo* and *in vitro* translation work greatly contributed to the understanding of phage multiplication by providing evidence for the existence of three cistrons on the viral genome of which one was shown to code for a protein involved in RNA replication, another for coat protein and a third for maturation protein (71, 111). Thus, after some years of extensive investigations the knowledge about RNA phage replication had grown enormously, whereas that of the eukaryote RNA viruses made, and still makes, only slow progress. Therefore, it is not surprising that most of the research on animal and plant RNA virus replication is strongly coloured and influenced by the bacteriophage work.

Although, on the one hand we have to be very aware of all the risks and pitfalls involved in extrapolating prokaryote results to the eukaryote systems, on the other hand, the findings obtained with the RNA phages are too important and influential to neglect. Therefore, I will describe first the most prominent results of the RNA phage work, confining myself mainly to the Qβ replicase. Thereafter, I will review the current status of research on the *in vitro* replication

of animal and plant viruses containing a single-stranded RNA genome of the plus type (12).

2.1.1 *In vitro replication of Qβ RNA*

Soon after the discovery of the RNA bacteriophages it became evident from hybridization experiments and from studies using inhibitors of DNA-dependent RNA synthesis and DNA synthesis, that DNA was not involved in phage RNA synthesis (51, 64, 104, 222) and that progeny viral RNA had to be generated from parental RNA via an RNA-dependent RNA polymerase. Then, attempts were undertaken by several groups to detect and isolate a phage induced RNA-dependent RNA polymerase, the so-called RNA replicase (224), from infected cells (10, 100, 255). The first success was achieved in 1963 by Spiegelman's group with the MS2 replicase (100), in 1965 followed by the isolation of the replicase from Qβ (101, 223), a phage discovered in Japan by Watanabe (247). Both partially purified enzymes exhibited a virtually complete dependence on exogenous template and showed a remarkable preference for their own, homologous RNA. No significant enzyme activity could be detected under optimal conditions with several other heterologous RNA species, including tRNA and rRNA of the host cell, TMV RNA and STNV RNA.

The Qβ replicase was chosen for further studies because of its higher stability than the MS2 replicase (221). In general, the replicases of group I phages (f2, R17, MS2, fr and M12) appeared to be unstable and difficult to obtain in a template-dependent form (10, 73, 253, 254) whereas group III phage replicases (Qβ, VK, ST) have proved rather easy to purify (162, 228).

One of the most exciting results in the history of the Qβ replicase was reported by Spiegelman's group in 1965. The enzyme appeared to be capable of mediating *in vitro* virtually unlimited synthesis of infectious self-replicating progeny viral RNA in an autocatalytic reaction (103, 173, 174, 223). From then on, until about 1969, the enzyme has been applied primarily to study the mechanism of phage RNA replication *in vitro* and to elucidate all the intermediate steps occurring between the start of the reaction with viral (+) strand RNA as the template and the generation of progeny (+) strands. These studies, which have been reviewed extensively (9, 71, 122, 196, 225, 228, 250, 252) can be summarized as follows.

In the first step a replicase molecule binds to the single-stranded (+) strand and initiates synthesis of a (-) strand reading the (+) strand from the 3'end to the 5'end and elongating the new chain in the 5' to 3' direction. After the onset

of RNA synthesis other replicase molecules can attach to and initiate on the
(+) strand giving rise to replication complexes (replicative intermediates,
RI's), which consist of a single-stranded template, one or more single-stranded
nascent (-) strands and replicase molecules. This (+) strand-directed synthesis
of (-) strands requires in addition to the complete Qβ replicase a host-supplied
protein, the so-called "host factor I" (45, 84, 85, 124, 133) which will be dis-
cussed in more detail in 2.1.4.

Then, in the second step, which does not need the involvement of the host
factor, the newly synthesized single-stranded (-) strands are used as templates
for the generation of progeny (+) strands which are also synthesized and re-
leased in a single-stranded form. The replicative intermediates, consisting of
several single-stranded nascent chains dangling from their parental chain via
the replicase molecules are labile structures which can easily collapse into
double-stranded forms (replicative forms, RF's) upon isolation with deprotein-
ization agents (21, 252). These biologically inactive double-stranded RNA mol-
ecules also accumulate in infected cells as byproducts late in the infection
cycle (20, 125, 251).

2.1.2 *Template specificity of Qβ replicase*

One of the remarkable properties of all the bacteriophage RNA replicases iso-
lated thus far is their template specificity (73, 74, 99-101). Only Qβ (+) strand
RNA (101), Qβ (-) strand RNA (77), RNA molecules described as "variants" of Qβ
RNA (160), a "6S" RNA present in Qβ-infected *E. coli* (18) and RNA of the closely
related RNA phages ST and VK (162) are accepted as natural templates by Qβ repli-
case. Nonviral and unrelated viral RNAs are ignored. In addition, the Qβ repli-
case can utilize poly(C) or ribocopolymers containing cytidylic acid as synthetic
templates for the synthesis of the complementary strand (70, 109, 161). RNA syn-
thesis directed by these synthetic polymers remains limited however to the syn-
thesis of the complementary strand (161)

A peculiar feature of the Qβ replicase is its ability to perform RNA synthesis
in the absence of exogenous RNA after an initial lag phase, generating a variety
of self-replicating molecules (119, 158, 233). From two of these RNA molecules,
respectively MDV-1 RNA (midivariant 1) and microvariant RNA, 221 and 114 nucleo-
tides long, the complete nucleotide sequence has been determined (157-159).
Both of these RNAs which comprise less than 10% of the length of the Qβ RNA
genome have few sequences in common but are nevertheless recognized and repli-

cated by the Qβ replicase in the absence of host factor.

The experiments by Küppers and Sumper (134) designed to elucidate the minimal requirements for RNA template recognition by the Qβ replicase seem to offer an attractive explanation for these phenomena. Examinating the nucleotide sequences of the 3' termini of RNAs used by the Qβ replicase, Küppers and Sumper first noticed a striking feature. Qβ (-) strand, MDV (+) strand, MDV (-) strand and the (+) and (-) strand of a "6S" RNA all contain a C-C-C sequence at a defined distance from the 3' terminus which itself also contains a C-C-C sequence. Using the idea of two C-clusters cooperating in the recognition process, they synthesized a variety of oligonucleotides which were then assayed for template activity. The results demonstrate that any oligo- or polynucleotide able to offer a C-C-C sequence at the 3' terminus and a second C-C-C cluster a defined distance from the 3' end is an efficient template. Thus by chemical modification non-template RNAs such as poly(A) and poly(U) could be converted to template RNAs. The only exception to the presence of two C-clusters is the Qβ (+) strand RNA. However, this RNA cannot be replicated by the Qβ replicase alone. An additional protein, host factor, is required for the proper template activity of this RNA molecule (see also 2.1.3.1 and 2.1.4) (85).

As the authors pointed out: "Only those sequences able to offer the two C-clusters in the correct steric position can act as templates. Since naturally occurring RNAs have in general a fixed tertiary structure this mechanism efficiently discriminates between templates and non-templates. On the other hand, RNA sequences with little or no tertiary structure allowing more flexibility can nearly always fulfill the initiation conditions if they have a C-cluster at the 3' end and a second C-cluster somewhere further on" (134).

In contrast to the high template specificity of the Qβ replicase exhibited in the presence of Mg^{2+}-ions, the enzyme can be compelled to copy heterologous RNAs, including nonviral (rRNA, mRNA) and viral RNAs, by the addition of Mn^{2+}-ions (102, 171, 175). The Mn^{2+}-promoted replication of the Qβ (+) strand proceeds in the absence of host factor, in contrast to the Mg^{2+}-directed synthesis. Another way of relaxing the template specificity of the Qβ replicase consists of the use of poly(A)-containing RNAs as templates in the presence of high concentrations of primers, such as oligo(rU) or oligo(dT) (76). Under these special conditions nearly full-length complementary strands of rabbit globin mRNA have been obtained (75, 245).

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2.1.3 Structure of Qβ replicase

In 1970 research on the Qβ replicase received a new impulse by the demon-
stration of the subunit nature of the enzyme. At the same time, Kondo *et al.*
(129) and Kamen (120) reported a new purification procedure resulting in highly
purified replicase preparations which upon SDS-polyacrylamide gelelectrophoresis
were shown to consist of four different polypeptides. The molecular weights of
these proteins, designated as I, II, III and IV, are 70,000, 65,000, 45,000 and
35,000 respectively (121). Only one (subunit II) of these subunits was shown to
be coded by the phage genome, whereas surprisingly the three other ones were
identified as host-specified polypeptides (120, 129). These findings initiated
new types of experiments designed to identify the nature of the host-derived
subunits and to unravel the functional involvements of the four subunits in the
process of RNA synthesis.

2.1.3.1 Subunit II

Subunit II of the Qβ replicase is the phage-coded polypeptide (120, 129),
which is responsible for the polymerizing activity of the enzyme (136). It is
the central element of the replicase around which the other subunits fulfill
ancillary functions. In addition to this polymerizing activity, subunit II is
indispensable for the specific binding of the replicase with Qβ (-) strands
(122). Binding to (+) strand proceeds in collaboration with other proteins (see
below). Subunit II is the only polypeptide in which the Qβ replicase differs
from the bacteriophage f2 replicase. The latter enzyme also contains three
host-derived subunits in addition to the phage-coded polypeptide, which are
identical to those of the Qβ replicase (72-74). So, the template specificity
of the Qβ replicase which is different from the f2 replicase must at least in
part reside in subunit II.

2.1.3.2 Subunit I

In 1972 subunit I, which is one of three host-derived subunits of the Qβ
replicase, was shown to be identical to the so-called translational inter-
ference factor i, a protein which inhibited *in vitro* ribosome binding at the
RNA phage coat cistron ribosome binding site when added in excess to presatu-
rated ribosomes (93, 94). In 1974, this factor i, and so subunit I was ident-

ified as the ribosomal protein S1 from the *E. coli* 30S ribosomal subunit (115, 246).

As a part of the translation machinery, ribosomal protein S1 is required, together with initiation factor IF3, for the proper recognition and binding of mRNA to the 30S ribosomal subunit (53, 230, 240, 241). The ribosomal protein S1 is an RNA-binding protein which preferentially binds to pyrimidine-rich single-stranded regions in RNA (19, 43, 105, 118, 156) and is located in the mRNA binding site of the 30S ribosome subunit (78) directly adjacent to the 3' end of the 16S rRNA (52, 126). This 3' end seems to be involved in the specific mRNA binding via an RNA-RNA interaction (52, 126, 213, 214, 229, 243), although recently the importance of this RNA-RNA interaction has been challenged (226, 238). Thus, ribosomal protein S1, initiation factor IF3 and the 3' end of the 16S rRNA act together in the specific mRNA recognition. Ribosomal protein S1 may promote, in conjunction with IF3, a local unfolding of the mRNA and/or the rRNA, thereby exposing bases near the 3' terminus of the 16S rRNA for interaction with the complementary sequence in the mRNA (19, 128, 230, 234, 239). Recently, the literature on the function of ribosomal protein S1 in protein synthesis has been reviewed extensively by Van Dieyen (238).

As a part of the Qβ replicase, S1 is required for the binding and initiation of RNA synthesis on Qβ (+) strands but not for RNA synthesis directed by Qβ (-) strands, "6S" RNA, poly(C) and C-containing ribopolymers as templates (123, 136, 250). In addition, RNA chain elongation and termination on Qβ (+) strands are unaltered in the absence of subunit I (122).

Binding of Qβ replicase to Qβ (+) strands is a rather complex process involving the cooperative interaction between subunit I, subunit II, host factor and specific regions of the template (122). RNA synthesis starts at the 3' terminus of the (+) strand, but this region itself has a very low affinity for the replicase (202, 244, 250). First, the replicase is positioned correctly on the RNA by tight binding via its "selective RNA binding site", to a region in the RNA located at about the middle of the molecule, between about 2100 and 2700 nucleotides from the 5' end. This specific binding is promoted by subunit I and II. In the second step, host factor and GTP (217) mediate in the positioning of the 3' terminus of the template into the less specific "chain initiation site" of the enzyme (122, 210, 248, 250) so that chain initiation can occur. Thus according to this model (122, 202, 250) template recognition is mainly based on the relative positions of the internal binding site and the 3' terminus of the RNA (so on its tertiary structure) and not on very specific interaction between

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the enzyme and precisely defined nucleotide sequences.

2.1.3.3 Subunit III + IV

In 1972 Blumenthal and coworkers (23) demonstrated that subunit III and IV
were identical respectively to the *E. coli* protein synthesis elongation factors
EF-Tu and EF-Ts. The EF-Tu and EF-Ts,·which occur coupled in the soluble frac-
tion of the cell as the EF-T factor, catalyze the following steps (147).

(1) EF-Tu-Ts + GTP + aminoacyl-tRNA $\rightleftharpoons$ a.a.-tRNA-Tu-GTP + Ts

(2) a.a.-tRNA-Tu-GTP $\xrightarrow{\text{ribosome}}$ (a.a.-tRNA-mRNA-ribosome) + Tu-GDP + Pi

(3) Tu-GDP + Ts $\rightleftharpoons$ Tu-Ts + GDP

So, the EF-Tu in the EF-Tu·Ts complex forms a ternary complex with GTP and amino-
acyl-tRNA thereby releasing free EF-Ts. The ternary complex is then bound to the
ribosome A-site during which transfer GTP is hydrolyzed to GDP and P_i. After the
binding of the aminoacyl-tRNA to the ribosomes and the hydrolysis of GTP, EF-Tu
and GDP are removed from the ribosomes as a EF-Tu-GDP complex. This complex binds
to free EF-Ts, displacing GDP and regenerating EF-Tu.Ts.

In the beginning, the known functions of these protein synthesis factors seemed
to fit with possible functions in the Qβ replicase. For example, since Qβ repli-
case only initiates new chains with GTP, the GTP binding activity of EF-Tu could
be used by the replicase to supply the first nucleotide as a "primer" whose
3'-OH is extended by replicase subunit II. The 5' end of the nascent chain
might then be released from the replicase by EF-Ts, allowing chain elongation
to proceed (122). According to this model EF-Tu·Ts should be required for chain
initiation but not for subsequent chain elongation.

Landers, Blumenthal and Weber (136) demonstrated that subunits I and II are
able to continue polymerization at a normal rate but do not initiate subsequent
rounds of synthesis after removal of EF-Tu and EF-Ts from the preinitiated
replicase-RNA complex.

These findings were supported by Hori *et al.*(110) who showed first that Qβ
replicase induced in the *E. coli* mutant HAK 88 carrying a thermosensitive
elongation factor EF-Ts is thermolabile with regard to GTP binding ability but
not with template binding. Furthermore, by making use of the finding that upon
glycerol gradient centrifugation at low ionic strength, Qβ replicase dissociates
into two enzymatically inactive complexes, one consisting of subunits I + II and
the other of subunits III + IV (120), Hori *et al.* reconstitued Qβ replicase from
its subunit I + II complex and EF-Tu·Ts complex from respectively wild type and

temperature sensitive mutant cells. This reconstituted replicase also appeared to be thermolabile with regard to chain initiation but not elongation.

These studies demonstrated the involvement of subunits III and IV in the initiation step of RNA synthesis but the results did not imply that the functions of the replicase-associated elongation factors correspond to any of the host functions of EF-Tu and EF-Ts performed in protein biosynthesis. The Qβ replicase-associated elongation factors respond at least in a different way to several treatments than the free factors. For example, it was shown by Landers *et al.* (136) and Brown and Blumenthal (34) that the ability of EF-Tu in Qβ replicase to bind aminoacyl-tRNA and support protein synthesis could be eliminated by treatment with either TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone), ZPCK (L-1-carbobenzoxylamido-2-phenylethyl chloromethyl ketone) or NEM (N-ethylmaleimide) without affecting the polymerase activity. In addition, GTP binding to EF-Tu and EF-Ts catalyzed GDP-exchange are strongly inhibited by high ionic strength when these factors are part of the replicase although these activities of the free factor are not affected (24, 136). Finally, free EF-Tu.Ts is completely dissociable by GTP while EF-Tu.Ts in the replicase is slightly (136).

To investigate the function of subunit III and IV more precisely Blumenthal and coworkers (34, 35) designed several elegant experiments which were based on the findings that replicase can be reconstituted *in vitro* from the separate subunits (22, 23, 136), whereas in addition the catalytic activities of EF-Tu (GTP/GDP binding and aminoacyl-tRNA binding) and of EF-Ts (catalysis of GDP exchange with EF-Tu.GDP) normally displayed during protein biosynthesis can be measured when the factors form a part of the replicase (22, 23, 136). Thus, replicase can be reconstituted from elongation factors which have been altered so that they are no longer effective in protein synthesis and can then be tested for enzymatic activity.

Firstly, Brown and Blumenthal (34) showed that inactivation of the aminoacyl-tRNA binding site in native or in replicase-associated EF-Tu by treatment with NEM or TPCK does not affect Qβ replicase activity. Secondly, by treating the replicase with kirromycine, an antibiotic that inhibits protein synthesis by modifying the GTP binding site of EF-Tu but not the aminoacyl-tRNA binding site, they showed that the treated enzyme displays an unaltered RNA polymerase activity but is no longer able to supply EF-Tu to an *in vitro* protein synthesizing system. Finally they provided evidence that the EF-Tu.Ts complex, rather than the individual polypeptides, functions in the replicase. Thus,

reagents that prevent formation of the EF-Tu.Ts complex, like kirromycine and GDP, inhibit the reconstitution of replicase from separate subunits whereas, in addition, replicase in which the native EF-Tu and EF-Ts were replaced (22) by an EF-Tu.Ts complex covalently crosslinked by treatment with dimethyl suberimate, exhibits normal RNA synthesis. Since the elongation factors are now unable to perform their protein synthesis related functions (nucleotide binding and exchange activity) but are still capable of functioning in the Qβ replicase, EF-Tu and EF-Ts apparently function as a complex in the replicase and do not function in protein synthesis. Probably subunits III and IV play some structural role in maintaining the active conformation of the enzyme complex.

2.1.4 Host factor

In 1968 it was found that Qβ replicase itself is unable to use Qβ (+) strands as template and requires an additional *E. coli* protein, the so-called host factor I (HF) (9, 84). This HF, which is present in uninfected as well as in infected cells, appeared to be involved in the Qβ (+) strand-directed synthesis of (-) strands but not in the synthesis directed by Qβ (-) strand and other templates. The protein has been purified to homogeneity and was shown to be a heat stable 72,000 molecular weight oligomer consisting of six identical 12,000 molecular weight polypeptide chains (43, 45, 85, 133). Carmichael *et al.* (45) demonstrated that HF, of which about 2500 copies are present per cell, is associated with ribosomes, probably with the 30S subunit, but is not related to any of the known 30S or 50S ribosomal proteins. In uninfected cells the function of HF is still unclear.

As a part of the replication machinery, HF appears to be required for a step at or prior to initiation of Qβ (-) strand synthesis (85, 133) since a specific antibody to HF only inhibited Qβ (+) strand-directed synthesis if added before initiation but did not affect the rate of elongation (44). Binding studies have shown that HF binds very tightly and specifically to two sites in Qβ RNA which are both single-stranded in nature and contain adenylate-rich sequences (210). One of these binding sites is located near the 3' end of the RNA. Since the 3' end itself has no affinity for the replicase (202), HF probably facilitates the interaction of the replicase with the template by mediating in the correct positioning of the 3' terminus of the template into the initiation site of the enzyme (see 2.1.3.1).

2.2 EUKARYOTE VIRUS RNA REPLICASES

As reviewing the Qβ replication may be considered as representative for pro-
karyote viruses containing a single-stranded RNA genome, a discussion about
eukaryote virus RNA replication requires to be limited owing to the wide var-
iety of virus groups and diversity in replication mechanisms. Therefore, con-
cerning the animal viruses, the following will pass over RNA tumorviruses,
paramyxoviruses, myxoviruses and rhabdoviruses and will only deal with picorna-
viruses and togaviruses, whose genome consists of a single-stranded RNA of the
plus type which is replicated via an RNA-dependent RNA polymerase (12, 33, 114,
169, 179, 212, 257). These two virus groups, the picornaviruses with polio-
myelitis virus, mengovirus, encephalomyocarditis virus, rhinovirus, and foot-
and-mouth-disease virus and the togaviruses with Sindbis virus, Semliki Forest
Virus, eastern equine encephalitis virus and western equine encephalitis virus
as representative members, may be envisaged as relevant animal virus counter-
parts of CPMV regarding purification and properties of replicase.

As far as plant RNA viruses are concerned the diversity in replication mech-
anisms is apparently limited despite marked differences in genome constitution.
Most plant viruses contain a single-stranded RNA genome of the plus type -
whether or not distributed among one or more separate nucleoprotein particles -
which is presumed to be replicated by RNA replicase, at least in part, viral
coded and induced in the host cell after infection (33, 117, 216).

2.2.1 *Animal virus RNA replicases*

2.2.1.1 Membrane-bound replicases

At about the same time the RNA phages were discovered, information about the
mechanism of replication of small animal RNA viruses had just started to emerge
(57-59, 82, 218). It was found for several picorna- and togaviruses, including
mengovirus (108, 182, 193), poliovirus (13, 82, 192, 211, 265), Sindbis virus
(142), encephalomyocarditis virus (68) and Semliki Forest Virus (235) that virus
multiplication occurred in the absence of host specific nucleic acid synthesis
and that the site of virus RNA replication was located in the cytoplasm (83,
97, 140). It thus became likely that virus replication was mediated by a virus-
induced enzyme capable of copying the viral RNA genome. Such an RNA-dependent
RNA polymerase was detected in 1962 by Baltimore in mengovirus-infected L-cells

(15, 16) and was soon followed by the discovery of RNA polymerases induced by poliovirus (14, 107), encephalomyocarditis virus (49, 112, 113), foot-and-mouth-disease virus (184), rhinovirus (258), Sindbis virus (227) and Semliki Forest Virus (150).

Comparison of these viral RNA polymerases reveals that they share several properties.

(1) The replicase molecules do not occur free in the cytoplasm but are found associated with cytoplasmic membranes (3, 40-42, 55, 86, 91, 113, 177, 237). The appearance of replicase and the initiation of viral RNA synthesis takes place in close temporal relation with an extensive *de novo* proliferation of cytoplasmic membranes following virus infection (2, 40-42, 54, 87, 92, 164, 181, 219, 237). These membranes, which were shown to be composed of smooth and rough membranes, seem to fulfill different functions during virus multiplication (40, 41, 199, 237). The rough membranes are the site of viral RNA translation, whereas the RNA replication complex consisting of replicase bound to nascent chains and their templates (40-42) is associated with the smooth membranes. Thus, the virus-directed synthesis of new distinct membranous structures apparently provides the proper frame on which virus biosynthesis is organized.

(2) The replicases are bound to endogenous template RNA and do not respond to or require the addition of exogenous RNA. RNA synthesis *in vitro* only consists of elongation and completion of molecules which were initiated already *in vivo*.

(3) Enzyme activity is insensitive to actinomycin D, DNase and orthophosphate and requires all four ribonucleoside triphosphates in addition to Mg^{2+}-ions; Mn^{2+}-ions can only poorly substitute.

(4) The *in vitro* synthesized RNA comprises all three types of virus-specific RNA also found *in vivo*, namely the replicative intermediate (RI), replicative form (RF) and single-stranded RNA (5, 11, 56, 65, 90, 155, 183, 220, 227). A precursor-product relationship among these *in vitro* synthesized RNAs has been demonstrated by pulse-chase experiments; the multistranded RI is the immediate precursor of the single-stranded progeny viral RNA, whereas double stranded RNA (RF) accumulates as a byproduct (21, 90, 141, 154, 183). Thus, in these respects the crude replicase systems apparently reflect replication *in vivo*, including the synthesis of poly(A) covalently bound to virus specific RNA (65, 220).

2.2.1.2 Solubilization and partial purification

Although the crude replicase systems have proved to be useful and reliable for the analysis of virus replication, their use is limited. The membrane-bound replicases are bound to endogenous template and do not initiate *de novo* RNA chains. Thus, important questions about the mechanism of RNA replication have to remain unanswered. In addition, the cytoplasmic membrane preparations appear to contain too many contaminating proteins to permit the elucidation of the (subunit) structure of the replicase and to unravel possible host-supplied protein components involved in viral RNA replication as has been demonstrated for prokaryote replicases.

Because of these curtailments, the need for a purified replicase displaying template dependence became manifest. This demands the dissociation of the replicase from the membranes and the removal of the endogenous template RNA. Starting from this point, however, the isolation of a purified replicase proceeds slowly for several reasons. Firstly, treatment of membranes with ionic and/or nonionic detergents causes the release of a "soluble" replication complex(es) which sedimented heterogenously at high S values (60-300 S) suggesting that the solubilized replicase is still attached to small membrane fragments (3-5, 39, 65, 69, 91, 137, 199, 236, 237, 258). Secondly, the replicase remains tightly bound to endogenous template RNA upon solubilization. Attempts to remove the template from the soluble replication complex were unsuccessful or had to be so severe that the resulting soluble and template-dependent replicase became extremely unstable (137, 199, 236, 237). Furthermore, extensive purification of the animal virus replicases is hampered by the low content of replicase in infected cells. As has been pointed out by Traub *et al.* (236) animal cells infected with picornaviruses, contain about 200 times less replicase activity per gram of cells as Q_β-infected *E. coli* cells. Despite these oppositions from nature, the partial purification for poliovirus (148, 197, 198), Semliki Forest Virus (47, 48) and encephalomyocarditis virus (236) replicase has been described and is believed to permit the possible identification of the virus-specified polypeptide(s) of the replicase.

2.2.1.2.1 Poliovirus replicase

Purification of the poliovirus replicase (148, 197, 198) was designed to isolate the enzyme bound to its endogenous template in an active ribonucleo-

protein complex. This has been accomplished by precipitation of the detergent-solubilized replicase with 2 M LiCl, a monovalent salt known to disrupt ribosomes and to precipitate single-stranded nucleic acids, and sedimentation through a sucrose gradient. This sucrose gradient-purified polymerase activity, showing an apparent sedimentation coefficient of 25 S, was found to contain predominantly one virus-specific noncapsid polypeptide with a molecular weight of 58,000.

Very recently Flanegan and Baltimore (79) detected a poly(A).oligo(U)-dependent poly(U) polymerase in the membrane fraction of HeLa cells infected with poliovirus. This primer-dependent RNA polymerase was solubilized with detergents (nonidet P-40 and sodium deoxycholate) and freed of endogenous RNA by treatment with 2 M LiCl. Analysis by glycerol gradient centrifugation showed that the enzyme activity sedimented at 4 S suggesting a molecular weight of about 65,000. This novel virus-related enzyme activity seems to fit the model of primer-dependent poliovirus RNA replication, recently proposed by Nomoto *et al.* (170) as an explanation for the presence of a small basic protein covalently linked to the 5' end of the virion (+) strand (80, 138, 178), the nascent strands of the replicative intermediate (80, 170) and the (-) strands (170). It will be of great interest to determine whether this primer-dependent poly(U) polymerase is identical to, or a subunit of, the poliovirus RNA replicase.

2.2.1.2.2   Semliki Forest Virus replicase

A different approach, but also based on the presence and properties of the template RNA to which the replicase remains tightly bound upon solubilization, has been applied by Kennedy and coworkers for the purification of Semliki Forest Virus replicase (47, 48). After solubilization with Triton N-101 and sucrose gradient centrifugation to give a 25 S solubilized replicase complex, the enzyme-template complex was subjected to affinity chromatography through an oligo(dT)-cellulose column to which 42 S virus RNA was bound by means of its poly(A) tail. Since the template in the 25 S enzyme-template complex was mainly of negative polarity, a part of the replicase complex was hydrogen-bounded to the immobilized 42 S RNA. The replicase purified throughout this step appeared to contain two virus-coded polypeptides with molecular weights of 90,000 and 63,000.

2.2.1.2.3 Encephalomyocarditis virus replicase

Encephalomyocarditis virus replicase is the only animal viral RNA replicase isolated until now which is template-dependent (236). This has been accomplished by high-salt dextranepolyethylene glycol phase treatment of the solubilized replication complex. It was found, however, that even minute amounts of phospholipids prevented the complete dissociation between replicase and its template by this phase separation, thereby necessitating the use of severe agents like SDS and Genetron 113 (1,1,2-trichlorotrifluoroethane) to solubilize the replicase and to remove all lipid material. The template-free replicase was further purified by gradient sievorptive chromatography on DEAE-Sephadex and glycerol gradient centrifugation. Unfortunately, the enzyme appeared to be extremely unstable, so that no reliable enzymic studies could be performed.

2.2.1.2.4 Properties of *in vitro* synthesized RNAs

In general, after detergent treatment of the membranes, the polymerase activity of the solubilized replicases is reduced and functionally impaired. Unlike the membrane-bound replicases, the solubilized enzymes mostly produce double-stranded RNA and little or no single-stranded RNA (4, 38, 48, 183, 197, 258). It has been suggested that the template and products of the enzyme are sequestered *in vivo* in the membrane-bound enzyme complex and protected against exposure to nuclease. By dissolution of the membranes the RNA then becomes accessible to RNases (154, 258). This could implicate that the association *per se* of the enzyme with the membrane is not prerequisite for the replicase to function properly (141). According to Caliguiri and Tamm (38, 42) the impairment of the poliovirus replicase after dissolution of the membranes is caused by the disruption of the membrane structure which normally could provide the proper matrix for the replicase to operate. However, recent studies by Butterworth *et al.* (37) make this type of argument unlikely. By analyzing the spontaneous *in vitro* association behaviour of the solubilized replicase complex with phospholipid bilayer membranes (liposomes) of defined composition, they were able to show that the activity of the polymerase was not affected by the physical state (fluidity) of the phospholipid membrane and that its active site was not intimately associated with the hydrocarbon portion of the membrane. This suggests that the polymerase is a possible peripheral membrane protein rather than an integral membrane-bound protein (215).

2.2.2 Plant virus RNA replicases

2.2.2.1 Crude replicase preparations

2.2.2.1.1 Isolation and general properties

On the analogy of the RNA bacteriophages and the picorna- and togaviruses, the replication of plant viruses is presumed to be mediated by a virus-coded RNA replicase (or at least a component of this enzyme) and to proceed in a similar manner. This belief was supported by the early findings that the multiplication of TMV, BPMV and TYMV was not inhibited by actinomycin D, an antibiotic known to inhibit DNA-dependent RNA synthesis (17, 88, 188, 201). From recent studies it is known now that there is actually an early, albeit still unresolved, actinomycin D-sensitive step in TMV (60, 143), PVX (172), BPMV (143), CCMV (143), AMV (1) and CPMV (143, 200) replication and that the earlier conclusion is only valid when the antibiotic is administrated several hours after infection. Nevertheless, the idea about the existence of an RNA replicating enzyme present in RNA virus-infected plant cells was further supported by the occurrence of virus-specific double-stranded RNA intermediates in the replicative cycles as has been demonstrated for the first time in encephalomyocarditis virus-infected animal cells by Montagnier and Sanders in 1963 (163). Indeed, virus-specific double-stranded RNAs were found in plants infected by TMV (36, 149, 189, 215), TYMV (149, 189), BMV (106), AMV (180) and CPMV (242) to mention a few.

The search for a plant virus replicase was initiated seriously in 1965 by Bové and coworkers (26-28), who demonstrated the presence of a virus-specific actinomycin D resistant RNA-dependent RNA polymerase activity in TYMV-infected chinese cabbage leaves (see also 7, 190). This finding has been followed by the detection of RNA-dependent RNA polymerases of a wide variety of single-stranded RNA viruses, including TMV (30, 31, 191), BMV (206), BBMV (204, 205), CMV (89, 151-153), TRSV (176, 194), AMV (25, 139, 249), TNV (81, 232), PEMV (185) and CPMV (61, 262).

From these studies, which deal mainly with the isolation and properties of the crude replicase preparations, it is evident that the plant virus-induced RNA polymerases resemble their animal virus counterparts in several respects. First, the enzyme activity is dependent on the presence of all four ribonucleoside triphosphates and requires Mg^{2+}-ions in preference to Mn^{2+}-ions. Second,

replicase activity is resistant to actinomycin D, rifampicin, α-amanitin, DNase and orthophosphate but sensitive to pyrophosphate. Third, the enzyme is firmly associated with endogenous template RNA and does not respond to the addition of template. Fourth, most of the replicases are bound to endogenous template RNA in a replicase viral RNA complex and associated with a particulate fraction from the cytoplasm sedimenting between 1,000 and 31,000 xg.

The precise localization of the replicase, however, has been known for only a very few viruses. Extensive *in vivo* and *in vitro* studies have shown that the chloroplasts comprise the site of TYMV replication and the TYMV replicase template complex is associated with the chloroplast membrane envelope to which numerous virus-specific double membrane vesicles are bound (135). The replicase from PEMV, the only plant RNA virus that is known to multiply in the nucleus (63) has recently been shown to be associated with the nucleus and virus-specific vesicles probably originating from the nuclear membrane (63, 185, 186). CPMV replicase appears to be primarily associated with membrane vesicles in a cytopathic structure specific for virus infected cells (6, 62, 231). This structure also contains virus-specific double-stranded RNA and most probably represents the site of CPMV RNA replication (6, 62).

As has been pointed out above, the plant virus replicases generally are present in a membrane-rich fraction. However, some exceptions have been reported, for example, the RNA polymerase induced by TRSV in cucumber cotyledon cells, has been found in a cytoplasmic 17,000 xg supernatant (194). In addition to the membrane-bound replicase (152) CMV-infected cucumber cotyledons also contain a soluble RNA polymerase (46, 151). Both, the particulate as well as the soluble activity, are absent from healthy plant extracts (152). Probably both activities reflect different, possibly incomplete forms of the putative RNA polymerase-viral RNA template complex responsible for the *in vivo* replication of CPMV RNA (152). Another soluble replicase has been reported for systemically TMV-infected young tobacco leaves by Brishammer and Juntti (32). This contrasts with the presence of a membrane-bound replicase in directly inoculated leaves as described by Zaitlin's group (30, 264). However, since Brishammer's homogenization procedure comprises the use of a buffer containing 10 mM EDTA (in addition to 5 mM Mg^{2+}), whereas Mg^{2+}-ions appear to be involved in the binding of CPMV replicase (259-261), TMV replicase (256, 263) and AMV replicase (25) to their respective membranes, the replicase probably has been released from the membranes during homogenization.

2.2.2.1.2 Characteristics of *in vitro* synthesized RNAs

In contrast to the membrane-bound animal virus RNA replicases which are able
to synthesize single-stranded viral RNAs as well as double-stranded intermediates,
hardly any single-stranded viral RNA is synthesized by the membrane-bound plant
virus replicases. Most of the newly made RNA is present in RNase resistant
double-stranded form (RF) and complementary to endogenous template RNA (30, 95,
116, 135, 139, 152, 168, 205, 206, 232, 249, 264).

For one of the best studied membrane-bound replicases, TYMV replicase, Bové
and coworkers have demonstrated by a variety of techniques that the product of
the replicase reaction occurs as a full-length, double-stranded RNA and that
the newly made RNA is of the plus type (27, 135). This, however, does not mean
that the newly synthesized (+) strand is of full-length size and the result of
de novo initiation. On the contrary, as has been emphasized by Lafleche *et al*.
(27, 135, 165), the replicase molecules are bound to endogenous preexisting (-)
strands and resume *in vitro* to continue and complete the synthesis of (+) strands
previously initiated in the cell.

In general, the RNA synthesized *in vitro* appears to be virus-specific and
mainly (+) stranded (30, 116, 165, 168, 208, 249, 264), although some evidence is
available that also (-) strands are produced by the crude TMV replicase isolated
early after infection (264), and by AMV replicase (139). Synthesis of a small
amount of single-stranded RNA has been described for the crude replicases from
BMV- (130, 131, 207), BBMV- (116) and CPMV-infected plants (262). In the BMV and
BBMV systems a part of the labeled RNase-resistant RNA could be chased into
RNase-sensitive viral RNAs, provided protective exogenous RNA was added through-
out the isolation and assay of the replicase. Without this precaution the pre-
sence of endogenous nucleases precluded the release and detection of single-
stranded (+) strands. Single-stranded RNA species synthesized by the membrane-
bound CPMV replicase were shown to be of the same size as the virion RNAs,
demonstrating that termination and release of newly made chains can occur *in
vitro*.

2.2.2.2 Partial purification

So far, the crude plant virus replicase systems appeared to be of limited
value for the examination of virus replication *in vitro*. The failure of the
membrane-bound replicases to respond to added template, in addition to the

presence of contaminating nucleases offered considerable restrictions. Furthermore, the crude preparations precluded the structural identification of the replicases. To overcome these difficulties, the enzymes have to be detached from the membranes, liberated from their template to which they are tightly bound and purified further. Solubilization procedures, mostly involving non-ionic detergents, have been described for replicase from TYMV (29, 165, 168), TMV (203, 256, 263, 264), BMV (96, 132, 209), TNV (81), CMV (152), AMV (25, 139), TRSV (176, 194) and CPMV (259-261). However, reports concerning additional purification steps are very scarce and clearly reflect the great difficulties encountered in the purification of plant virus RNA replicases.

2.2.2.2.1 TYMV replicase

Most of the TYMV replicase, solubilized by the nonionic detergent Lubrol W, consisted of enzyme molecules still bound to (-) strand template (165, 168). Preparations of TYMV replicase strictly dependent on added template RNA were attained by high-salt dextrane polyethylene glycol phase separation of the enzyme-template complex (168). The replicase, present in the polyethylen glycol phase and freed of its template, was further purified by ammonium sulphate fractionation, DEAE-cellulose column chromatography and sucrose gradient centrifugation (165-167). The molecular weight of the native replicase was estimated by sucrose gradient centrifugation to be about 400,000 (166, 167). TYMV (+) strand RNA was accepted as template and directed the synthesis of (-) strand which was not released but remained hydrogen bounded to the template in a double-stranded structure (167, 168, 195). A part of these *in vitro* synthesized (-) strands appeared to be of full-length size. At first, the TYMV replicase was reported to lack template specificity (168) whereas recently (167, 195) some preferential recognition of the enzyme for TYMV (+) and (-) strand RNA seems to exist.

Although TYMV replicase is one of the most purified plant viral replicases, the degree of purification is still inadequate for the structural characterization and identification of putative subunits. Unfortunately, TYMV replicase becomes unstable after DEAE-cellulose chromatography and loses its activity upon storage in liquid nitrogen (165).

2.2.2.2.2 TMV replicase

Another membrane-bound virus RNA replicase which has been solubilized by a detergent, Nonidet P40, and partially purified is TMV replicase (264). The solubilized enzyme became template-dependent upon glycerol gradient centrifugation and sedimented to approximately the same position as human gamma globulin, suggesting a molecular weight of about 160,000. The replicase was stimulated by several RNAs and did not exhibit a preference to TMV RNA. As with TYMV replicase no synthesis of RNA-resistant single-stranded RNA occurred. Only the (-) strand complementary to the (+) strand used as template was synthesized.

Recently, membrane-bound TMV replicase has been solubilized without detergents (256, 263) by making use of a procedure developed by Zabel *et al.* (259-261) for CPMV replicase. After subsequent purification by glycerol gradient centrifugation and DEAE-Sephadex chromatography the degree of purification did not allow the structural identification of the replicase (256).

Brishammer and Juntti (32) describe the partial purification of TMV replicase from systemically infected leaves in which the enzyme is presumed to be soluble *in vivo* and is found in the soluble fraction (100,000 xg, 60 min). After gel filtration and affinity chromatography on a RNA-Sepharose column, an enzyme is obtained which is template-dependent, shows a slight preference to TMV RNA and has an apparent molecular weight of about 130,000. Again the *in vitro* synthesized product was largely resistant to RNase and comprised no single-stranded RNA released from the (+) stranded template.

2.2.2.2.3 CMV replicase

CMV replicase, present in the soluble fraction from CMV-infected cucumber cotyledons has been made template-dependent and purified about 100-fold by DEAE-Sephadex, phosphocellulose and single-stranded DNA-agarose column chromatography (46). The replicase showed a high activity with poly(C) as template in addition to the activity with CMV RNA and several other unrelated RNAs. The poly(C)-dependent poly(G) polymerase activity could be separated from the CMV RNA polymerase activity by gradient elution from a DNA-agarose column. However, after this step most of the enzyme activity was lost. Gel electrophoresis patterns from the replicase and from corresponding fractions from healthy leaves devoid of RNA polymerase activity were still very similar showing that the replicase was far from being pure.

2.2.2.2.4 BMV replicase

Membrane-bound BMV replicase has been solubilized by Triton X-100 (96, 98)
or Nonidet P40 (132, 209) and partially purified by sucrose gradient centri-
fugation. However, further purification of this enzyme, which showed a native
molecular weight of about 150,000, was hampered by the great instability of
the enzyme (96). A polypeptide with a molecular weight of 34,500 has been
found in barley leaves infected with BMV (98). Since this protein was syn-
thesized only during an early stage of infection and was associated with the
cell fraction, containing replicase activity, it is believed to represent a
possible component of the replicase.

2.2.2.2.5 AMV replicase

Solubilization of AMV replicase has been attained either by washing the mem-
branes with a Mg^{2+}-deficient buffer (25, 259-261) or by sedimentation of the
membranes through a sucrose cushion (139). No further purification has been
described yet.

2.2.2.2.6 TNV replicase

Solubilization and partial purification of TNV replicase was achieved by
Triton X-100 and subsequent ammonium sulphate fractionation and glycerol gradi-
ent centrifugation. From the latter, a molecular weight of about 200,000 has
been estimated for the solubilized enzyme (81). However, in this case handling
the enzyme has also been impeded by its lability.

In summary, only a few plant virus RNA replicases have been solubilized and
made template-dependent. The free replicase is able to accept (+) strands as
template and to catalyze the synthesis of (-) strands, which remain bound to
the template in a RNase-resistant double-stranded form. Further purification
of the template-free replicase has been described for only a very few viruses
and has provided replicase preparations which are far from being homogenous.
In addition, extensive purification is hampered by the lability of most repli-
cases and the low content and specific activity.

2.2.2.3 RNA-dependent RNA polymerases from uninfected plants

Although it is tempting to assume that plant virus RNA replication is mediated by a virus-coded RNA polymerase, it is still not proven. On the one hand this stems from the fact that no pure replicase is available and therefore no poly-peptide chain can be assigned to a virus gene, whereas on the other hand, com-plications arise by the presence of low levels of RNA-dependent RNA polymerase activity in uninfected plants, including chinese cabbage (8), broad bean (249), tobacco (25, 67, 81, 139, 232, 256, 263), cowpea (P. Zabel, R. Huber-Spanier and A. van Kammen, unpublished results), and wheat germ (P. Zabel, R. Huber-Spanier and A. van Kammen, unpublished results). In addition to these plant systems, an RNA-dependent RNA polymerase activity has also been detected in *Halobacterium cutirubrum* (145, 146) and in rabbit reticulocytes (66).

Whether the plant enzymes are involved in virus replication remains to be elucidated. Usually the host enzyme is found in the soluble fraction (8, 67, 139, 232, 256, 263) although the activity has also been described for the mem-brane fraction from tobacco leaves (25, 81, 139, 232). Surprisingly the activ-ity of the host enzyme is stimulated upon virus infection (8, 67, 81, 232, 256) and parallels the course of virus infection.

Recently, the soluble RNA-dependent RNA polymerases from noninfected and TMV-infected tobacco leaves have been purified 200-300 fold (C.P. Romaine and M. Zaitlin, manuscript in press). Both enzymes exhibited identical behaviour upon ammonium sulphate fractionation, Sephadex-G100 gel filtration and DEAE-BioGel and phosphocellulose ion-exchange chromatography. Moreover, both enzymes were found to be indistinguishable with respect to a number of enzymatic properties (i.e. kinetics, lack of template-specificity, cofactor requirements, salt-sensitivity and nature of the RNA products). According to these data, the in-creased soluble RNA polymerase activity following TMV infection is due to a stimulation of a preexisting host enzyme rather than to the synthesis of a novel virus-specific enzyme. Whether this enzyme is structurally related to the known membrane-bound TMV-specific RNA replicase is an intriguing question but awaits for the further purification of the replicase.

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3.   In Vitro Replication of Cowpea Mosaic Virus RNA

I. Isolation and Properties of the Membrane-Bound Replicase[1]

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A fraction which contained the membrane-bound cowpea mosaic virus RNA replicase was isolated from cowpea mosaic virus-infected cowpea leaves. The replicase activity appeared on day 1 after inoculation, then increased to reach a maximal on day 4. The increase in enzyme activity preceded the most-rapid virus multiplication. The membrane-bound replicase activity was almost completely insensitive to actinomycin D and DNase. The corresponding fraction from healthy leaves had no RNA-dependent RNA polymerase activity. The viral RNA synthesis in vitro proceeded linearly for 20 min and required all four ribonucleoside triphosphates and Mg^{2+} ions. Mn^{2+} was a poor substitute for Mg^{2+}. The reaction was optimal at pH 8.2. During the whole period of RNA synthesis the in vitro synthesized RNA was at least 70% resistant against RNase in $2\times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate), but completely digestible by RNase in $0.1\times$ SSC. Analysis of the products by sucrose gradient centrifugation followed by treatment of separate fractions with RNase demonstrated that both single- and double-stranded RNA were present. Double-stranded RNA sedimented at about 20S, with a shoulder at 16S to 17S. A minor part of the double-stranded RNA sedimented below 10S. Single-stranded RNA sedimented with the same rate as the two viral RNAs, 26S and 34S.

The genome of cowpea mosaic virus (CPMV) consists of two single-stranded RNA molecules which are separately encapsidated. Purified preparations of the virus contain two nucleo-protein particles (M and B) of similar size and with the same capsid protein composition but with different contents of RNA. Empty protein capsids (T) that lack RNA are also found. Both nucleoprotein particles, or both RNAs, are necessary for virus multiplication (6, 21).

The molecular weights of the RNAs sedimenting at 26S and 34S are 1.37×10^6 for M RNA and 2.02×10^6 for B RNA (L. Reijnders, A. M. J. Aalbers, A. van Kammen, and R. W. J. Thuring, 1974, Virology, in press). Hybridization studies and genetic analysis of some mutants have shown that the RNAs have no base sequences in common and that each RNA represents a unique piece of the CPMV genome (6, 21). Double-stranded RNA, which is specific for CPMV, has been isolated from CPMV-infected cowpea plants. Two double-stranded RNAs have been found, one corresponds to double-stranded B RNA and the other to double-stranded m RNA, suggesting that each RNA is synthesized separately (18, 19). Recently, the replication of CPMV RNA has been shown to be

associated with vesicular membranes of a cytopathic structure in CPMV-infected cells (1; G. A. De Zoeten, J. M. H. Assink, and A. van Kammen, Virology, in press). We attempted to isolate the CPMV-specific RNA replicase from infected leaves to gain more insight into the replication mechanism and the regulation of the synthesis of the two viral RNAs. We describe the isolation and properties of a crude CPMV RNA replicase which is bound to membranes. The enzyme complex catalyzed in vitro incorporation of [³H]UMP into both double-stranded as well as single-stranded viral RNAs.

MATERIALS AND METHODS

Materials. Unlabeled ribonucleoside triphosphates, phosphoenol pyruvate (trisodium salt), RNase A (from bovine pancreas, $5\times$ crystallized type 1A), and RNase T1 (from *Aspergillus oryzae* grade III) were purchased from Sigma Chemical Co., St. Louis, Mo. Pancreatic DNase I (electrophoretically pure) was obtained from Worthington Biochemical Corp. Freehold, N.J.; [5-³H]UTP (ammonium salt) was obtained from The Radiochemical Center, Amersham; pyruvate kinase was obtained from Boehringer Mannheim GmbH (Mannheim, Germany), and Extran was obtained from Merck & Co. Actinomycin D was a generous gift from Merck, Sharp and Dohme. Solvene-100 sample solubilizer was obtained from Packard Instrument Co.

Buffers. Buffer A contained 0.05 M Tris-hydrochloride (pH 7.4), 0.01 M KCl, 0.001 M EDTA, and 0.003 M β-mercaptoethanol. Buffer B contained 0.05 M Tris-hydrochloride (pH 8.2), 0.01 M KCl, 0.01 M MgCl$_2$, 0.001 M EDTA, 5% (vol/vol) glycerol, and 0.003 M β-mercaptoethanol. Buffer C contained 0.01 M Tris-hydrochloride (pH 7.2), 0.1 M NaCl, 0.01 M EDTA, and 0.5% sodium dodecyl sulfate (SDS).

Virus and plants. *Vigna unguiculata* (L.) Walp. var. "Blackeye Early Ramshorn" were grown in the greenhouse. Eight to ten days after sowing, the primary leaves were inoculated with crude sap from plants infected with a yellow strain isolate of CPMV. After inoculation, the plants were transferred to a growth chamber and further grown at 30 C with 75% relative humidity and continuous light. The leaves were harvested 4 days after inoculation (Fig. 1).

Virus was purified as described by Van Kammen (20), and the RNA was extracted with SDS and a mixture (1:1, vol/vol) of chloroform and phenol-cresol (12). The virus growth curve was determined as described by Van Griensven (Ph.D. thesis, Agricultural University, Wageningen, The Netherlands 1970).

Isolation of membrane-bound CPMV replicase. All operations were carried out at 4 C; the glassware used in the preparation of extracts and the assays of enzyme activity was heated at 150 C overnight. Centrifuge tubes were stored in 2% Extran and washed extensively with double-distilled water before use.

Portions (3 g) of freshly harvested, infected leaves from which the midribs were removed, were rinsed with double-distilled water, wiped with absorbent tissue, and homogenized with 35 ml of buffer A in a chilled mortar. The homogenate was filtered through two layers of "Miracloth," and the filtrate was centrifuged at 1,000 × *g* for 10 min in a Sorvall SS34 rotor. The supernatant was adjusted to 20% (vol/vol) glyc-

FIG. 1. *Time course of appearance of bound CPMV replicase activity in Vigna leaves infected with CPMV. Eight-day-old Vigna plants were inoculated and incubated with noninoculated control plants in a growth chamber. At daily intervals primary leaves were harvested and the bound replicase was isolated and assayed.*

erol and centrifuged at 31,000 × *g* for 30 min; the resultant supernatant was discarded. The green pellet was washed twice by suspending it in buffer B with the aid of a Thomas homogenizer and sedimenting it at 31,000 × *g* for 30 min; it was finally suspended in 0.5 ml of buffer B for each gram of tissue used. This suspension was used as the crude bound replicase.

Replicase assay. The standard assay mixture (total volume of 0.28 ml) contained 0.05 M Tris-hydrochloride (pH 8.2), 5% glycerol, 0.01 M MgCl$_2$, 0.01 M KCl, 0.01 M (NH$_4$)$_2$SO$_4$, 0.1 μmol each of ATP, GTP, and CTP, 0.01 μmol of UTP, 5 μCi of [^{3}H]UTP (final specific activity of 0.4 Ci/mmol), 0.001 M EDTA, 1 μmol of phosphoenolpyruvate, 10 μg of pyruvate kinase, actinomycin D (20 μg/ml), 3 mM β-mercaptoethanol, and enzyme preparation. The enzyme preparation was preincubated with actinomycin D for 5 min at 30 C, and then the reaction was started by the addition of the other ingredients. Incubation was at 30 C for 30 min or for the designated period of time in a shaking waterbath. The reaction was stopped by the addition of two 50-μliter samples to 5 ml of ice-cold 5% trichloracetic acid which contained 2% Na$_4$P$_2$O$_7$, 2% NaH$_2$PO$_4$, and 0.05% uridine. Bovine serum albumine (350 μg per sample) was added as a carrier, and after 15 min at 0 C the acid-insoluble precipitates were collected on Whatman GF/A filters, washed five times with 5-ml portions of the trichloracetic acid-phosphate-uridine solution, washed twice with 5 ml of 80% ethanol, and finally washed with 5 ml of diethyl ether (4). The filters were treated with 0.75 ml of a toluene-water mixture (9:1) for 2 h at 50 C to solubilize the precipitates and were subsequently counted with 10 ml of toluene-permablend scintillation cocktail containing acetic acid (2 ml/liter) (2). Under these conditions the counting efficiency for tritium was 40% in a Packard Tricarb scintillation counter.

Except where noted otherwise, the results are expressed as picomoles of [^{3}H]UMP incorporated per milligram of protein per 30 min of incubation time. The values given are corrected for zero time values. Protein was measured by the procedure of Lowry et al. (9) with crystalline bovine serum albumin as a standard.

Sucrose-SDS density gradient centrifugation. The reaction mixture was made 2% in SDS, 1% in Brij 58, 1% in deoxycholate (DOC), and 10 mM in EDTA (SDS-Brij-DOC-EDTA detergent mixture) and incubated at 30 C for 20 min. A sample of the extract was directly layered onto a 34-ml linear 15 to 30% (wt/vol) sucrose density gradient in buffer C and centrifuged in an SW27 rotor at 22,500 rpm for 18 h at 20 C. Fractions were collected from the bottom. Two equal samples were taken from each fraction. One was treated with RNase (4 μg of RNase A per ml + 4 U of T1 RNase per ml) in 2.5 ml of 2× SSC for 30 min at 30 C; the other was incubated for an equivalent period without RNase. Both samples were then made 5% in trichloracetic acid, and, after the addition of carrier protein, they were processed as described above. CPMV RNAs with sedimentation coefficients of 26S and 34S were used as external marker.
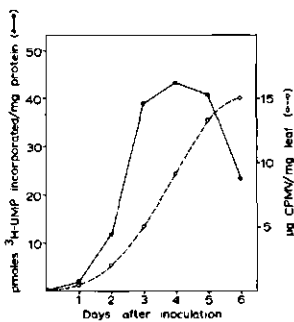
RESULTS

When an homogenate of CPMV-infected leaves was centrifuged at 31,000 × g, after a preliminary low-speed centrifugation to remove nuclei and chloroplasts, the sedimented material contained an RNA polymerase activity which was not present in the corresponding fraction from healthy leaves. The activity depended on the period between inoculation and harvesting of the leaves (Fig. 1). The virus-specific RNA polymerase activity, which will be referred to as bound CPMV RNA replicase activity, was detectable on day 1 after inoculation and increased rapidly to reach a maximum after 4 days. The activity then declined. The increase of CPMV RNA replicase preceded the rapid multiplication of CPMV in the leaves (Fig. 1). The replicase activity which developed depended on the age of the primary leaves at infection. Thus the activity which developed in 16-day-old primary leaves was only 60% of the activity developed in 8- to 10-day-old leaves, although the rates of increase were the same. In all subsequent experiments, primary leaves were inoculated 8 to 10 days after sowing and were harvested 4 days later.

Characteristics of the replicase reaction in vitro. (i) Requirements of the bound CPMV replicase. The omission of each of the three unlabeled ribonucleoside triphosphates (ATP, GTP, or CTP) from the reaction mixture caused a strong decrease in [³H]UMP incorporation activity (Table 1). If all three unlabeled ribonucleoside triphosphates were left out, the replicase activity was almost completely suppressed. It was necessary to wash the constituents of the 31,000 × g pellet twice before incubation to obtain this high degree of dependency. When the washing procedure was omitted, the incor-

poration of [³H]UMP in the absence of the three added unlabeled ribonucleoside triphosphates was about 20% of that in the complete reaction mixture. These results suggest that there is a considerable endogenous nucleotide pool in the unwashed 31,000 × g pellet.

The replicase was routinely assayed in the presence of actinomycin D. The RNA synthesis was only slightly higher in the absence of actinomycin D (Table 1). Moreover, when actinomycin D was replaced by DNase (30 μg/ml) a very similar incorporation of [³H]UMP occurred. These results demonstrate that the 31,000 × g fraction of the leaves had very little DNA-dependent RNA synthesizing activity. In contrast, the strong RNA polymerase activity in a fraction of the homogenate which sedimented in 10 min at 1,000 × g was almost completely inhibited by actinomycin D. Clearly, the fractionation procedure achieved a good separation between the virus-specific and the host-specific RNA synthesis.

The bound CPMV RNA replicase required Mg²⁺ ions for its activity (Table 1), and the optimal concentration was in the broad range of 8 to 20 mM (Fig. 2). Higher concentrations of Mg²⁺ (up to 40 mM) caused only a small decrease in enzyme activity. Mn²⁺ could only partially replace Mg²⁺ (Table 1 and Fig. 2). At optimal concentrations the replicase activity was five times higher with Mg²⁺ than with Mn²⁺ ions. RNA synthesis in the presence of 10 mM Mg²⁺ was inhibited 40% by 2 mM Mn²⁺ (Table 1).

(ii) Effect of monovalent ions and pH. The enzyme activity was not affected by up to 60 mM (NH₄)₂SO₄, but higher concentrations caused inhibition (Fig. 3). The activity was not

TABLE 1. *Requirements of the bound CPMV replicase*

Reaction conditions	Percent of control
Complete[a]	100
– ATP	22
– GTP	20
– CTP	24
– (ATP, GTP, CTP)	6
– Mg²⁺	5
– Mg²⁺ + Mn²⁺ (2 mM)	20
+ Mg²⁺ (10 mM) + Mn²⁺ (2 mM)	59
– Actinomycin D	108
– Actinomycin D + DNase (30 μg/ml)	98

[a] The complete reaction mixture was as described in Materials and Methods.
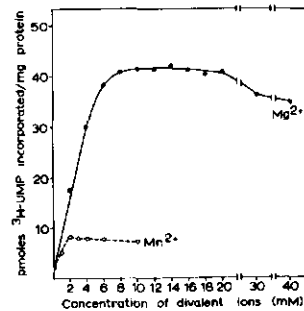

FIG. 2. *Effect of Mg²⁺ and Mn²⁺ ions on the bound CPMV replicase activity. Assay conditions were as described except for the concentration of divalent cation.*

affected by KCl at concentrations of up to 0.4 M. The enzyme activity was maximal at pH 8.2. Between pH 7.9 and 8.5 the enzyme displayed at least 90% of its activity (Fig. 4).

(iii) The time course of the bound CPMV replicase reaction. RNA synthesis by the CPMV RNA replicase was linear for 20 min, but declined to zero 30 to 40 min after incubation (Fig. 5). The host cell activity in the $31,000 \times g$ fraction of healthy leaves was negligible (Fig. 5).

The products of the bound-replicase reaction. (i) Synthesis of RNase-resistant RNA. The product of the RNA replicase reaction was characterized by determining the degree of RNase resistance of the synthesized RNA at various reaction times. Equal samples of the reaction mixture were incubated in 2 ml of $2\times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate) (high salt) and $0.1\times$ SSC (low salt) with 25 μg of RNase A and 25 U of RNase T1 per ml for 30 min at 30 C. At least 70% of the RNA synthesized throughout the reaction consisted of mate-
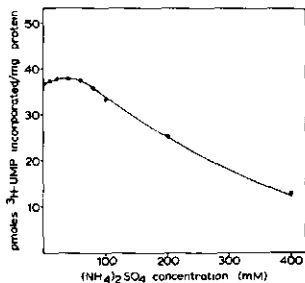

FIG. 5. *Time course of RNA synthesis by the bound CPMV replicase. A batch enzyme reaction mixture was incubated under standard assay conditions. At the indicated times, two 50-μliter samples were removed, immediately added to 5 ml of 5% trichloroacetic acid containing 2% $Na_4P_2O_7$, 2% NaH_2PO_4, and 0.05% uridine, and further processed as described. ○, Activity of a corresponding fraction from healthy leaves.*

FIG. 3. *Effect of $(NH_4)_2SO_4$ on the bound CPMV replicase activity. Assay conditions were as described except for the concentration of $(NH_4)_2SO_4$.*
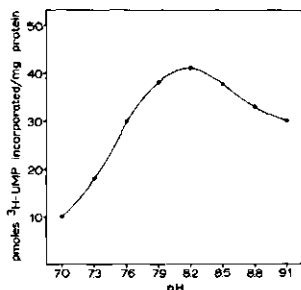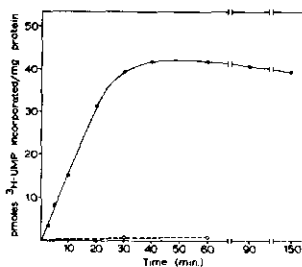

FIG. 4. *Effect of pH on the bound CPMV replicase activity. Assay conditons were as described except for the pH of the assay mixture.*

rial resistant to RNase in high salt (Fig. 6). In contrast, all of the RNA was made soluble by RNase in low salt. In some experiments the portion of RNase-resistant material was somewhat higher during the first 5 min of incubation than later. As a control, samples of the reaction mixture were incubated in $2\times$ SSC without RNase. No loss of acid-precipitable counts was observed in these incubations. These results suggest that newly made RNA is, for the most part, present in partially double-stranded replicative structures.

(ii) Analysis by sucrose-SDS-density gradient centrifugation. The products of the replicase reaction were also analysed by sucrose gradient centrifugation. A complete reaction mixture was incubated for 30 min and then treated with the SDS-Brij-DOC-EDTA detergent mixture for 20 min at 30 C. A sample was layered onto a linear 15 to 30% sucrose gradient containing 0.5% SDS. A typical sedimentation profile of the labeled products is shown in Fig. 7. The bulk of the synthesized RNA sedimented at about 20S, with a shoulder at 17S. There was some material sedimenting slower than 10S and two minor peaks with sedimentation coefficients of 26S and 34S. The S values of the two fastest sedimenting peaks corresponded with those of CPMV middle-component and bottom-component RNA, respectively, used as external markers on a separate gradient. The RNA in the fractions of the sucrose gradient was tested for RNase resistance. The RNA sedimenting at 20S, 17S, and slower was found to be partially resistant to RNase; the 26S and 34S RNAs were completely hydrolyzed (Fig. 7). As the S values
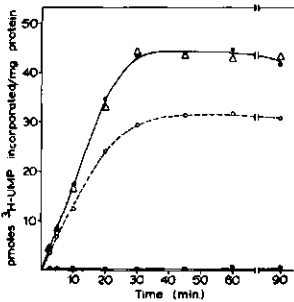
FIG. 6. *Time course of total RNA synthesis and of the fraction resistant to RNase. A 2-ml reaction mixture was incubated under standard assay conditions. At the indicated times two 50-μliter portions were removed. One sample was immediately assayed for total acid-insoluble radioactivity (●); the second portion was incubated in 2 ml of 2× SSC containing 25 μg of RNase A per ml and 25 U of RNase T1 per ml for 30 min at 30 C (○); the third portion was heated with RNase A (25 μg/ml) and RNase T1 (25 U/ml) in 2 ml of 0.1× SSC (■); and the last sample was incubated in 2 ml of 2× SSC without RNase (△). The reactions were stopped by adding trichloroacetic acid to a final concentration of 5%. Carrier protein was added, and the acid-insoluble precipitates were processed.*
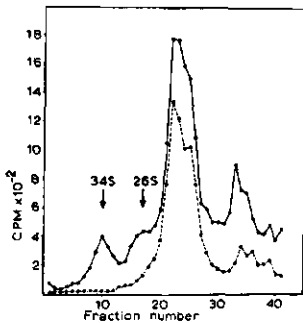

FIG. 7. *Sucrose-SDS density gradient centrifugation of replicase reaction product. The reaction mixture contained (in 0.84 ml) 0.75 ml of enzyme preparation, 0.75 μmol each of ATP, GTP, and CTP, 0.075 μmol of [³H]UTP (final specific activity of 0.8 Ci/mmol), Macaloid (0.5 mg/ml, previously incubated at 40 C for 90 min), actinomycin D (20 μg/ml), and the other ingredients as described. The reaction mixture was incubated for 30 min at 30 C and subsequently deproteinized by adjustment to 2% SDS, 1% Brij 58, 1% DOC, and 10 mM EDTA. A portion of the extract (0.75 ml) was layered onto a 34-ml 15 to 30% linear sucrose gradient in buffer C and centrifuged at 20 C and 22,500 rpm for 18 h in an SW 27 rotor. Fractions*

of 20S and 17S agree reasonably with those expected for double-stranded bottom-component and middle-component RNA, respectively, it was concluded that the membrane-bound CPMV replicase produced, in vitro, mainly double-stranded RNA but also an appreciable amount of single-stranded virus RNA. The sedimentation profile shown in Fig. 7 was very characteristic and reproducible for the RNA products obtained by treatment of the reaction mixture with the SDS-Brij-DOC-EDTA detergent mixture and direct layering onto the sucrose-SDS-gradients. A similar profile was obtained when the RNA was extracted from the reaction mixture with SDS and a mixture of chloroform and phenol-cresol (1:1) (12). Extraction of the RNA by SDS and phenol-cresol without chloroform resulted, however, in low recoveries, and very little of the material extracted had a sedimentation coefficient greater than 15S.

DISCUSSION

Our results show that CPMV-infected cowpea leaves contain an RNA-dependent RNA polymerase activity which appears to be closely bound with cytoplasmic membranes, and which is able to synthesize in vitro both double-stranded and single-stranded viral RNA species. The fraction of cell constituents sedimenting at 31,000 × g for 30 min from a leaf homogenate contains most of the cytoplasmic membranes, and with CPMV-infected leaves it also contains the specific RNA polymerase activity. Recently it was demonstrated that the cytoplasm of CPMV-infected leaf cells contains a characteristic cytopathic structure consisting of vesicular membranes embedded in amorphous electron-dense material (De Zoeten et al., Virology, in press; J. M. H. Assink, Ph.D. thesis, Agricultural University, Wageningen, The Netherlands, 1974). It was further shown that the replicative form of CPMV RNA is associated with the vesicles of the cytopathic structure in which a rapid in vivo incorporation of [³H]uridine took place. The replication of CPMV RNA therefore appeared to be associated with the vesicles of the cytopathic structure. The occurrence of in vitro RNA polymerase activity in the membrane fraction of CPMV-infected leaves confirmed this assumption. The RNA polymerase activity was virus specific as shown by its appearance and increase after inoculation of cowpea leaves

were collected from below and two equal portions were assayed for total RNA (●) and RNase-resistant RNA (○).

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with CPMV. Furthermore, the RNA polymerase incorporated [³H]UMP into RNase-resistant double-stranded RNA and also produced labeled single-stranded middle- and bottom-component CPMV RNA. By chopping the infected leaves, it has been possible to isolate the cytopathic structures more or less intact (De Zoeten et al., Virology, in press) The structures sedimented at $1,000 \times g$ for 15 min together with the nuclei and chloroplasts. Homogenization by mortar and pestle resulted in a replicase-containing fraction which was only slightly contaminated by nuclear or chloroplast RNA polymerase activity. More than 90% of the RNA polymerase activity in the $31,000 \times g$ pellet was resistant to actinomycin D and insensitive to DNase, indicating that it contained only a minor contamination from the DNA-dependent RNA polymerase of the cell. In contrast, the $1,000 \times g$ pellet from a homogenate of both infected and healthy leaves contained a high [³H]UMP incorporation activity, which was almost completely inhibited by actinomycin D.

The CPMV-replicase had some features in common with other plant viral RNA replicases. (i) The enzyme was found in a membrane fraction (3, 8, 15). (ii) The rapid increase of the replicase activity preceded the maximal virus multiplication (13, 16). (iii) The enzyme required Mg^{2+} ions for its activity, and Mn^{2+} ions are a poor substitute (5, 11, 22). (iv) The RNA synthesis proceeded linearly for about 20 min and then leveled off (3, 8, 17). (v) Most of the in vitro synthesized RNA was resistant to RNase in high salt (2× SSC) (8, 14, 22). It is notable that the membrane-bound CPMV replicase also produced some single-stranded middle- and bottom-component RNA. It seems premature to conclude that these single-stranded viral RNAs were the result of complete de novo synthesis in vitro. The possibility should be kept in mind that the replicase only completed by elongation the synthesis of viral RNA strands which were then released from the replicase-template complex. Such single-stranded viral RNAs would have radioactivity over a variable length at one end, depending on the site where the elongation of the pre-existing strand was started. This has not been demonstrated nor excluded in our experiments. Neither is there any indication for reinitiation of RNA chain synthesis. Laflèche et al. (8) demonstrated with turnip yellow mosaic virus that, in vitro, the RNA replicase only elongated pre-existing RNA strands. No release of single-stranded RNA occurred in that system. The same probably holds for the in vitro tobacco mosaic virus RNA replicase reaction, as

suggested by Bradley and Zaitlin (3). The synthesis of single-stranded plant viral RNAs in vitro has been reported for the membrane-bound brome mosaic virus RNA replicase (7).

However, the labeled product of the CPMV replicase consisted predominantly of partially RNase-resistant RNA sedimenting at 20S and 17S. Previous studies (18, 19) have demonstrated that there are two size classes of double-stranded RNA, corresponding with double-stranded middle- and bottom-component RNA of CPMV. The in vitro labeled 20S and 17S RNase-resistant RNA therefore represented replicative structures corresponding to each of the two viral RNAs.

The significance of the partly RNase-resistant RNA sedimenting below 10S is not clear. Such RNA has not been noted in preparations of double-stranded RNA from CPMV-infected leaves (18, 19). The RNA sedimenting below 10S might have arisen as a by-product of the in vitro synthesis. For example, the RNA synthesized in vitro by the RNA polymerase induced by cucumber mosaic virus in cucumber cotyledons consisted of low-molecular-weight double-stranded RNA; however, no high-molecular-weight RNA was found, making the relationship of these RNA species to the replication mechanism obscure (10). The slowly sedimenting RNase-resistant RNA might also be due to some limited degradation of the 20S material during the extraction procedure. The nature of the products was found to depend to some extent on the extraction procedure. We preferred to extract the RNA from the reaction mixture before sucrose gradient centrifugation with the SDS-Brij-DOC-EDTA detergent mixture because it gave highly reproducible results and the recovery of labeled RNA was very high (more than 90%). In preliminary experiments, the RNA was extracted from the reaction mixture by means of phenol-cresol and SDS. However, this method gave low recoveries and, in addition, no RNA sedimented faster than 15S. This might indicate that degradation can occur to a variable extent during extraction. The true nature of the partially RNase-resistant RNA sedimenting slower than 10S and also the other RNA components found in the gradient require further investigation. However, our results show that CPMV-infected Vigna leaves can provide an available in vitro system to analyze the replication of a multicomponent plant RNA virus.

ACKNOWLEDGMENTS

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4.   In Vitro Replication of Cowpea Mosaic Virus RNA

II. Solubilization of Membrane-Bound Replicase and the Partial Purification of the Solubilized Enzyme

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A method for the solubilization of membrane-bound Cowpea mosaic virus RNA replicase has been developed bypassing the use of detergents. Solubilization has been achieved by washing the $31,000 \times g$-pellet containing the bound replicase with a Mg^{2+}-deficient buffer. This procedure had several advantages as compared to treatments with nonionic or ionic detergents: (i) the solubilized enzyme was stable at 4 C, (ii) more than 80% of the replicase could be solubilized without loss of total enzyme activity, (iii) the replicase was rather selectively released resulting in a two- to threefold increase in specific activity per se, and (iv) most of the green color from chloroplast fragments present in the crude replicase fraction remained membrane bound resulting in only slightly colored preparations of solubilized enzyme. The solubilized replicase has been further purified by DEAE-Bio Gel column chromatography. RNA synthesis directed by the DEAE-purified enzyme was template dependent and proceeded at a linear rate for at least 9 h.

The purification of the RNA replicases (RNA-dependent RNA polymerases) of the bacteriophages Qβ and f2 has proven to be of great value in the elucidation of the mechanism of phage RNA replication and its mode of regulation (3, 15, 16, 26, 31, 32, 38). In addition, by analysis of the subunit structure of the Qβ replicase the involvement of specific host proteins in bacteriophage replication has been revealed (see reference 5).

The study of eukaryotic virus RNA replication is greatly hampered by the lack of a pure and stable RNA replicase. Many attempts have been undertaken to purify the RNA replicases from cells infected with animal and plant RNA viruses. The first step in the purification of the eukaryotic replicases, comprising the detection and characterization of RNA-dependent RNA polymerase activity in virus-infected cells, has been reported for several animal and plant viruses (4-6, 8, 9, 21, 23, 27, 30, 36, 39). Most of the eukaryotic RNA replicases appeared to be bound to cytoplasmic membranes in tight association with endogenous RNA template (2, 9, 11, 14, 17, 18, 22, 29, 30). The further purification and characterization, however, depends on the availability of a soluble and template-dependent enzyme and thus demands that the replicase is released from the membranes and the template. From here the purification of

eukaryotic replicases has met with many difficulties. The replicases are usually released from the membranes with the aid of nonionic and/or ionic detergents (2, 11, 14, 18, 19, 22, 24, 29, 34, 40). After solubilization the animal virus replicases still contain template RNA (2, 11, 14, 18, 22, 29, 34) which can only be removed laboriously, resulting in unstable enzyme preparations (29, 34).

In the case of plant virus RNA replicases, solubilization and removal of endogenous RNA could be attained for several viruses resulting in the partial purification and characterization of the replicase (10, 13, 24, 25, 40). Extensive purification of plant virus replicases, however, is also hampered by the lability of the enzymes.

Recently we reported the isolation and characterization of the membrane-bound cowpea mosaic virus (CPMV)-RNA replicase (39). In this paper we describe a procedure to release the CPMV replicase from the membranes that is very mild, does not involve the use of detergents, and yields a stable enzyme. After DEAE-Bio Gel column chromatography of the solubilized enzyme, a stable and template-dependent replicase is obtained.

MATERIALS AND METHODS

Materials. DEAE-Bio Gel A (control no. 13270 and 13758) was obtained from BioRad Laboratories, Rich-

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mond, Calif., dithioerythritol (DTE) from Sigma Chemical Co., St. Louis, and phenylmethylsulphonyl-fluoride (PMSF) from Merck and Co. The source of all other chemicals has been previously mentioned (39). Actinomycin D was a generous gift from Merck, Sharp, and Dohme.

Buffers. Buffer A consisted of 0.05 M Tris-hydro-chloride (pH 7.4), 0.01 M KCl, 0.001 M EDTA, and 0.003 M β-mercaptoethanol. Buffer B contained 0.05 M Tris-hydrochloride (pH 8.2), 25% (vol/vol) glycerol, 0.05 M KCl, 0.001 M EDTA, 0.01 M DTE and 0.5 mM PMSF. Buffer C was identical to buffer B except for the addition of 0.01 M $MgCl_2$. Buffer D contained 0.05 M Tris-hydrochloride (pH 8.2), 50% (vol/vol) glycerol, 0.05 M KCl, 0.001 M EDTA, 0.01 M DTE, and 0.5 mM PMSF. Buffer E contained 0.01 M Tris-hydro-chloride (pH 7.2), 0.1 M NaCl, 0.01 M EDTA, and 0.5% sodium dodecyl sulfate.

Virus and plants. The growth of *Vigna unguiculata* (L.) Walp var. "Blackeye Early Rams-horn" plants and their infection with a yellow strain isolate of CPMV has been described (39). The pri-mary leaves were harvested on day 4 after inoculation (39). Virus was purified as described by Van Kammen (35).

Isolation of CPMV-RNAs. CPMV nucleoprotein components (B and M) were separated by sucrose density gradient centrifugation in a Spinco Ti15 zonal rotor. RNA was isolated from the separate com-ponents by phenol-cresol-chloroform-sodium dodecyl sulfate extraction as described previously (39). B- and M-RNA were purified by fractionation through a 34-ml linear 15 to 30% (wt/vol) sucrose gradient in buffer E at 20 C for 18 h at 22,500 rpm in an SW27 rotor.

Before centrifugation the RNA sample was heated at 65 C for 3 min and then quickly cooled at 0 C to prevent aggregation.

Isolation of membrane-bound CPMV-replicase. The details have been described elsewhere (39). In brief, 48 g of freshly harvested infected leaves were homogenized in eight portions of 6 g each with 35 ml of buffer A in a mortar. The filtered homogenate was centrifuged at 1,000 × g for 15 min. The supernatant was adjusted to 20% (vol/vol) glycerol and centrifuged at 31,000 × g for 30 min. The resulting pellet containing the bound replicase was directly used for the solubilization procedure.

Solubilization of membrane-bound replicase. An outline of the procedure is depicted in Fig. 1. The 31,000 × g pellet was resuspended in buffer B (1 ml for each gram of leaf tissue used) with the aid of a Thomas homogenizer and incubated for 60 min under continuous stirring on a magnetic stirrer at 4 C. The suspension was centrifuged at 4 C for 60 min at 31,000 × g in a Sorvall SS34 rotor. The clear, slightly green colored supernatant was carefully removed with a Pasteur pipette. To avoid contamination with the upper fluffy layer of the pellet, the last few milliliters were left in the centrifuge tube. The supernatant was saved and the residue was resuspended in buffer B (0.25 ml for each gram of leaf tissue used) and incubated for 30 min as described above. After centrifugation for 60 min at 31,000 × g the superna-tant 2 was carefully removed with a Pasteur pipette.

Homogenate
    1000 x g, 15 min
    Supernatant
    Adjust to 20% (v/v) glycerol
    31,000 x g, 30 min
    Pellet (Membrane-bound replicase)
    Resuspend in buffer B (1ml/g fresh weight)
    and incubate 60 min at 4C under
    continuous stirring
    31,000 x g, 60 min
    Pellet   Supernatant 1 (Solubilized
    Resuspend in   replicase)
    buffer B (0.25 ml/g
    fresh weight)
    and incubate 30 min
    at 4C under continuous
    stirring
    31,000 x g, 60 min
    Supernatant 2 (Solubilized replicase)
    Combine supernatant 1 and 2
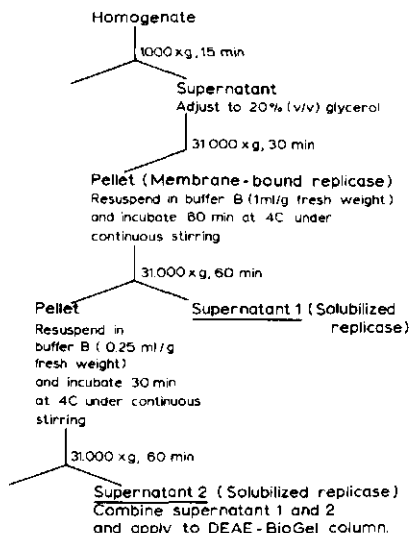   and apply to DEAE-BioGel column.

Fig. 1. *Scheme for the solubilization of mem-brane-bound CPMV replicase.*

Again great care was taken to avoid contamination with the material floating on top of the dark green sediment which was rather loosely packed now. The 31,000 × g pellet, washed with the Mg^{2+}-containing buffer C remained solid and firmly bound to the centrifuge tube. The two supernatants were combined and used directly for the ion-exchange chromatogra-phy. The pellet was resuspended in buffer B (0.25 ml for each gram of leaf tissue used) for testing of re-sidual activity.

DEAE-Bio Gel column chromatography. Thirty milliliters of a packed DEAE-Bio Gel slurry was washed with 200 ml of buffer B, poured into a column, and equilibrated with buffer B. The combined super-natants containing the solubilized enzyme were ap-plied to the column (1.6 by 15 cm) and the unad-sorbed material was washed out with buffer B. Then the bound material was eluted with a linear 0.05 to 0.4 M KCl gradient in the same buffer and 2.7-ml fractions were collected at a flow rate of about 16 ml/h. The fractions containing template-dependent replicase activity were pooled and dialyzed against 2 liters of buffer B saturated with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 31,000 × g for 20 min, dissolved in 2 ml of buffer B, dialyzed for 2 h against 1 liter of buffer B to remove residual $(NH_4)_2SO_4$, and finally dialyzed overnight against buffer D. The enzyme solution was divided in aliquots (200 µl) and stored frozen in liquid nitrogen.

The DEAE-Bio Gel was regenerated and reequili-brated by washing the slurry on a Büchner funnel with buffer B containing 0.5% Sarkosyl and 1.0 M KCl until the green color had disappeared, and finally washed with buffer B.

Replicase assay. The standard assay mixture (total volume of 0.240 ml) contained 0.05 M Tris-hydrochloride (pH 8.2), 5 to 10% glycerol, 0.01 M MgCl$_2$, 0.025 M KCl, 0.013 M (NH$_4$)$_2$SO$_4$, 0.001 M EDTA, 0.25 μmol each of ATP, GTP, and CTP, 0.01 μmol of UTP, 5 μCi of [^{3}H]UTP (specific activity 12 to 14 Ci/mmol), 1 μmol of phosphoenol pyruvate, 10 μg of pyruvate kinase, 5 μg of actinomycin D, 0.004 M DTE, 25 μg of CPMV-RNA, and enzyme. Assay mixtures were incubated for 30 min at 22 C and the reactions were terminated by the addition of 3 ml of ice-cold 10% of trichloroacetic acid containing 4% Na$_4$P$_2$O$_7$ and 4% NaH$_2$PO$_4$. After the addition of bovine serum albumin (350 μg per sample) the mixture was left on ice and then the acid-insoluble precipitates were collected on Whatman GF/A filters, washed five times with 5-ml aliquots of 5% trichloroacetic acid containing 2% Na$_4$P$_2$O$_7$ and 2% NaH$_2$PO$_4$, five times with 1 N HCl containing 0.1 M Na$_4$P$_2$O$_7$, twice with 80% ethanol, and finally with ether. The filters were then processed as described previously (39). In the case of a time course experiment a batch enzyme reaction mixture was incubated in the dark, because of the presence of actinomycin D, and at the times indicated two 50-μl samples were taken and immediately spotted on numbered GF/C filters. The filters were collected in 5% trichloroacetic acid containing 2% Na$_4$P$_2$O$_7$ and 2% NaH$_2$PO$_4$, and washed batchwise with two changes each, respectively, of the trichloroacetic acid-phosphate solution, hydrochloric acid-phosphate solution, ethanol, and ether as described above.

RESULTS

Solubilization of the membrane-bound replicase. Solubilization of membrane-bound proteins can be attained by several methods (28, 33). To investigate the most suitable method to release CPMV replicase from the membranes, the effect of different detergents and high-ionic strength was examined first.

In these experiments the 31,000 × g pellet containing the membrane-bound replicase was isolated, resuspended in 1 ml of buffer C for each gram of leaf tissue used, and treated for 30 min at 4 C with (i) detergents such as Nonidet P40, Triton X-100, Brij 58, Lubrol W, Tween 80, or deoxycholate in concentrations ranging from 0.1 to 2% or with mixtures of the different detergents; (ii) detergents in combination with high salt (0.25 to 2.0 M KCl), and (iii) high salt (0.25 to 2.0 M KCl). The suspensions were then centrifuged for 60 min at 31,000 × g. Several treatments appeared to be very effective in dissolving proteins from the 31,000 × g pellet as shown by the reduced size and protein content of the residual pellet. However, there was a considerable loss of replicase activity, recoverable in pellet plus supernatant, sometimes up to 50%. Moreover the results were disappointing with regard to subsequent purification because of the

lability of the solubilized replicase. Therefore, we examined another method known to release proteins from membranes (28, 33), namely, subjection of membranes to divalent cation depletion.

The 31,000 × g pellet containing the membrane-bound replicase was isolated, resuspended in the Mg^{2+}-deficient buffer B (Fig. 1), and incubated for 60 min at 4 C under continuous stirring. After centrifugation it was found that the distribution of the replicase activity had changed drastically. Table 1 compares the distribution of enzyme activity between pellet and supernatant after washing with Mg^{2+}-deficient or Mg^{2+}-containing buffer. More than 90% of the replicase activity remained bound to membranes in the case of the Mg^{2+}-containing buffer wash. However, after washing the 31,000 × g pellet with the Mg^{2+}-deficient buffer B, 70 to 80% of the replicase was released to supernatant 1.

The pellet obtained after the Mg^{2+}-deficient buffer wash showed about the same size as the control and contained almost all of the green material; in contrast, detergents dissolved the membranes almost completely.

When the washing procedure was repeated, a further 40 to 50% of the remaining replicase activity was released. So, by washing twice with the Mg^{2+}-deficient buffer B, more than 80% of the replicase activity was solubilized (Table 1). One of the main features of this solubilization procedure was the rather selective release of replicase. The specific activity of the solubilized enzyme had increased two- to threefold with respect to the membrane-bound enzyme. Moreover, no loss in total enzyme activity occurred as was the case with detergents. Another

TABLE 1. *Distribution of CPMV replicase activity after washing the 31,000 × g pellet containing the membrane-bound replicase with Mg^{2+}-deficient or Mg^{2+}-containing buffer*[a]

Replicase activity after	Washing with Mg^{2+}-deficient buffer		Washing with Mg^{2+}-containing buffer	
	Pellet	Supernatant	Pellet	Supernatant
1st wash	20–30%	70–80%[b]	92%	8%
2nd wash	10–20%	80–90%[c]		

[a] The 31,000 × g pellet containing the membrane bound replicase was isolated from 24 g of CPMV-infected Vigna leaves and washed with either 24 ml of buffer B or 24 ml of buffer C as described in Fig 1. The second wash with the Mg^{2+}-deficient buffer was performed with 6 ml of buffer B.

[b] Supernatant 1.

[c] Supernatant 1 plus 2.

striking feature of the solubilized enzyme proved to be its stability. The enzyme could be stored in buffer B at 0 to 4 C for several days without significant loss in activity. RNA synthesis by the solubilized enzyme was slightly stimulated by the addition of template RNA and continued for at least 60 min (Fig. 2). The membrane-bound enzyme did not respond to the addition of template and catalyzed UMP incorporation for about 30 to 40 min as was shown previously (39).

DEAE-Bio Gel chromatography. The combined 31,000 × g supernatants containing the released enzyme were used for chromatography on a DEAE-Bio Gel column (Fig. 3). Most of the green color still present in the soluble enzyme preparation moved through the column and characterized the flow-through material. The bulk of the replicase activity eluted at about 0.10 to 0.14 M KCl and comprised the first absorbancy at 280 nm (A_{280}) peak resolved by the KCl gradient. The second A_{280} peak contained mostly nucleic acids as judged by the A_{260}-A_{280} ratio which was greater than 2.0. In most experiments the replicase peak was not symmetrical and skewed to the right yielding a shoulder or sometimes a minor peak (Fig. 3, fractions 52 to 56). This minor peak appeared to contain replicase molecules slightly contaminated with template RNA as indicated by some residual enzyme activity in the absence of added template. However, RNA synthesis directed by the bulk of the replicase was template

dependent (Fig. 4). Thus, chromatography of the solubilized replicase on a DEAE-Bio Gel column affords an almost complete separation of replicase from nucleic acids concomitant with the removal of a considerable amount of con-
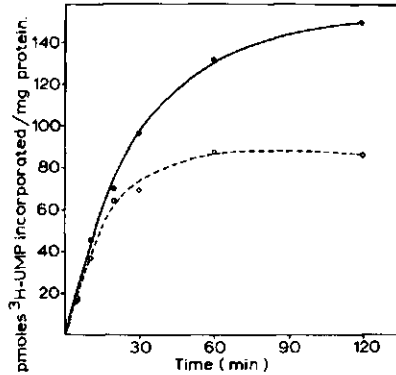

FIG. 2. *Time course of RNA synthesis by the solubilized CPMV replicase before DEAE-Bio Gel chromatography. Membrane-bound replicase was isolated and solubilized as described in Fig. 1. An enzyme reaction mixture (0.72 ml) was incubated under standard assay conditions in the dark in the presence (●) or absence (○) of unfractionated CPMV RNA (75 μg). At the times indicated, two 50-μl aliquots were removed and assayed for acid-insoluble radioactivity.*
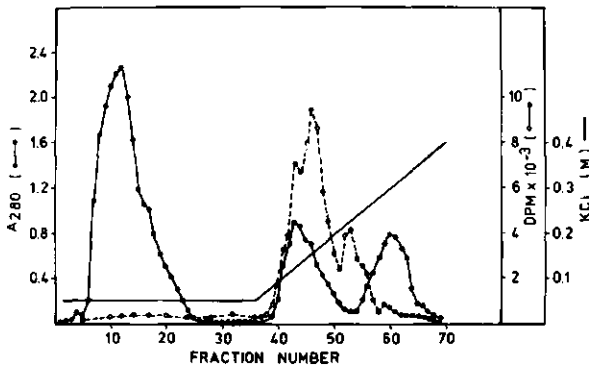

FIG. 3. *DEAE-Bio Gel column chromatography of solubilized CPMV replicase. Solubilized replicase (57 ml, 43 mg of protein) was prepared from 48 g of infected Vigna leaves as described in Fig. 1 and applied to a DEAE-Bio Gel column (1.6 by 15 cm) equilibrated with buffer B. After the flow-through material had emerged, the column was developed with a 108-ml linear 0.05 to 0.4 M KCl gradient in buffer B. Fractions of 2.7 ml were collected at a flow rate of 16 ml/h; 100-μl aliquots of each fraction were assayed for replicase activity for 60 min (○); (●) A_{280} nm; (——) KCl molarity.*
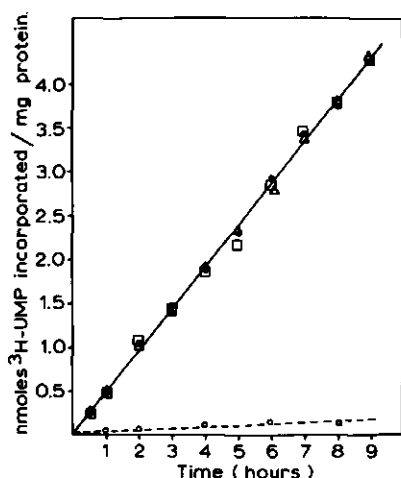
FIG. 4. *Time course of RNA synthesis by the solubilized CPMV replicase after DEAE-Bio Gel chromatography. Membrane-bound replicase from 48 g of infected Vigna leaves was solubilized and purified by DEAE-Bio Gel column chromatography. The appropriate fractions were pooled, precipitated by dialysis against buffer B saturated with $(NH_4)_2SO_4$, and further processed as described in Materials and Methods. A 1.2-ml reaction mixture containing 0.125 ml of enzyme solution (230 µg of protein) was incubated in the dark under standard conditions in the presence of respectively 100 µg of CPMV-B-RNA (●), 100 µg of CPMV-M-RNA (△), 50 µg of CPMV-B-RNA plus 50 µg of CPMV-M-RNA (□), or in the absence of RNA (○). At the times indicated, two 50-µl aliquots were removed and assayed for acid-insoluble radioactivity.*

tamination eluting in the flow through. The profile (Fig. 3) was quite reproducible for more than 10 different experiments and was obtained with two different batches of DEAE-Bio Gel (no. 13270 and 13758). One batch (no. 13639) was inferior because all the nucleic acids co-eluted with the proteins in one peak at low ionic strength.

One of the most striking properties of the DEAE-purified enzyme was its capacity to perform RNA synthesis at a linear rate for at least 9 h (Fig. 4), demonstrating a remarkable stability of the DEAE-purified enzyme. The same kinetics of RNA synthesis were obtained with enzyme stored in liquid nitrogen for at least 1 month.

No significant differences occurred when either B-RNA, M-RNA or both RNAs from CPMV were used as template.

DISCUSSION

The solubilization procedure with the Mg^{2+}-deficient buffer B has several features which makes it superior to methods using detergents. (i) More than 80% of the membrane-bound replicase is solubilized without loss of total enzyme activity. (ii) The released enzyme is stable in buffer B for several days at 0 to 4 C without special precautions. (iii) The supernatant containing the released replicase is only slightly colored. Almost all of the green material is retained in the dark green pellet. In contrast, detergents dissolved the membrane pellet almost completely. (iv) The release of the replicase is rather selective, resulting in a two- to threefold increase in specific activity per se. Furthermore, this method is not limited in use to only CPMV-infected leaves. The same procedure has been applied successfully for the solubilization of tobacco mosaic virus replicase and alfalfa mosaic virus replicase from the membranes of infected tobacco leaves (P. M. Romaine and M. Zaitlin; C. M. Clerx-van Haaster and J. F. Bol, personal communication).

Divalent cation depletion of membranes is a well-established method to solubilize ATPases (1, 12, 28, 33). These proteins, which can be easily and selectively detached from the membranes, are thought to be bound to the surface of the lipid bilayer or to surface proteins (28). Most of these proteins contain a large excess of acidic side chains and a low content of hydrophobic residues (28, 33). The divalent cations are thought to be required for binding because they neutralize the repulsive electrostatic charges of the membrane and form salt bridges between carboxyl groups of the proteins and phosphate groups of the phospholipids (28). Mg^{2+} ions were also found to be essential in binding DNA-dependent RNA polymerase to chloroplasts from maize (7). Bottomley et al. (7) found that very low Mg^{2+} concentrations were critical for solubilization of the enzyme. From studies concerning the in vivo replication of the bacteriophage MS2, Haywood (20) concluded that replicase components or factors required for complementary-strand synthesis are bound to membranes even in the absence of divalent cations and that the polymerase is no longer bound to these factors during the synthesis of the bulk of the progeny single-stranded RNA.

Our results demonstrate that CPMV repli-

case can be released from the membranes with a Mg^{2+}-deficient buffer and therefore may resemble the ATPases with respect to the kind of binding. Whether the replicase is bound directly to the membranes or to RNA chains which, in turn, are attached to the membranes is not clear. We have to emphasize that the leaves were homogenized in a Mg^{2+}-deficient buffer. In spite of this the replicase was found to be associated with the membrane. Just after the isolation of the $31,000 \times g$ pellet the addition of Mg^{2+} ions appeared to be essential for the prolonged association with the membranes. So the absence of Mg^{2+} ions during the isolation of the $31,000 \times g$ pellet did not essentially alter the attachment to the membranes probably because of a high Mg^{2+} pool in the plants. It even facilitated the solubilization procedure because the replicase could now be released directly in contrast to the ATPase-containing membranes which need to be washed five times or more before they release the enzyme (28).

Purification and removal of endogenous template RNA by DEAE-Bio Gel chromatography did improve the ability of the replicase to perform RNA synthesis considerably. Before the DEAE step, RNA synthesis directed by the soluble enzyme was only partially stimulated by RNA and leveled off after about 60 min, whereas RNA synthesis directed by the DEAE enzyme continued for at least 9 h. Whether the DEAE enzyme is capable of reinitiating and producing full-length single-stranded minus and plus strands has still to be determined. Experiments relating to these questions are presently in progress.

ACKNOWLEDGMENTS

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5. PROPERTIES OF CPMV REPLICASE

   In the preceding chapter, we have described a simple and mild procedure for
the solubilization of membrane-bound CPMV replicase. Furthermore, it has been
shown that the solubilized replicase could be purified further by DEAE-BioGel
column chromatography to provide a template-dependent enzyme preparation dis-
playing high stability. Thus, we have got a replicase preparation at our dis-
posal that might be valuable for the analysis of RNA replication *in vitro* and
could offer stable material for the development of additional purification
steps.

   In this chapter we will present a series of experiments primarily designed
to characterize the replicase. We have examined, firstly, the assay conditions
which are favorable for replicase activity. We then tested the response of the
enzyme to various synthetic and natural RNA templates to see whether CPMV
replicase exhibits template specificity. In addition, some preliminary studies
have been carried out on the binding of the replicase to CPMV RNA and the size
of the *in vitro* synthesized products.

5.2 MATERIAL AND METHODS

5.2.1 *Materials*

   Cowpea seeds (*Vigna unguiculata* (L) Walp. var. "Blackeye Early Ramshorn")
were obtained from W. Atlee Burpee Co., Philadelphia, Pa.. Ribonucleoside tri-
phosphates (ATP, GTP, CTP and UTP), rifampicin, cordycepin (3'-deoxyadenosine),
dithioerythritol (DTE), phosphocreatine, creatine phosphokinase (140 units/mg
protein), RNase A (from bovine pancreas, 5 x crystallized type (A), and RNase
T1 (from *Aspergillus oryzae* grade II, 480,000 units/mg protein), were purchased
from Sigma Chemical Co.. DEAE-BioGelA (control no. 13758) was provided by BioRad
Laboratories, Sephadex G25-Fine by Pharmacia, poly(A), poly(U), poly(G) and

poly(C) by Boehringer Mannheim GmbH, DNase I by Worthington and PMSF by Merck and Co.. Soluene-350 sample solubilizer, Instafluor and Instagel were obtained from Packard Instrument Co.; disodiumtriisopropylnaphthalene sulphonate (TPNS) from Serva and sodiumdodecyl sulphate (specially pure SDS) from BDH. $(5-^{3}H)$ UTP (11-24 Ci/mmol), $(5-^{3}H)$ CTP (17 Ci/mmol), $(8-^{3}H)$ GTP (15 Ci/mmol) and $(2-^{3}H)$ ATP (20 Ci/mmol) were purchased from The Radiochemical Center, Amersham. Sarkosyl NL 97 and actinomycin D were kindly donated by Ciba Geigy and Merck Sharp and Dohme respectively. α-Amanitine was a generous gift from Dr. H. Stunnenberg (Department of Genetics, Wageningen, The Netherlands).

5.2.2 RNAs

CPMV and CPMV RNA were isolated as described by Klootwijk *et al.* (19). Radish Mosaic Virus (RaMV) RNA was isolated from RaMV by the same method used for CPMV RNA. RaMV was kindly donated by Dr. R. Hull (Norwich, England). Semliki Forest Virus (SemFV) RNA was a gift from Dr. M. Pranger (University of Utrecht, The Netherlands); Dr. J. Klootwijk (Free University of Amsterdam, The Netherlands) donated 17S rRNA from yeast and Dr. L. van Vloten-Doting (University of Leiden, The Netherlands) the RNAs from Brome Mosaic Virus (BMV), Tobacco Streak Virus (TSV), Satellite Tobacco Necrosis Virus (STNV), Tobacco Mosaic Virus (TMV), Cowpea Chlorotic Mottle Virus (CCMV), Cucumber Mosaic Virus (CMV), Alfalfa Mosaic Virus (AMV) and Turnip Yellow Mosaic Virus (TYMV).

5.2.3 Virus and plants

The growth of *Vigna unguiculata* (L.) Walp. var. "Blackeye Early Ramshorn" plants and their infection with a yellow strain isolate of CPMV (41, 43) was carried out as described in chapter 3. The primary leaves were harvested on day 4 after inoculation and used directly for the isolation of the replicase.

5.2.4 Replicase purification procedure

The DEAE-purified replicase was prepared as described in chapter 4 with some modifications as discussed in detail in chapter 6.

5.2.5 *Replicase assay*

The standard assay mixture contained in a final volume of 0.24 ml: 0.05 M Tris-hydrochloride (pH 8.2), 5 to 10% glycerol, 0.008 M Mg-acetate, 0.005 to 0.02 M KCl, 0.06 M $(NH_4)_2SO_4$, 0.001 M EDTA, 5 μg of actinomycin D, 0.8 to 2 mM DTE, 0.25 μmol each of ATP, GTP and CTP, 0.01 μmol of UTP, 5 μCi of ^{3}H-UTP (specific activity 11-25 Ci/mmol; 1 mCi/ml), CPMV RNA and enzyme as indicated in the figure and table legends. In some experiments (see figure and table legends) the final volume of the reaction mixture was 0.12 ml with half of the amounts of ribonucleoside triphosphates and actinomycin D specified above. Unless specified otherwise, CPMV RNA is a mixture of B-RNA and M-RNA.

The reaction mixtures were incubated at 30^OC for 60 min and assayed for trichloroacetic acid-precipitable counts by spotting samples on Whatman 3MM filter discs which were immediately immersed and collected in ice-cold 5% trichloroacetic acid containing 2% $Na_4P_2O_7$ and 2% NaH_2PO_4 and washed batchwise as described in chapter 4. Each filter was treated with 0.75 ml of Soluene-350 for 1 h at 50^OC to solubilize the precipitates and was subsequently counted with 7 ml of Instafluor. Under these conditions the counting efficiency for tritium was 40% in a Packard Tricarb scintillation counter. The values given are all corrected for zero time values.

5.2.6 *Protein determination*

Protein was measured by the method of Lowry *et al.* (24) using bovine serum albumin as a standard. Because several buffer constituents strongly interfere with this assay, protein was first precipitated with 10% ice-cold trichloroacetic acid and then washed with ice-cold acetone. The precipitates were collected by centrifugation, dissolved in 1 N NaOH and then used in the protein assay.

5.2.7 *Product analysis*

Enzyme reactions were terminated by the addition of 1/3 volume of a detergent mixture containing 4% (wt/vol) TPNS, 8% (wt/vol) Sarkosyl, 0.04 M Tris-hydrochloride (pH 7.2), 0.04 M EDTA, 0.4 M NaCl and then the mixture was filtrated through a Sephadex G25-Fine column (0.9 x 13 cm), equilibrated with 1% (wt/vol) TPNS, 2% (wt/vol) Sarkosyl, 0.01 M Tris-hydrochloride (pH 7.2), 0.01 M EDTA,

0.1 M NaCl, to remove unincorporated ^{3}H-UTP. Fractions of 0.2-0.25 ml were collected and 0.01-0.025 ml samples assayed for radioactivity by counting with 5 ml Instagel containing 0.5 ml of H_2O. The void volume comprising the *in vitro* synthesized products was pooled and layered onto a 11 ml 15-30% (wt/vol) linear sucrose gradient in 0.01 M Tris-hydrochloride (pH 7.2), 0.1 M NaCl, 0.01 M EDTA and 0.5% SDS and centrifuged in a Beckman SW41 rotor at 40,000 rpm for 6 h at 20°C. Fractions were collected through a hole punctured in the bottom of the tube directly in counting vials. After the addition of 0.5 ml of H_2O they were counted with 5 ml of Instagel. ^{32}P-labeled CPMV RNAs (26S and 34S) were used as internal sedimentation markers.

5.2.8 Replicase-RNA binding assay

Binding of replicase to ^{32}P-labeled CPMV RNA was assayed on basis of the ability of nitrocellulose filters to retain RNA-protein complexes. Incubation mixtures were prepared on ice and contained in a total volume of 0.24 ml all the ingredients required for the polymerase assay except the four ribonucleoside triphosphates and actinomycin D. ^{32}P-RNA and replicase were used at concentrations specified in the figure legends. The reaction was started by the addition of replicase and incubated at 30°C. After incubation for 15 min two separate 100 μl portions were taken from each incubation mixture and filtered slowly through membrane filters (Millipore HAMK 02412), which had been pre-soaked for at least 30 min in washing buffer containing 0.05 M Tris-hydrochloride (pH 8.2), 25% (vol/vol) glycerol, 0.05 M KCl and 0.001 M EDTA. After the sample had passed through, the filters were washed with 2 x 3.5 ml of ice-cold washing buffer, dried and counted with Instafluor.

In the absence of replicase 2-3% of the input counts were retained on the filter while replicase at saturating concentrations was able to retain about 50-55% of the RNA. In preliminary experiments we have found that a low background (2-3%) only occurred when Mg^{2+}-ions were omitted from the washing buffer. The reason for this is unknown but we think that the unusual nature of the 5' end of CPMV RNA (19, 20) might be responsible for the sticking of the RNA to the filters in the presence of Mg^{2+}-ions.

In competition experiments replicase was held fixed so as to be at one-half plateau and various amounts of unlabeled competitor RNA were mixed with the ^{32}P-labeled CPMV RNA before replicase was added.

5.3. RESULTS

5.3.1 *Assay conditions favorable for replicase activity*

5.3.1.1 Reaction requirements

Table I shows the general characteristics of CPMV replicase. The synthesis of RNA showed an absolute requirement for RNA and a divalent cation. Enzyme activity was not inhibited by actinomycin D, cordycepin, α-amanitin, rifampicin, DNase and orthophosphate. In contrast, RNA synthesis was completely suppressed by pyrophosphate and RNases. Thus, these results are consistent with the properties of an RNA-dependent RNA polymerase.

TABLE I. PROPERTIES OF CPMV REPLICASE

Reaction conditions	percent of control
Complete[a]	100[b]
$- Mg^{2+}$	1.8
$- Mg^{2+} + Mn^{2+}$ (3 mM)	42.9
$+ Mg^{2+}$ (8 mM) $+ Mn^{2+}$ (3 mM)	24.0
$-$ RNA	1.2
$+ PP_i$ (4 mM)	0
$+ P_i$ (4 mM)	92.4
$+$ phosphocreatine (10 mM) + phosphokinase (10 µg/ml)	85.8
$+$ RNase A (10 µg/ml) + RNase T_1 (10 units/ml)	1.5
$+$ EGTA (0.1-5 mM)[c]	100
$-$ actinomycin D	118.8
$-$ actinomycin D + cordycepin (50 µg/ml)	116
$-$ actinomycin D + α-amanitin (25 µg/ml)	123
$-$ actinomycin D + rifampicin (50 µg/ml)	110.6
$-$ actinomycin D + DNase (30 µg/ml)	109

a) The complete reaction mixture (0.12 ml) containing 1.5 µg of protein and 10 µg of CPMV RNA was assayed for 60 min at 30°C as described in Material and Methods.
b) 100% = 7.6 pmoles of ^{3}H-UMP incorporated.
c) EGTA = ethylenglycol-2-(2-aminoethyl)-tetracetic acid.

5.3.1.2 Effect of Mg^{2+}- and Mn^{2+}-ions

Using $MgCl_2$ as the source of Mg^{2+}-ions, optimal replicase activity was obtained at 12 mM whereas higher concentrations diminished the RNA synthesis (Fig. 1). When Mg^{2+}-ions were added as acetate salt, RNA synthesis proceeded optimal at 8 mM and displayed a higher rate than the $MgCl_2$-catalyzed incorporation at all concentrations tested, suggesting an inhibitory action of chloride ions (Fig. 1). The replicase preferred Mg^{2+}- to Mn^{2+}-ions for optimal activity (Fig. 1). Replacement of Mg^{2+}-ions by Mn^{2+}-ions caused a 2.5-fold decrease in RNA synthesis at the optimum $MnCl_2$ concentration (3 mM). Addition of Mn^{2+}-ions to a Mg^{2+}-activated reaction reduced the ^{3}H-UMP incorporation by about 75% (Table I).
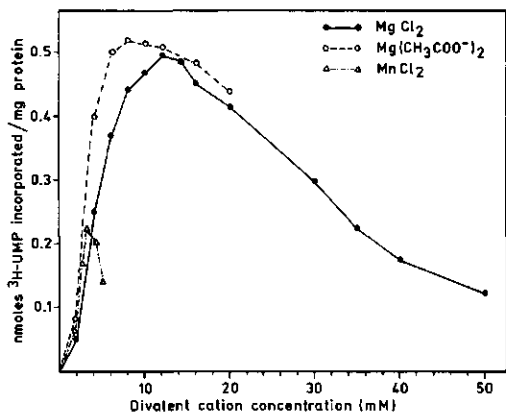

Figure 1. Effect of Mg^{2+}- and Mn^{2+}-ions on CPMV replicase activity. Reaction mixtures (0.24 ml) containing 42 µg of protein, 10 µg of CPMV RNA and various amounts of Mg^{2+} or Mn^{2+} were assayed for 60 min at 22°C as described in Materials and Methods.

5.3.1.3 Effect of K^+- and $(NH_4)^+$-ions

Monovalent cations were either stimulatory or inhibitory depending on the type of salt (Fig. 2). Low concentrations of $(NH_4)_2SO_4$ slightly stimulated the enzyme activity. Optimal activity was achieved with 60 mM but above this concentrations the enzyme activity strongly decreased. RNA synthesis was not affected by K-acetate concentrations up to 100 mM. Higher concentrations however caused inhibition (Fig. 2). Potassium ions added as the chloride salt strongly decreased the enzyme activity (Fig. 2). Replicase activity was optimal at the lowest KCl concentration, suggesting again the inhibitory effect of Cl^--ions on RNA synthesis. It should be emphasized that it is rather the RNA synthesizing reaction which is sensitive to Cl^--ions and not the replicase

per se, since the replicase could be centrifuged and stored at high salt
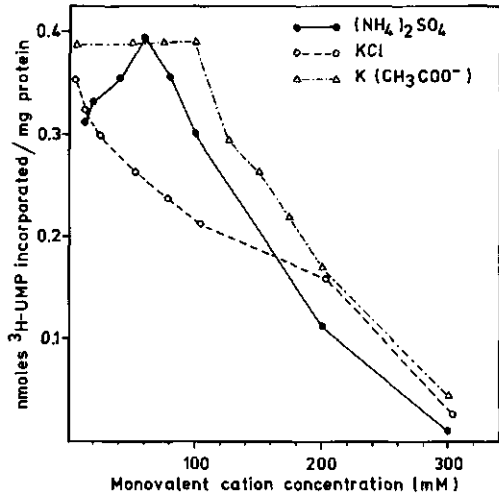(250 mM KCl) and remained active when assayed at low salt concentrations.

Figure 2. Effect of K^+- and NH_4^+-ions
on CPMV replicase activity. Reaction
mixtures (0.24 ml) containing 42 µg
of protein, 10 µg of CPMV RNA, 12 mM
$MgCl_2$, 13 mM $(NH_4)_2SO_4$ and various
amounts of KCl (3-303 mM) were as-
sayed for 60 min at 22^oC as described
in Material and Methods. The reaction
containing various amounts of $(NH_4)_2SO_4$
(13-300 mM) was carried out in the
presence of 42 µg of protein, 10 µg
of CPMV RNA, 12 mM $MgCl_2$ and 13 mM
KCl. The reaction mixtures (0.12 ml)
containing various amounts of K-ace-
tate (0-300 mM) were assayed in the
presence of 24 µg of CPMV RNA, 8 mM
Mg acetate, 2.5 mM KCl and 60 mM
$(NH_4)_2SO_4$.

5.3.1.4 pH

The replicase exhibited a rather broad pH optimum ranging from pH 7.6 to
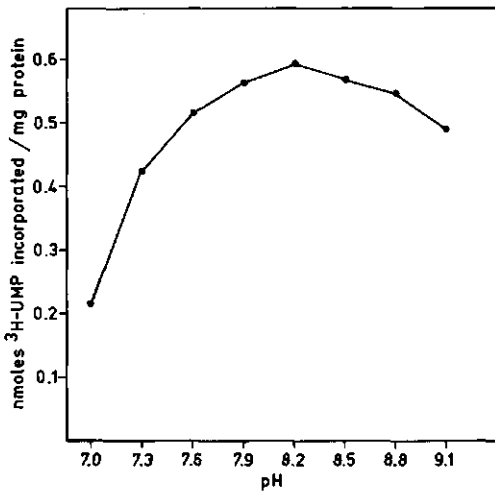9.1. Maximal activity occurred at pH 8.2 (Fig. 3).

Figure 3. Effect of pH on CPMV repli-
case activity. Reaction mixtures
(0.24 ml) containing 42 µg of pro-
tein, 10 µg of CPMV RNA, 12 mM $MgCl_2$,
3 mM KCl and 60 mM $(NH_4)_2SO_4$, were
assayed at various pHs for 60 min at
22^oC as described in Material and
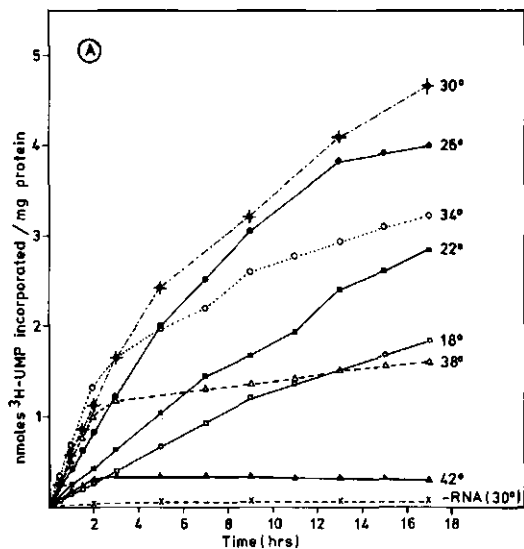Methods. The Tris buffer was ad-
justed to the required pH with HCl.

Figure 4. Effect of the temperature on CPMV replicase activity. (A) Reaction mixtures (1.44 ml) containing 83 µg of protein and 40 µg of CPMV RNA were incubated under standard conditions at the specified temperatures. At the times indicated, duplicate aliquots were removed and assayed for acid insoluble radioactivity as described in Material and Methods.

(B) Reaction mixtures (0.96 ml) each containing 92 µg of protein and 80 µg of CPMV RNA were incubated under standard conditions at the specified temperatures. At the times indicated, duplicate aliquots were removed and assayed for acid insoluble radioactivity as described in Material and Methods.

(C) The amount of ^{3}H-UMP incorporated during the first hour by the two different replicase batches used in (A) and (B), is plotted as a function of the temperature. ●——●replicase batch (A); o———o replicase batch (B).
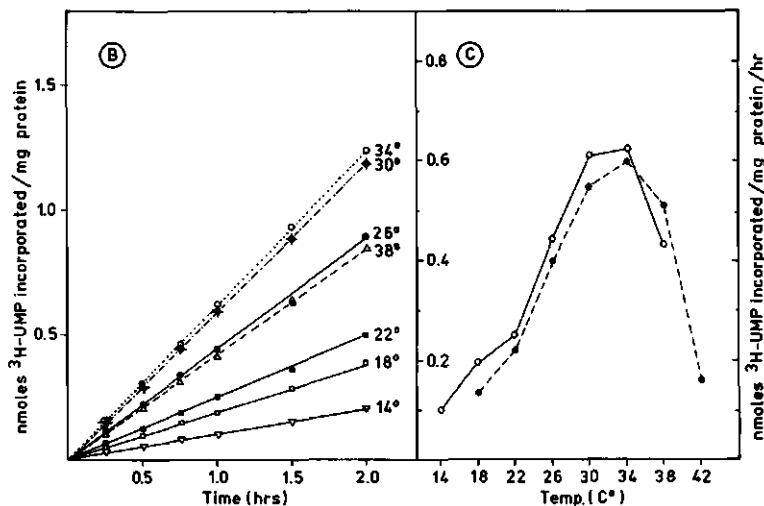
5.3.1.5 Temperature

To measure the effect of the temperature on the replicase activity, the time course of RNA synthesis was determined at different temperatures ranging from $18^{o}C$ to $42^{o}C$ (Fig. 4A). At $18^{o}C$ to $30^{o}C$ RNA synthesis proceeded for at least 15 hours demonstrating the pronounced stability of the enzyme, while at $38^{o}C$ and $42^{o}C$ RNA synthesis stopped after 3 hours. At $30^{o}C$ the enzyme was still able to perform RNA synthesis after 17 hours. The rate of RNA synthesis was linear for at least 2 hours (Fig. 4B) at all temperatures tested. Fig. 4C shows that during this period RNA synthesis proceeds optimal at 30 to $34^{o}C$.

5.3.1.6 RNA synthesis as a function of enzyme concentration

As shown in Fig. 5, the rate of RNA synthesis increased linearly with the amount of enzyme added from 0.5 to 120 µg of protein per 0.12 ml. It is clear from these data that the enzyme remains stable upon dilution.
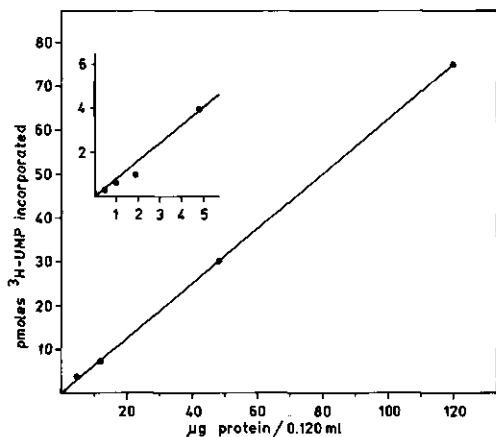

Figure 5. RNA synthesis as a function of enzyme concentration. Reaction mixtures (0.12 ml) containing 11.25 µg of CPMV RNA and increasing amounts of protein were assayed for 60 min at $30^{o}C$ as described in Material and Methods. Enzyme dilutions were made in 0.05 M TrisHCl pH 8.2, 25% glycerol, 0.05 M KCl, 0.001 M EDTA, 0.01 M DTE and 0.5 mM PMSF. To each assay 25 µl of enzyme solution was added.

5.3.1.7 RNA synthesis as a function of template concentration

Replicase activity increased linearly with template concentration up to about 0.4 µg (0.24 pmol) of CPMV RNA per 15 µg of protein (Fig. 6). Template saturation occurred at about 5-10 µg of RNA per 15 µg of protein. From the linear double reciprocal plot (not shown) it can be calculated that about 0.4 µg of CPMV RNA (0.24 pmol) is required to obtain 1/2 V_{max}.

TABLE II. RIBONUCLEOSIDE TRIPHOSPHATES REQUIREMENT

Reaction conditions	% Incorporation					
	^{3}H-CTP	^{3}H-GTP	^{3}H-ATP	^{3}H-UTP	^{3}H-UTP[2]	^{3}H-UTP[3]
Complete[1]............	100	100	100	100	100	100
– CTP.................	–	14.1	4.6	19.9	18.4	24
– GTP.................	5.2	–	16.6	27.1	24.4	20
– ATP.................	1.8	4.6	–	21.8	23.7	22
– UTP.................	2.9	3.5	13.2	–	–	–
– (GTP, ATP, UTP)......	1.5	–	–	–	–	–
– (CTP, ATP, UTP)......	–	0.5	–	–	–	–
– (CTP, GTP, UTP)......	–	–	2.6	–	–	–
– (CTP, ATP, GTP)......	–	–	–	20	7.5	6

1) Complete reaction mixtures (0.12 ml) containing 20 µg of protein, 10 µg of CPMV RNA, 0.005 µmol of labeled ribonucleoside triphosphate and 0.125 µmol each of the other three unlabeled ribonucleotides were assayed at 30°C for 60 min as described in Material and Methods.
2) 10 µg of TMV RNA was used as template instead of CPMV RNA.
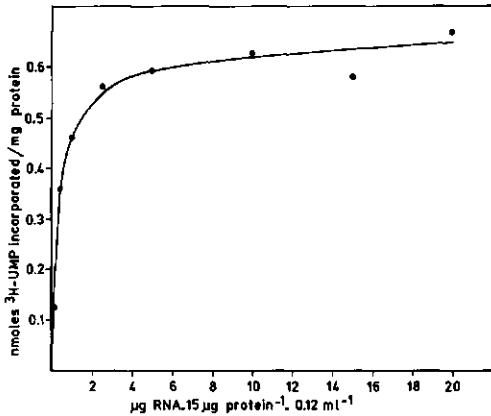3) Data for the membrane-bound replicase taken from chapter 3.

Figure 6. Template saturation curve. Reaction mixtures (0.12 ml) containing 15 μg of protein and increasing amounts of CPMV RNA were assayed for 60 min at 30°C as described in Material and Methods.

5.3.1.8 Ribonucleoside triphosphate requirement

Analysis of the replicase activity by monitoring the incorporation of either ^{3}H-CTP, ^{3}H-GTP or ^{3}H-ATP in the absence of one or all three other, unlabeled ribonucleoside triphosphates revealed that the replicase required all four ribonucleoside triphosphates (Table II). Incorporation with only ^{3}H-CTP, ^{3}H-GTP or ^{3}H-ATP present in the reaction mixture was only 0.5-3% that of the complete reaction mixture. However, when we measured the incorporation of ^{3}H-UTP in the absence of ATP, GTP and CTP, there was still a considerable residual activity (20%) (Table II).

Because of the poly(A) tracts present at the 3' end of the CPMV RNA genome, it was conceivable that initiation of complementary strand synthesis preferentially occurs at the poly(A) tail of the template (+) strand and results in the synthesis of (-) strands which will be relatively enriched in poly(U) when nucleotide sequences adjacent to the poly(A) segment are only partially transcribed. This implies that a TMV RNA-directed incorporation of ^{3}H-UTP might respond differently to the omission of the three other unlabeled ribonucleotides because of the poly(A)-deficient nature of TMV RNA. Table II shows that indeed much less activity was left (7.5%) when only ^{3}H-UTP was present with TMV RNA as template. It was found in chapter 3 that ^{3}H-UTP incorporation activity of the membrane-bound CPMV RNA replicase, which mainly elongates (+) strands complementary to endogenous (-) strands, and thus not involves poly(A)-directed poly(U) synthesis, is only 6% in the absence of all three unlabeled ribonucleoside triphosphates (Table II).

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These results and the ones to be presented in 5.3.3 may suggest that the poly(A) tract is involved in the initiation step of CPMV replication.

5.3.2 *Template requirements*

5.3.2.1 Template activity of poly(A), poly(U), poly(C) and poly(G)

In an attempt to study the template specificity of CPMV replicase, several synthetic, viral and nonviral RNAs were assayed in time course experiments for their capacity to direct the synthesis of RNA. Since both CPMV RNAs contain a poly(A) tract at their 3' ends (9) we first investigated the template activity of poly(A) at two different concentrations (20 µg/0.24 ml and 100 µg/0.24 ml). At the highest concentration the incorporation of ^{3}H-UMP was
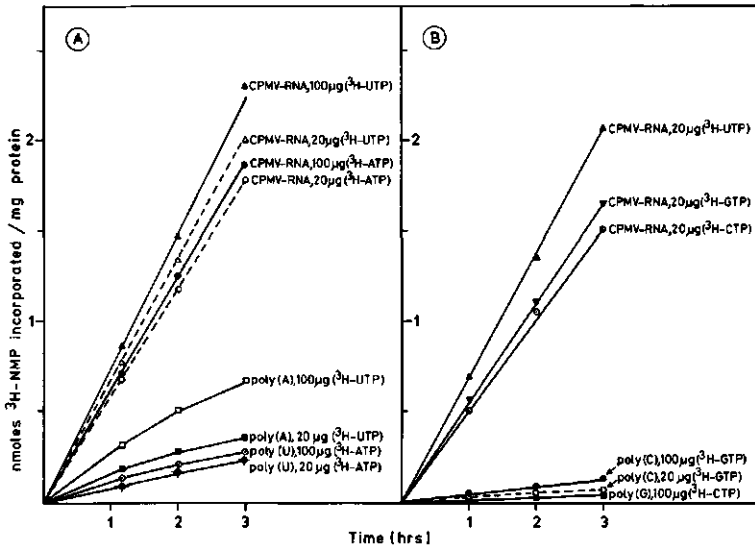

Figure 7. Time course of RNA synthesis directed by poly(A), poly(U), poly(G) and poly(C). Reaction mixtures (0.24 ml) containing 48 µg of protein, 0.01 µmol of labeled ribonucleoside triphosphate and 0.25 µmol each of the other three unlabeled triphosphates were assayed at 30°C as described in Material and Methods in the presence of various polynucleotide templates as indicated. Duplicate samples (30 µl) were taken at intervals for determination of acid-insoluble radioactivity.

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about 30% of that with CPMV RNA as template (Fig. 7A) while at the lower
concentration (20 µg/0.24 ml) the poly(A)-directed incorporation of ^{3}H-UMP
amounted to about 17% of that of CPMV. These results demonstrate that CPMV
RNA replicase can utilize poly(A) as a template although the efficiency is
very low, since on a molar basis very high amounts of this artificial template
are required.

The template activity of poly(U) as measured by the incorporation of ^{3}H-AMP
was low (10-15%) and only manifest at high concentrations (Fig. 7A). Addition
of poly(G) both at high (100 µg /0.24 ml) and low (20 µg/0.24 ml) concentrations
did not stimulate the incorporation of ^{3}H-CMP (2%) (Fig. 7B). The template
activity of poly(C) as measured by the incorporation of ^{3}H-GMP was negligible
(< 7%) (Fig. 7B).

5.3.2.2 Template activity of various RNAs

After having established that synthetic polynucleotides were either inactive
or very inefficient templates, in comparison with CPMV RNA, we then examined
the template activity of a variety of nonviral and viral RNAs all added in
equimolar amounts. The kinetics of RNA synthesis are shown in Fig. 8. All the
RNAs tested, except tRNA from yeast (not shown), were able to stimulate CPMV
replicase. The RNA synthesis directed by the RNAs from the plant viruses TMV,
TSV and RaMV and 17S rRNA from yeast was comparable (80-110%) to the reaction
catalyzed by CPMV RNA. The rRNA from *E. coli* and the RNAs from TYMV, CCMV, BMV,
STNV, AMV and SemFV were about 45-60% as effective whereas CMV RNA showed the
lowest efficacy (20-25%). The same results were obtained with two different
batches of replicase. With most templates tested, the RNA synthesis continued
at a linear rate for 4 h.

From these results it is evident that the DEAE-purified replicase from CPMV
does not display specificity with natural templates, under conditions which
are optimal for ^{3}H-UMP incorporation. Since however, the enzyme was tested at
saturating RNA concentrations and thus an apparent V_{max} was measured the possi-
bility still existed that at limiting RNA concentrations and therefore under
more stringent conditions the enzyme might display a more fastidious behaviour
in the selection of templates. To test this hypothesis the RNA synthesis was
measured as a function of the template concentration using TSV RNA and CPMV
M-RNA (Fig. 9A). From the double-reciprocal plots shown in Fig. 9B, it can be
calculated that, respectively, 0.10 pmol/0.12 ml and 0.15 pmol/0.12 are required

to obtain $\frac{1}{2} V_{max}$ for the reaction catalyzed by CPMV M-RNA and TSV RNA suggesting a remarkably similar affinity of the enzyme for both templates.
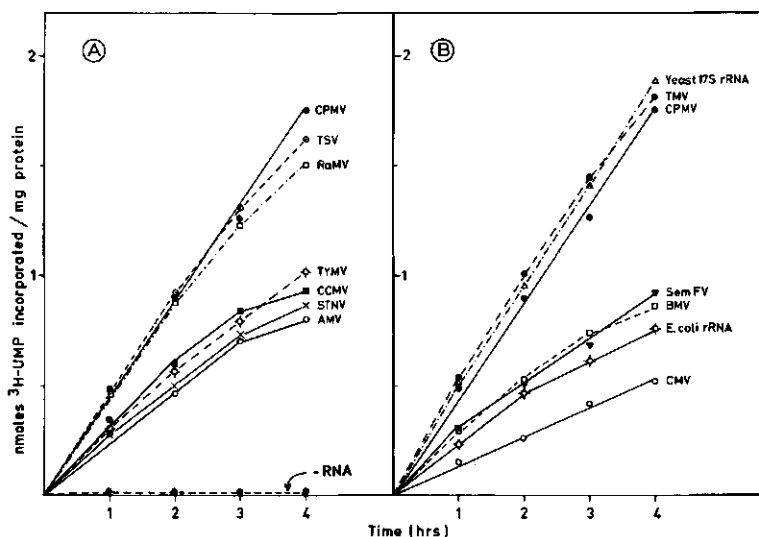

Figure 8. Time course of RNA synthesis directed by various template RNAs. Reaction mixtures (0.24 ml) containing 22 µg of protein were assayed at 30°C in the absence (●---●(A)) or presence of 6 pmoles of the indicated template RNAs as described in Material and Methods. At the specified times, duplicate aliquots (35 µl) were removed and assayed for acid-insoluble radioactivity.
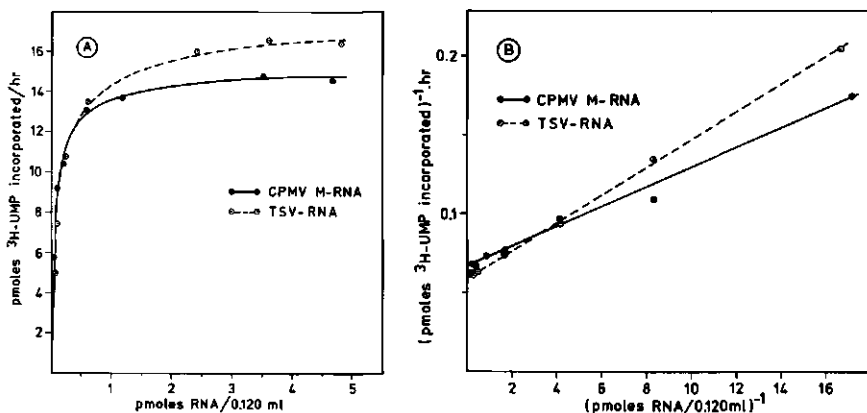

Figure 9. Template saturation curves. (A) Reaction mixtures (0.12 ml) containing 15 µg of protein and increasing amounts of CPMV M-RNA (●——●) or TSV RNA (⊙----⊙) were assayed for 60 min at 30°C as described in Material and Methods. (B) Double-reciprocal plots of the data.

5.3.3 Replicase RNA template binding

Since free nucleic acids run through nitrocellulose membrane filters whereas protein-nucleic acid complexes are retained, membrane filters have been employed widely to assay for protein-nucleic acid interaction (2, 13, 32, 45).

When increasing amounts of replicase are added to a constant amount of ^{32}P-labeled CPMV RNA increasing amounts of ^{32}P-RNA are retained on the filter until a plateau is reached (Fig. 10). A reciprocal plot of the binding curve data yields a straight line (Fig. 10). Since the DEAE-purified replicase preparation used for this experiment is far from being pure (see chapter 6) it was conceivable that the binding results from non-specific interaction of contaminating proteins. Therefore, we examined the RNA binding capacity of a corresponding DEAE-purified protein preparation from uninfected leaves. As shown in Fig. 10, this protein preparation completely failed to bind CPMV RNA.
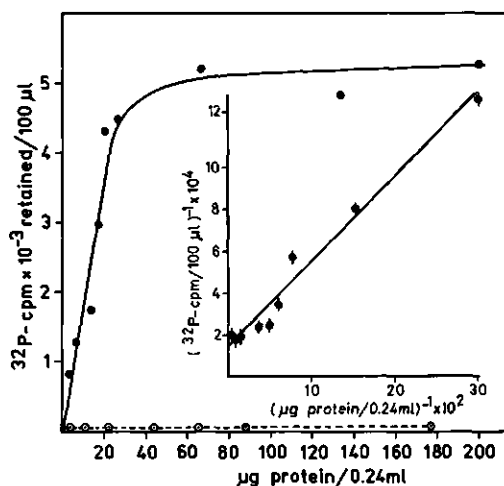

Figure 10. Binding of CPMV replicase and the corresponding DEAE-purified protein fraction from uninfected Vigna leaves to ^{32}P-CPMV RNA. Binding assays (0.24 ml) containing 1 µg of ^{32}P-CPMV RNA (24,000 cpm/µg) and increasing amounts of either CPMV replicase (●——●) or "healthy" protein (○----○) were prepared and incubated as described in Material and Methods After incubation at 30°C for 15 min, two 100 µl samples were taken, filtered through millipore filters, washed and counted. A blank value of 227 cpm obtained from a binding assay run in the absence of protein has been subtracted. The insert shows the double-reciprocal plot of the data.

These data clearly demonstrate that the retention of the CPMV RNA is specific for the replicase preparation and most likely involves the binding of the polymerase to the binding site(s) on the RNA. Thus, by performing competition binding assays, it became possible to examine whether the poly(A) tract of the CPMV genome comprises a potential binding site for the replicase. Therefore, increasing amounts of unlabeled poly(A) were added to a constant amount of ^{32}P-labeled CPMV RNA under conditions of RNA excess, whereupon the replicase was added and the amount of ^{32}P-RNA retained on the filter was determined. Fig. 11
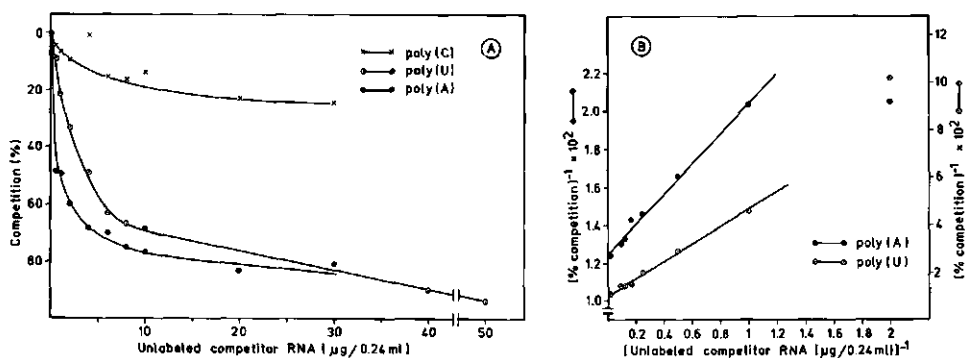
Figure 11. Competition between CPMV RNA and either poly(A), poly(U) or poly(C) for binding to CPMV replicase. (A) Binding assays (0.24 ml) containing 1 μg of ^{32}P-CPMV RNA (18,800 cpm/μg), varying amounts of unlabeled polynucleotide, as specified, and 16.5 μg of protein were prepared and incubated as described in Material and Methods. ^{32}P-CPMV RNA was mixed with the unlabeled polynucleotides prior to the addition of replicase. After incubation at 30°C for 15 min duplicate 100 μl samples were taken, filtered through millipore filters, washed and counted. (B) Double-reciprocal plots of the data.

shows the competition between poly(A) and CPMV RNA for the replicase. About 1 μg of poly(A) added to 1 μg of ^{32}P-CPMV RNA was able to reduce the labeled complex with 50% whereas only 20% of the labeled complex was formed in the presence of about 20 μg of poly(A).

On the basis of these observations it can be presumed that the addition of poly(U) as a competitor might inhibit the binding of the replicase as a result of hybrid formation with the poly(A) tail, which might render the binding site inaccessible to the replicase. Unfortunately, these binding assays could not be performed under high salt conditions which are optimal for hybrid formation, since the binding *per se* of the replicase to the CPMV RNA appeared to be sensitive to salt; at 0.2 M KCl complex formation was found to be inhibited about 50%. Nevertheless the results of the competition binding assay show that poly(U) is capable to inhibit complex formation (Fig. 11). In the presence of 50 μg of poly(U) only 10% of the labeled complex was formed. From the reciprocal plots (Fig. 11B) it can be calculated that, respectively, 0.64 μg of poly(A)/0.24 ml and 3.75 μg of poly(U)/0.24 ml are required to obtain half of the maximal competition.

In contrast to the strong decrease in complex formation caused by either poly(A) or poly(U), poly(C) displayed a much lower competition ability. In the presence of 30 μg of poly(C), about 70-75% of the replicase CPMV RNA complex

82

remained.

5.3.4 *Analysis of in vitro products*

In a preliminary effort to investigate the size and nature of the *in vitro* synthesized RNA, the products were analyzed by sucrose gradient centrifugation. Replicase was allowed to synthesize RNA for 1, 2 and 3 hours using CPMV RNA as template under rate-saturating concentrations. Reactions were terminated by the addition of detergent mixture, chromatographed on a Sephadex G-25 column to remove nonincorporated precursors and sedimented through SDS-sucrose gradients. Another sample was incubated for 1 hour and treated with RNase A plus RNase T_1 prior to sucrose gradient centrifugation.

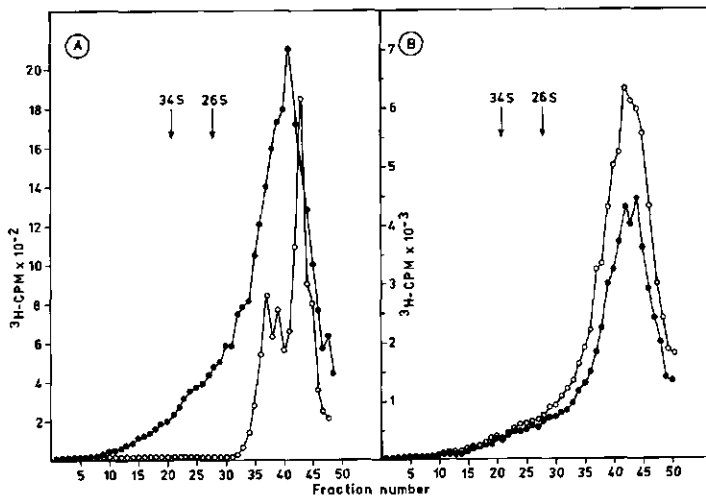

Figure 12. Sucrose gradient centrifugation of replicase reaction products. Standard reaction mixtures (0.12 ml) containing 30 µg of protein and 20 µg of CPMV RNA were incubated at 30°C for either (A) 1 h (●——●) (B) 2 h (●——●) or 3 h (o——o). The reactions were terminated by the addition of detergent mixture as described in Material and Methods. After purification by Sephadex G-25 chromatography, the product RNA was sedimented through a 11 ml linear 15-30% (wt/vol) sucrose gradient in TNES in a Spinco SW41 rotor at 20°C for 6 h at 40,000 rpm. Fractions were collected dropwise from the bottom of the tubes directly in counting vials and counted with 5 ml of Instagel containing 0.5 ml H_2O. Another reaction mixture (A) (o——o) was incubated for 1 h, then rapidly chilled to stop RNA synthesis, adjusted to 0.3 M NaCl and incubated with RNase A (5 µg/ml) and RNase T_1 (5 U/ml) for 30 min at 30°C prior to detergent treatment, Sephadex G-25 chromatography and sucrose gradient centrifugation. ^{32}P-labeled CPMV RNAs (34S and 26S) were used as internal sedimentation markers.

From the sedimentation profiles presented in Fig. 12 it is apparent that RNA species sedimenting at about 16S, accumulated throughout the course of enzymatic synthesis and constituted the bulk of the newly formed product. In front of this 16S peak some very heterogenous sedimenting RNA was found with sedimentation co-efficients up to 38S comprising 28% of the total amount of RNA synthesized in 1 hour. However, these RNA products did not increase but remained rather constant throughout prolonged incubations and comprised about 16% and 10% of the total amount of RNA synthesized in 2 and 3 hours respectively.

Treatment of the products with RNases prior to velocity sedimentation caused the complete disappearance of the fast sedimenting RNA (20S-38S) and a marked reduction in the quantity of the 16S material (Fig. 12A). About 63% of the RNA synthesized in 1 hour appeared to be sensitive to RNases. Since no effort was made to separate the labeled product RNAs from the unlabeled template strands and, thus, the RNAs were centrifuged under non-denaturing conditions, these results indicate that most of the *in vitro* synthesized RNAs are single-stranded and remain associated with template in replicative structures.
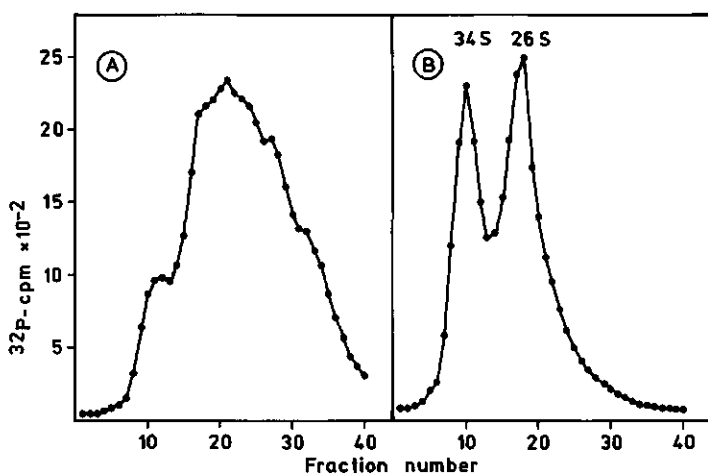
Figure 13. Degradation of ^{32}P-CPMV RNA by CPMV replicase. Standard reaction mixtures (0.12 ml) containing 30 µg of ^{32}P-CPMV RNA (3,000 cpm/µg) were in-cubated in the presence (A) or absence (B) of 30 µg of protein for 30 min at 30°C. After the addition of detergent mixture as described in Material and Methods the samples were directly analyzed by sucrose gradient centrifugation as described in the legend of Fig. 12. Radioactivity in the fractions was measured using Cerenkov counting. Sedimentation was from right to left.

To test the hypothesis that the absence of distinct peaks corresponding to single-stranded RNA products of genome-length was caused by nuclease(s) contaminating the replicase, ^{32}P-labeled CPMV RNA was incubated for 30 min with replicase and analyzed by sucrose gradient centrifugation. As shown in Fig. 13, the replicase indeed appeared to contain RNase(s) able to degrade the viral RNAs into several rather discrete products. Since hardly any label was present in the top fractions and the bulk of the digestion products sedimented still faster than 20S, this nuclease contaminant(s) probably involved an endonuclease(s) nicking the RNA at specific site(s). The degree of contamination was found to vary with different enzyme preparations. Attempts to inhibit the endogenous RNase(s) during *in vitro* RNA synthesis by the addition of the RNase inhibitors macaloid, polyvinylsulphate, heparine or dextransulphate, were unsuccessful, since the replicase appeared to be strongly inhibited by these reagents.

5.4 DISCUSSION

The major goal of this chapter has been to describe some of the basic properties of CPMV replicase. Although the DEAE-purified replicase preparations used for this characterization still contained a considerable amount of contaminating proteins, the template dependence in addition to the great stability of such preparations prompted us to study the template specificity. Prior to this analysis, we have determined the reaction conditions optimal for RNA synthesis, since knowledge of these conditions may contribute to the understanding of the replicase and facilitate prospective and more thorough *in vitro* studies on the replication mechanism.

A conspicious result described in this chapter is the apparent lack of template specificity of CPMV replicase. The enzyme was shown to be able to utilize quite efficiently a variety of unrelated viral and nonviral template RNAs under well-defined conditions with the exception of synthetic homopolymers. This was rather unexpected in view of the rigid template specificity exhibited by the bacteriophage replicases. In considering several possibilities which might account for the absence of template specificity of CPMV replicase, we should like to point out the following.

(i) The most simple possibility arising is that CPMV replicase, unlike the prokaryote replicases but like other eukaryote replicases (4, 6, 17, 22, 27, 42, 46), is indeed not template-specific, neither *in vitro*, nor *in vivo*. However, in our opinion the absence of template specificity does not

necessarily affect the enzyme adversely *in vivo*. Template specificity does not have to reside in the intrinsic properties of the enzyme itself, but may solely be inherent to the specific location in the cell. CPMV-infected *Vigna* leaves have been shown to contain a characteristic cyto-pathic structure consisting of numerous vesicular membranes embedded in a rather amorphous mass (1, 8). Both the virus-specific double-stranded RNA (8) as well as the replicase (40), and hence the replicative process of CPMV, were shown to be associated with the vesicular membranes of this cytopathic structure. Thus, the replicase together with its template are rather sequestered *in vivo* and not exposed to the supply of other RNA molecules.

One of the arguments used to account for the high template specificity of bacteriophage replicases (39) is that these enzymes are met in the cell by various cellular RNAs which are in excess of the phage RNA and do not need to be replicated. According to this argument, template specificity of the replicase should be a guarantee and prerequisite for the survival of the viral genome. However, this does not appear to be a sound argument, since most RNA molecules do not occur free in the cell but are associated with proteins as ribonucleoprotein particles (3, 44). Therefore, in this respect there is in fact no requirement for template specificity by the replicase.

(ii) Another explanation might be that during solubilization of the replicase from the membranes and subsequent purification essential protein factor(s) are lost. Thus, the replicase obtained is a core polymerase requiring an additional factor as a control element to discriminate between homologous and heterologous templates and to render the replicase incompetent for heterologous template recognition. This is strongly reminiscent of the role the sigma-factor plays in the selective transcription of DNA into RNA by the *E. coli* DNA-dependent RNA polymerase (5, 23). A very attractive candidate for the role of such a sigma-like replicase factor has been proposed recently by Nomoto *et al.* (28) for the small protein linked to the 5' end of the poliovirus genome (see also below). In view of the striking similarities between poliovirus RNA and CPMV RNA the template-dependent CPMV replicase may offer a model system to test this hypothesis.

It is remarkable, that the argument of the loss of a protein factor brought forward to explain the lack of template specificity of eukaryote replicases has only been used in reference to the Qβ replicase (22, 25,

42, 46). However, in that context, the argument is rather unsound for the following reason. The Qβ core polymerase is inactive with Qβ (+) strand as template in the absence of a host-supplied protein factor, in contrast to the eukaryote replicases which are all active with the homologous (+) strand. In addition, it has been recognized that the phage-coded subunit of Qβ replicase, which has the polymerizing activity of the enzyme complex, must be partially responsible for the template specificity, since this is the only subunit in which the Qβ replicase differs from the f2 replicase. So, by analogy with the Qβ replicase, using virus (+) strand RNA as a screening template during replicase purification, the highest demands are made upon the eukaryote replicase and loss of putative template specificity should be accompanied by lack of enzyme activity.

(iii) The third possibility to explain the absence of template specificity may be the use of nonspecific or inadequate reaction conditions during *in vitro* replication. In other words, the replicase itself is template specific, but does not get a chance to display it. In this respect it is quite conceivable that the assay conditions which are optimal for ³H-UMP incorporation, also promote non-specific interaction with and initiation on numorous sites along the heterologous template RNAs and that selective replication of CPMV RNA only occurs provided stringent requirements, concerning for instance divalent cations, ionic strength, enzyme to RNA ratio or possibly primers have been fulfilled.

It is generally assumed that eukaryote virus RNA replication resembles the bacteriophage replication, but, although this may be true in broad outline, it does not preclude small but essential differences. For instance, whereas bacteriophage replicases have been shown to be able to start a new polynucleotide chain *de novo*, CPMV replicase might require a primer to initiate properly. In this context it is quite interesting to recall the conspicuous 5' termini of CPMV RNA (19) as well as poliovirus (10, 18, 29), foot-and-mouth-disease virus (35) and encephalomyocarditis virus RNA (14), implying a different mechanism of initiation. In contrast to most other viral RNAs that are either "capped" at the 5' end with m⁷GpppN(m)p.... or have di- or triphosphate termini (36), the virion RNAs of poliovirus (12, 21, 28, 30), foot-and-mouth-disease virus (35), encephalomyocarditis virus (28) and most likely also CPMV (20) have been found to contain a small protein covalently linked to the 5' terminus. For the 5' end of poliovirion RNA the following structure has been ident-

ified: protein-pUUAAAACAG which is, except for the presence of the protein, identical to the polyribosomal poliovirus mRNA (12, 28, 30). The protein linked to the 5' end also appeared to be present on the nascent strands of the polio replicative intermediate (12, 28) and on the 5' end of (-) strands containing poly(U) complementary to the poly(A) of the (+) strand. These results strongly suggest that the 5' terminal protein, possibly with one or several covalently linked nucleotides (e.g. protein-pU$_{OH}$) might serve as a primer for initiating poliovirus RNA synthesis, whereby the protein is linked to the nascent chain.

The model of primer-dependent RNA replication has been supported by the experiments of Flanegan and Baltimore (11) demonstrating that HeLa cells infected with poliovirus, but not uninfected cells, contain a poly(A)· oligo(U)-dependent poly(U) polymerase. This enzyme activity, which appeared to have a molecular weight of about 65,000, may be identical to, or a component of the replicase. To our surprise, preliminary experiments (data not shown) which show that the CPMV replicase activity with CPMV RNA is stimulated in the presence of poly(U), apparently fit this primer-model. Additional support seems to be gained from *in vivo* studies with CPMV-infected *Vigna* protoplasts. Rottier *et al.* (34) have shown that CPMV replication can be blocked completely by actinomycin D, provided this inhibitor of DNA-dependent RNA synthesis is added very early during infection. From these studies it is tentative to conclude that a host-supplied RNA or protein molecule is indispensable for an early step in the replication of CPMV and therefore might act as a primer for the replicase. Recently, another example of the involvement of primers in the synthesis of viral RNA has been described. It concerns the *in vitro* synthesis of (+) strand RNA complementary to Influenza virion (-) strand RNA by the virion transcriptase, which was shown to require a specific dinucleoside monophosphate (ApG or GpG) as primer (7, 26, 31, 33)

In the foregoing we have discussed that template specificity does not have to be a prerequisite for the CPMV replicase to function properly in the replication process and does not preclude a specific interaction of the enzyme with its homologous template. That indeed a specific region of the CPMV genome, namely the poly(A) sequence at the 3' end, is recognized by the replicase is suggested by the competition binding experiments which demonstrate that poly(A) and poly(U), but not poly(C) can compete with CPMV RNA for the binding to the replicase. However, since the replicase also appears to accept TMV RNA and 17S rRNA from

yeast and thus RNA molecules with completely different features at their 3' ends, it remains to be elucidated whether binding of CPMV replicase to the poly(A) on CPMV RNA reflects the *in vivo* behaviour of the enzyme or is only coincidental. To determine unambiguously the role of the poly(A) on CPMV RNA, it will be of interest to test whether removal of the poly(A) tail from the CPMV genome is harmful for its template activity and infectivity.

Deadenylated encephalomyocarditis virus RNA (15) and poliovirus RNA (37, 38) were shown to be unable to replicate *in vivo*. Since on the one hand no progeny viral RNA could be detected after infection with poly(A)-deficient poliovirus RNA but on the other hand the translational capacity of this RNA *in vitro* was unaffected, it is tempting to conclude that the poly(A) on poliovirus RNA is required for binding of the replicase and initiation of RNA replication. It is noteworthy, that artificial poly(A) itself is an inefficient template for CPMV replicase. Thus, although the replicase is able to bind to the poly(A) on CPMV RNA, binding *per se* to A-rich sequences does not result in efficient utilization.

Taking into account the amount of artificial poly(A) required to compete with CPMV RNA, it is evident that the poly(A) tail on CPMV RNA is not the only part of the CPMV genome involved in replicase binding. Probably, the cooperation of other sites of the viral RNA genome is required for the correct positioning of the poly(A) tail into the initiation site of the enzyme so that chain initiation can start. In this model an oligo(U)-containing primer might facilitate the alignment of the replicase on the poly(A) tail of CPMV RNA.

Sucrose gradient analysis of the RNA synthesized *in vitro* revealed one distinct peak of 16S RNA constituting the bulk of the RNA products and, in addition, RNA species sedimenting heterogeneously from 20-38S. In contrast to several other template-dependent plant virus RNA replicases which have been shown to synthesize *in vitro* mainly RNase-resistant double-stranded RNA (4, 6, 16, 27, 46), a major part (63%) of the RNA synthesized by CPMV replicase was single-stranded. Since the 20-38S RNA completely disappeared upon RNase treatment prior to sucrose gradient centrifugation, these results suggest the formation of replicative intermediates in which nascent product RNAs are still attached to the genome RNAs. A major drawback of the DEAE-purified replicase preparations is the contamination with RNase(s) which degrade the template RNA and prevent the synthesis of full-length single-stranded virus RNAs.

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6. FURTHER PURIFICATION AND ELECTROPHORETIC ANALYSIS OF CPMV REPLICASE

6.1 INTRODUCTION

In chapter 4 we have demonstrated that the solubilization of a membrane-bound replicase is not necessarily the bottle-neck in the purification of a eukaryote virus RNA replicase. It has been shown that the release of CPMV replicase from membranes and subsequent DEAE-BioGel column chromatography could be achieved quite easily without loss of enzyme stability. However, it soon became evident that the DEAE-purified enzyme preparations, although suitable for several enzymatic studies, was not homogeneous and did not enable the identification of the enzyme. Therefore, aiming at the final purification of CPMV replicase, we set out to continue the purification. In this chapter we will demonstrate that glycerol gradient centrifugation provides a very efficient and gentle purification step but does not seem to have the final word in the purification of CPMV replicase. Polyacrylamide gel electrophoresis under denaturing conditions reveals a rather complex pattern from which no conclusions about the molecular structure of the enzyme can be drawn.

6.2 MATERIAL AND METHODS

6.2.1 *Materials*

Protein markers used for polyacrylamide gel electrophoresis were: myosin (a gift from Dr. H. Pelham, Cambridge, England; M.W. 200,000), β-galactosidase (from *E. coli* grade IV, Sigma Chemical Co.; M.W. 116,200), phosphorylase A (from rabbit muscle, Boehringer, Mannheim GmbH; M.W. 92,500), transferrin (from human, Sigma Chemical Co.; M.W. 80,000), bovine serum albumin (SchwarzMann; M.W. 68,000), γ-globulins (from human, cohn fraction II, Sigma Chemical Co.; M.W. 54,000 and 23,500), catalase (Boehringer Mannheim GmbH; M.W. 57,500), ovalbumin (Sigma Chemical Co.; M.W. 46,000), lactate dehydrogenase (from rabbit muscle, Boehringer Mannheim GmbH; M.W. 35,000) and tobacco mosaic virus coat

protein, U1 strain (donated by Dr. R. Huber; M.W. 17,500). Protein markers used
for glycerol gradient centrifugation were: catalase (Boehringer Mannheim GmbH;
M.W. 240,000), aldolase (Boehringer Mannheim GmbH; M.W. 147,000) and bovine
serum albumin (Schwarz Mann; M.W. 68,000). Acrylamide and methylene bisacryl-
amide were obtained from Serva, tetraethylmethylenediamine from Koch-Light and
sodiumdodecyl sulphate (specially pure SDS) from BDH. Coomassie Brilliant Blue
R250 was purchased from Sigma Chemical Co. Cibacron Blue F3GA-Sephadex G-100 was
a gift from Dr. W.J.H. van Berkel (Department of Biochemistry, Wageningen, The
Netherlands). The source of all other chemicals has been mentioned in the pre-
vious chapters.

6.2.2 Buffers

Buffer $TK_{10}EDP$ contained: 0.05 M Tris-hydrochloride (pH 7.4), 0.01 M KCl,
0.001 M EDTA, 0.01 M DTE and 0.5 mM PMSF.
Buffer $TG_{25}K_{50}EDP$ contained: 0.05 M Tris-hydrochloride (pH 8.2), 25% (vol/vol)
glycerol, 0.05 M KCl, 0.001 M EDTA, 0.01 M DTE and 0.5 mM PMSF.
Buffer $TG_{50}K_{50}EDP$ contained: 0.05 M Tris-hydrochloride (pH 8.2), 50% (vol/vol)
glycerol, 0.05 M KCl, 0.001 M EDTA, 0.01 M DTE and 0.5 mM PMSF.
Buffer $TK_{250}EDP$ contained: 0.05 M Tris-hydrochloride (pH 8.2), 0.25 M KCl,
0.001 M EDTA, 0.01 M DTE and 0.5 mM PMSF.
Buffer $TG_5K_{250}EDP$ contained: 0.05 M Tris-hydrochloride (pH 8.2), 5% (vol/vol)
glycerol, 0.25 M KCl, 0.001 M EDTA, 0.01 M DTE and 0.5 mM PMSF.
Buffer TGEDP contained: 0.025 M Tris-hydrochloride (pH 8.2), 25% (vol/vol)
glycerol, 0.001 M EDTA, 0.01 M DTE and 0.5 mM PMSF.
Buffer TGMEDP was identical to buffer TGEDP except for the addition of 5 mM
Mg(OAc).
PMSF and solid DTE were added and dissolved just before use of the buffer. The
former was added from a 0.2 M stock solution in 96% ethanol stored at $-20^{o}C$.

6.2.3 Virus and plants

The growth of *Vigna unguiculata* (L.) Walp. var. "Blackeye Early Ramshorn"
plants and their infection with a yellow strain isolate of CPMV was carried
out as described in chapter 3. The primary leaves were harvested on day 4 after
inoculation and used directly for the isolation of the replicase.

6.2.4 Replicase purification procedure

All steps were carried out at $0-4^{O}C$ unless specified otherwise.

6.2.4.1 Isolation of membrane-bound replicase

The isolation of the membrane-bound replicase was performed as described in
chapter 3 except that PMSF, a protease inhibitor, was included in the homogen-
ization buffer and DTE was used instead of β-mercaptoethanol. Briefly, portions
(12 g) of freshly harvested leaves from which the midribs were removed, were
homogenized with 35 ml of $TK_{10}EDP$ in a prechilled mortar. The homogenate was
squeezed through two layers of "Miracloth" (fraction 1) and centrifuged at
1,000 xg for 15 min in a Sorvall SS34 rotor. The green coloured supernatant
was carefully pipetted off, adjusted to 20% (vol/vol) glycerol (fraction 2)
and centrifuged at 31,000 xg for 30 min. The 31,000 xg pellet was resuspended
with the aid of a Thomas homogenizer in $TG_{25}K_{50}EDP$ (1 ml for each gram of leaf
tissue used; fraction 3) and used for the solubilization procedure. This crude
replicase suspension could be stored at $-70^{O}C$ for several months without loss
of enzymatic activity providing a convenient stage to interrupt the purification
if desired. After storage at $-70^{O}C$, the enzyme was slowly thawed at $0^{O}C$ where-
upon the solubilization procedure was started.

6.2.4.2 Solubilization of membrane-bound replicase

Solubilization of the replicase providing fraction 4 was carried out as de-
scribed in chapter 4.

6.2.4.3 DEAE-BioGelA column chromatography

A DEAE-BioGelA column (1.6 x 15 cm) was prepared and equilibrated with
$TG_{25}K_{50}EDP$ as described in chapter 4. The solubilized enzyme (fraction 4) was
applied to the column at a flow rate of about 20-25 ml/h. Up to 475 ml of
fraction 4 containing 0.6-0.9 mg protein/ml could be applied to this column
without loss of enzyme activity in the flow-through. During the flow of the
sample through the column the top layer turned brown about 0.5 cm. After appli-
cation of the sample, the column was washed with $TG_{25}K_{50}EDP$ to remove unbound
material. Then the bound material was eluted with a 0.05 to 0.4 M KCl gradient,

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generated by a LKB Ultrograd Gradient Mixer. When 200-400 g of leaf tissue had been used for the preparation of replicase, a 160-200 ml gradient was applied and 3-3.25 ml fractions were collected at a flow rate of 16-20 ml/h and assayed for polymerase activity using 25 µl samples. The eluate of the column was continuously monitored at 280 nm by means of a Uvicord II (LKB). The fractions containing the bulk of the polymerase activity were pooled (fraction 5) and dialyzed overnight against 1 l of $TG_{25}K_{50}EDP$ saturated with $(NH_4)_2SO_4$. After collection of the precipitate by centrifugation at 31,000 xg for 20 min, the precipitate was either prepared for storage in liquid nitrogen as described in chapter 4 or prepared for glycerol gradient centrifugation. In the latter case, the pellet was dissolved in 1 to 2 ml $TG_5K_{250}EDP$ and dialyzed for 4 h against 500 ml of $TG_5K_{250}EDP$.

6.2.4.4 Glycerol gradient centrifugation

The dialyzed enzyme solution (0.5 to 0.75 ml) was layered on a 11.4 ml linear 15 to 30% (vol/vol) glycerol gradient in $TK_{250}EDP$ and centrifuged in a poly-allomer tube of the Beckman SW41 rotor at 40,000 rpm for 17 h at $1^{o}C$. Up to 7.5 mg of protein may be layered on each gradient in a SW41 rotor. Fractions were collected through a hole punctured in the bottom of the tube, monitored continuously at 280 by means of a Uvicord II and assayed for polymerase activity using 10 µl aliquots. Peak fractions which were completely colourless, were pooled and dialyzed overnight against 2 changes of 300 ml each of $TG_{50}K_{50}EDP$. The dialyzed enzyme solution (fraction 6) was divided into aliquots (100 to 150 µl) and stored in liquid nitrogen.

6.2.4.5 Cibacron Blue F3GA-Sephadex column chromatography

Cibacron Blue F3GA-Sephadex G-100 was prepared according to the procedure of Böhme *et al.* (1) as modified by Van Berkel and Müller (manuscript in preparation). The resin was poured into a column and equilibrated with TGMEDP. Fraction 6 protein (about 400 µg) was dialyzed against TGEDP, adjusted to 5 mM Mg(OAc) and applied to the Cibacron-Sephadex column (0.9 x 9 cm) at a flow rate of 7 ml/h. After application of the sample the column was washed with TGMEDP to remove unbound material. The bound material was eluted with TGMEDP + 0.5 M $(NH_4)_2SO_4$. The unbound and bound material were each collected as one fraction.

6.2.5 SDS-polyacrylamide slabgel electrophoresis

Proteins were analyzed by electrophoresis on polyacrylamide slabgels according to the method of Laemmli (7) as modified by Marsden *et al.* (8) using 5% acrylamide in the spacer gel and a 7 to 15% linear gradient of acrylamide in the separating gel. Gels of 10.5 cm or 12.5 cm were used in respectively the apparatus described by Studier (10) and the Pharmacia Gelelectrophoresis Apparatus GE4. Spacer and separating gel buffers were those described by Laemmli (7) except that the ratio of acrylamide to bisacrylamide was 20 to 1. The upper buffer contained 0.05 M Tris, 0.055 M glycine and 0.1% SDS; the lower buffer contained 0.01 M Tris-hydrochloride pH 8.1 and 0.1% SDS. The sample buffer consisted of 0.05 M Tris-hydrochloride pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue.

Protein samples were adjusted with concentrated sample buffer to 0.05 M Tris-hydrochloride pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10 to 20% glycerol and 0.001% bromophenol blue and heated at 100°C for 2 min. When required, proteins were concentrated by precipitation with 10% trichloroacetic acid, washed with acetone and dissolved in sample buffer. After electrophoresis the gels were immersed in fixing solution containing 25% (vol/vol) isopropanol and 10% (vol/vol) glacial acetic acid prior to staining overnight with Coomassie Brilliant Blue R250 as described by Kedinger *et al.* (5). The gels were destained in a solution containing 7.5% acetic acid and 5% ethanol.

6.3 RESULTS

6.3.1 DEAE-BioGel chromatography

In our attempts to develop additional purification steps, we have used the DEAE-purified enzyme preparation as the point of departure. Because it was desirable to have large quantities of enzyme at our disposal from which samples could be taken for pilot studies, the purification procedure as described in chapter 4 was scaled up, allowing the processing of 400 g of leaf tissue. This was met without difficulties, except for the DEAE-BioGel column, which sometimes did not afford complete removal of endogenous template RNAs when eluted with a linear 0.05-0.4 M KCl gradient. Therefore, another gradient profile was chosen to overcome this disadvantage. It was found that the resolution of the DEAE-BioGel column could be improved considerably by use of a nonlinear KCl

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gradient as shown in Fig. 1. In contrast to the replicase peak eluted by the
linear gradient previously used, the replicase peak was now very sharp and
symmetrical and completely separated from the nucleic acids eluting at the end
of the gradient. The enzyme exhibited only negligible activity (< 2%) in the
absence of added template RNA. The recovery of enzyme activity from the column
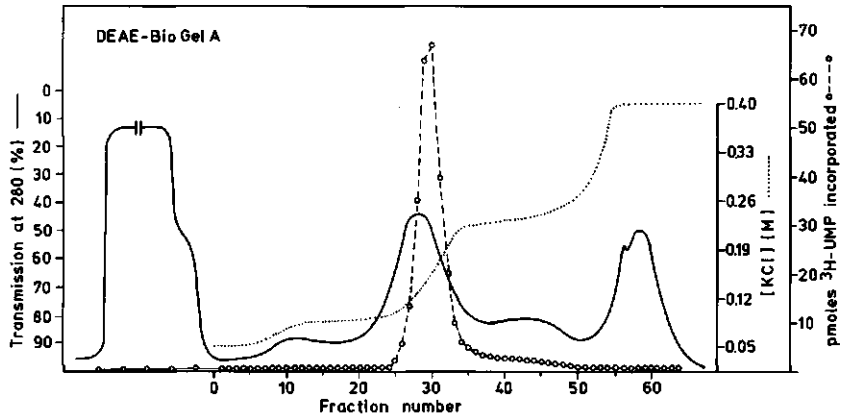was about 60-65% with a 4-5 fold purification.

Figure 1. DEAE-BioGelA column chromatography of solubilized RNA replicase.
Fraction 4 (350 ml, 210 mg of protein) was passed through a DEAE-BioGelA column
(1.6 x 15 cm) followed by $TG_{25}K_{50}EDP$ to wash out unbound material. The column
was eluted with 200 ml of a non-linear 0.05 to 0.4 M KCl gradient in the same
buffer. Fractions of 3.0 ml were collected and assayed for replicase activity
on 25 µl aliquots for 60 min under standard conditions. Enzyme activity (O---O)
is expressed as pmoles of ^{3}H-UMP incorporated per 25 µl of column fraction per
60 min; (.....) KCl molarity; (———) T_{280} (%).

6.3.2 Glycerol gradient centrifugation

Sedimentation of the DEAE-purified enzyme through a high salt (0.25 M KCl)
glycerol gradient appeared to be a very efficient and gentle step for the
further purification of CPMV replicase (Fig. 2). About 90-95% of the input
protein sedimented as one peak in front of and well separated from the repli-
case. As much as 80% of the input replicase activity was recovered and the
purification was about 7-10 fold. After this step the replicase was still
stable if stored at either -70°C or in liquid nitrogen.

From the positions of aldolase, catalase and BSA which were run as sedimen-
tation markers in a parallel gradient, a apparent molecular weight of about
150,000 has been estimated for CPMV replicase. However, more precise deter-
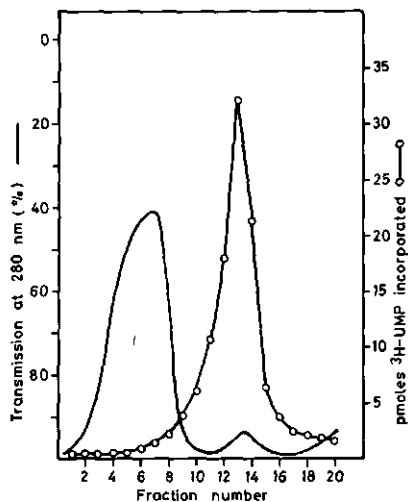
minations are needed to verify this estimate.

Figure 2. Glycerol gradient centrifugation of DEAE-purified replicase. DEAE-purified replicase (fraction 5; 0.75 ml, 7.5 mg of protein) was layered on 11.4 ml of a 15 to 30% glycerol gradient in $TK_{250}EDP$. The gradient was centrifuged at 40,000 rpm in a polyallomer tube of the SW41 rotor for 17 h at $1^{o}C$. Fractions of approximately 0.6 ml were collected through a hole in the bottom of the tube and assayed for replicase activity on 10 µl aliquots for 60 min under standard conditions. Enzyme activity (O——O) is expressed as pmoles of ^{3}H-UMP incorporated per 10 µl of gradient fraction per 60 min; (————) T_{280} (%). The bottom of the tube is at the left.

6.3.3 Overall purification

The overall purification of CPMV replicase from 84 g of *Vigna* leaves is summarized in Table I. After the glycerol gradient about 2 mg of protein per 100 g of leaf tissue is obtained with a final purification of approximately 150-200 fold.

It should be emphasized, however, that the enzyme activity is not an accurate measure of the extent of purification and only provides a rough estimate for the following reasons. First, replicase activity is difficult to assess in crude extracts due to the presence of other ribonucleotide polymerizing enzymes, like for example the DNA-dependent RNA polymerase, poly(U) polymerase and the soluble RNA-dependent RNA polymerase. Although the DNA-dependent RNA polymerases will be blocked by actinomycin D which is present in the replicase assay, it is uncertain whether this inhibition is complete in a crude plant extract. Second, nucleases are present in the earlier stages of the purification and interfere at variable extent with the assay. Third, measuring enzyme activity in fraction 1, 2 and 3 only comprises chain-elongation in preformed complexes, while the template-dependent reaction occurring in fraction 5 and 6 involves both chain initiation and chain-elongation. Fourth, assay conditions have been used which are optimal for the DEAE-purified enzyme (fraction 5) but are not necessarily

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TABLE 1. SUMMARY OF PURIFICATION OF CPMV REPLICASE[a]

Fraction no.	Fraction description	Volume (ml)	Total protein (mg)	Total activity (units)[b]	Specific activity (units/mg)	Fold purifi-cation	Yield (%)
1	Homogenate	265	1458	25,896	17.8	1	100
2	1000 xg supernatant	324	1134	22,585	19.9	1.1	87
3	membrane-bound replicase	84	160	14,051	87,8	4.9	54
4	solubilized replicase	94	89	12,369	139	7.8	48
5	DEAE-BioGel column pooled peak	22	13	6,897	530.5	29.8	27
6	Glycerol gradient pooled peak after dialysis	3.6	1.7	5,313	3125	175.6	21

a) From 84 g of *Vigna* leaves.
b) One unit of RNA replicase activity is defined as the amount of enzyme catalyzing the incorporation of 1 pmol of labeled UMP into acid-precipitable form in 30 min at 30°C. Fractions were assayed immediately after collection.

for the other fractions.

On a protein basis, the purification achieved is about 850-fold.

6.3.4 SDS-polyacrylamide gel electrophoresis

In order to visualize the purification process, the protein composition of
fractions at various stages of purification was analyzed by polyacrylamide gel
electrophoresis under denaturing conditions. Fig. 3 shows the gel patterns ob-
tained when approximately 25 µg of protein from the various fractions was sub-
jected to electrophoresis in the presence of SDS.

The membrane-bound replicase fraction appeared to contain a wide variety of
polypeptides with molecular weights ranging from 170,000 to very low values
(Fig. 3D). However, two clusters of bands with molecular weights of about 55,000
and 24,000 hereafter designated as the 55,000-cluster and the 24,000-cluster re-
spectively, constituted the bulk of polypeptides.

Solubilization of the replicase caused a significant change in the gel pattern,
thus illustrating the rather selective release of the replicase from the mem-
branes (Fig. 3F). The 24,000-cluster and two polypeptides with molecular weights
of about 64,500 and 29,000 respectively, were left almost completely in the mem-
brane-fraction in contrast to the 55,000-cluster and several larger polypeptides
(Fig. 3E and 3F).

The gel pattern of the DEAE-purified replicase fraction (Fig. 3G) was charac-
terized mainly by the 55,000-cluster constituting the bulk of polypeptides and
predominating all other bands. The relative proportion of polypeptide bands
above the 55,000-cluster was found to vary with different enzyme preparations.
In contrast to the membrane-bound replicase preparation, only a very few poly-
peptides with molecular weights less than 55,000 were left in the DEAE-prep-
aration.

In comparing the gel patterns of DEAE-purified replicase and the correspond-
ing protein fraction from healthy leaves, several polypeptide bands specific
for the replicase preparation were clearly visible (Fig. 4). One of these poly-
peptides with a molecular weight of approximately 170,000 appeared to be present
in all replicase preparations. Other polypeptide bands specific for the repli-
case preparation (see for example the 94,000 and 27,000 protein) were less con-
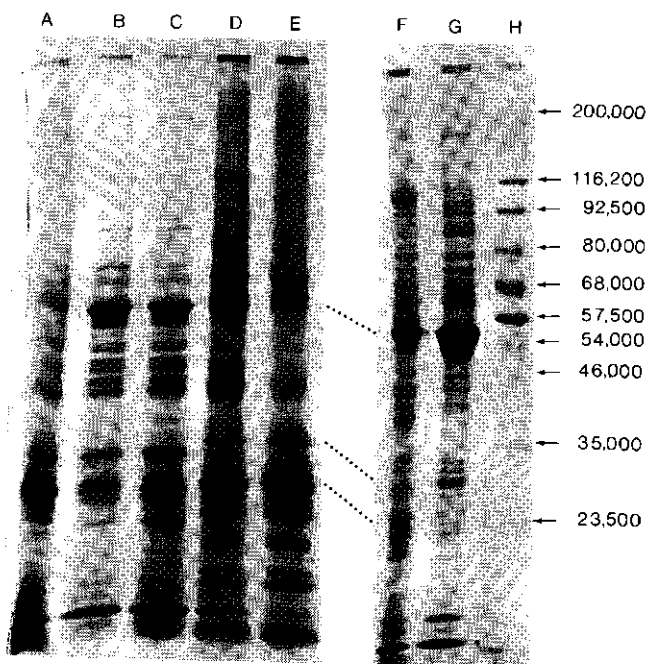sistent.

Figure 3. SDS-polyacrylamide slabgel electrophoretic analysis of CPMV replicase at various stages of purification. Samples of the following fractions containing approximately 25 µg of protein were prepared for electrophoresis on 7 to 15% gradient gels as described in Material and Methods.
A: leaf homogenate (fraction 1)
B: 1,000 xg supernatant (fraction 2)
C: 31,000 xg supernatant
D: 31,000 xg pellet: membrane-bound replicase (fraction 3)
E: residual 31,000 xg pellet after solubilization of replicase
F: solubilized replicase (fraction 4)
G: DEAE-purified replicase (fraction 5)
H: protein markers: myosin (M.W. 200,000), β-galactosidase (M.W. 116,200), phosphorylase (M.W. 92,500), transferrin (M.W. 80,000), bovine serum albumin (M.W. 68,000), catalase (M.W. 57,500), reduced γ-globulin (M.W. 54,000 and 23,500), ovalbumin (M.W. 46,000) and lactate dehydrogenase (M.W. 35,000).
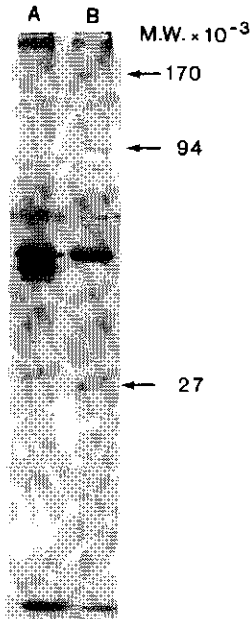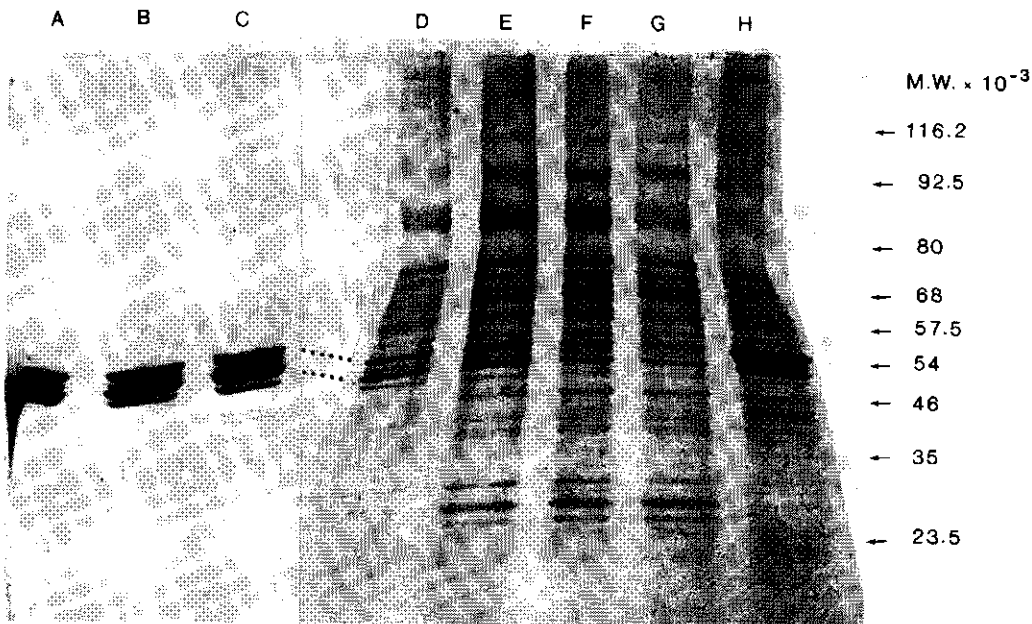
Figure 4. SDS-polyacrylamide slabgel electrophoretic analysis of CPMV replicase and protein from mock-infected leaves after DEAE-BioGel column chromatography. DEAE-purified replicase (about 10 μg of protein) and the corresponding DEAE-purified protein preparation (about 10 μg of protein) from mock-infected leaves were subjected to electrophoresis on 7 to 15% gradient gels as described in Material and Methods. A: DEAE-purified protein from mock-infected leaves.
B: DEAE-purified replicase.
The positions and the molecular weights of the polypeptides specific for the replicase are indicated.

Since glycerol gradient centrifugation had been found an efficient purification step able to remove about 90-95% of contaminating proteins, it was of special interest to visualize this step by polyacrylamide gel electrophoresis. Thus, various fractions throughout the gradient were subjected to gel electrophoresis. As shown in Fig. 5a, the material sedimenting ahead of the replicase and comprising the vast majority of protein in the glycerol gradient, appeared to consist almost exclusively of the 55,000-cluster resolved now into four components. As a result of this separation, the gel pattern of the replicase was no longer dominated by the 55,000-cluster. Thus, other polypeptides, hardly visible or even invisible in the DEAE-purified replicase preparation, started to define the gel profile of the replicase (Fig. 5a, lane E and F). In particular, two groups of polypeptides with molecular weights between about 95,000 and 60,000 and between 35,000 and 25,000 respectively, were rather pronounced. Assuming that this pattern might reflect the enzyme polypeptide composition in broad outline, we analyzed a protein preparation from mock-infected plants which had been subjected to the same isolation procedure. It was hoped that a comparison of both patterns might reveal the viral-coded subunit(s) of the replicase. However, from the results shown in Fig. 5b and Fig. 6 it is evident that
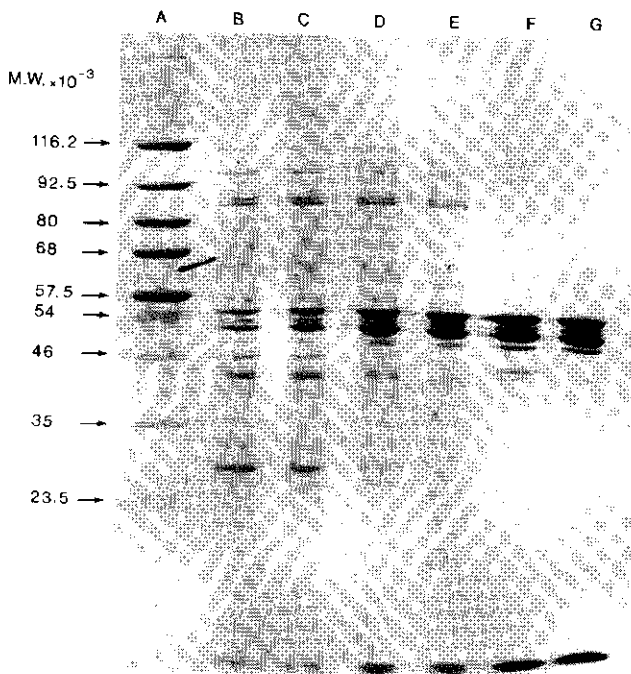
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(a)

Figure 5. SDS-polyacrylamide slabgel electrophoresis of fractions from the glycerol gradient.
(a) DEAE-purified replicase was analyzed by glycerol gradient centrifugation as described in Fig. 2. Samples of the following fractions from the gradient shown in Fig. 2, containing approximately 10 µg of protein were prepared for electrophoresis on 7 to 15% gradient gels as described in Material and Methods.
A: fraction 4.
B: fraction 6.
C: fraction 8.
D: fraction 12.
E: fraction 13.
F: fraction 14.
G: fraction 15.
H: DEAE-purified replicase.

(b)

(b) A DEAE-purified protein preparation from mock-infected leaves corresponding
   to the DEAE-purified replicase preparation was analyzed by glycerol gradient
   centrifugation (not shown) exactly as described in Fig. 2. The optical den-
   sity profile obtained was similar to that shown in Fig. 2. but no RNA poly-
   merase activity was detected throughout the gradient. Samples of the follow-
   ing fractions from this control gradient containing approximately 10 µg of
   protein were prepared for electrophoresis on 7 to 15% gradient gels as de-
   scribed in Material and Methods.
   A: protein markers.
   B: fraction 15.
   C: fraction 14.
   D: fraction 13.
   E: fraction 12.
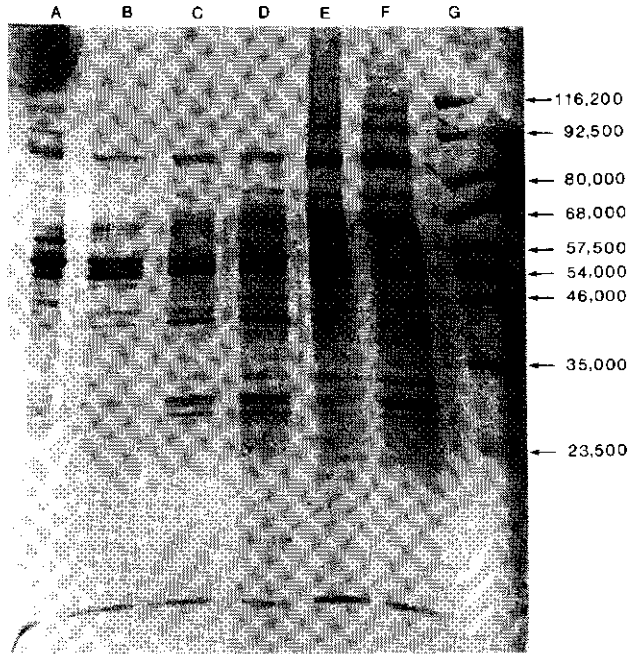   F: fraction 9.
   G: fraction 6.

Figure 6. Comparison of the electrophoretic patterns of CPMV replicase and protein from mock-infected leaves after glycerol gradient centrifugation. DEAE-purified replicase and the corresponding protein fraction from mock-infected leaves were analyzed by glycerol gradient centrifugation as described in Fig. 2 and 5. Samples of the following fractions containing approximately 10 µg of protein were prepared for electrophoresis on 7 to 15% gradient gels as described in Material and Methods.
A: DEAE-purified replicase.
B: fraction 12 from control glycerol gradient.
C: fraction 13 from control glycerol gradient.
D: fraction 14 from control glycerol gradient.
E: fraction 12 from glycerol gradient shown in Fig. 2.
F: fraction 13 from glycerol gradient shown in Fig. 2.
G: Protein markers.

the overall pattern of replicase and healthy material is rather similar, showing the same groups of polypeptides described above. A few polypeptides, with molecular weights of about 63,000, 65,000 and larger than 120,000 may be characteristic for the replicase preparation, but it is questionable whether these polypeptides are related to the replicase or are still derived from contaminating proteins.

That the polyacrylamide gel pattern of the replicase undergoes important changes upon further purification, became apparent from preliminary studies using chromatography on a Sephadex G-100 column to which Cibacron Blue F3GA, a sulphonated polyaromatic dye, had been coupled. Such a column has been shown to bind several enzymes utilizing nucleotide substrates or nucleotide coenzyme ligands (6, 9, 11-13). Upon chromatography of the replicase purified by glycerol gradient centrifugation on a Cibacron Blue-Sephadex column, about 90% of the protein applied to the column ran through. The remaining 10% of the protein was bound and could be eluted with 0.5 M $(NH_4)_2SO_4$. The replicase activity was distributed among the fractions containing unbound and bound material. About 55-60% of the input replicase activity appeared in the flow-through, whereas the replicase activity in the bound material varied considerable with different enzyme preparations between 20-85% of the input activity.

Analysis by gel electrophoresis of the flow-through and bound material revealed striking differences between both fractions (Fig. 7). In contrast to the flow-through material displaying a gel pattern almost identical to the replicase preparation purified by the glycerol gradient, the bound material was found to contain a considerable number of polypeptide bands which had not been observed previously, particularly in the region with molecular weights less than 46,000. On the other hand the 170,000 protein which appeared earlier to be specific for the replicase after DEAE-BioGel chromatography had disappeared. A preliminary comparison of the bound replicase fraction from the Cibacron-Sephadex column with a corresponding protein fraction from mock-infected leaves, demonstrated the presence of several polypeptides, which seem to be specific for the replicase preparation (Fig. 7). However, in our opinion, it is still premature to set much value to these polypeptides and to ascribe them to the replicase without further verification. More purification seems to be required to identify CPMV replicase.
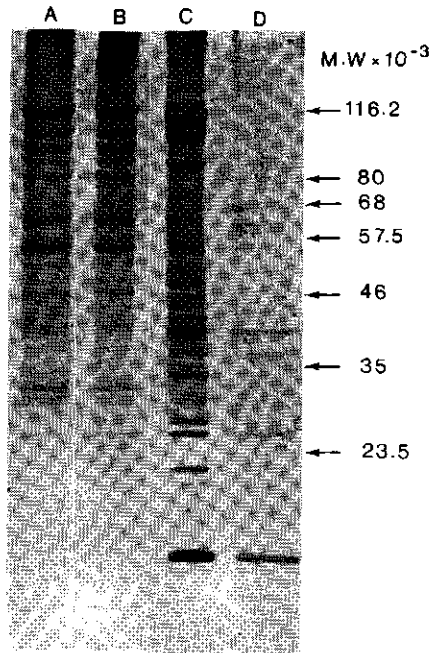
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Figure 7. SDS-polyacrylamide slabgel electrophoretic patterns of CPMV replicase
and protein from mock-infected leaves after Cibacron Blue F3GA-Sephadex column
chromatography. Glycerol gradient-purified replicase and the corresponding
protein preparation from mock-infected leaves were analyzed by Cibacron Blue
F3GA-Sephadex column chromatography as described in Material and Methods.
Samples of the following fractions were prepared for electrophoresis on 7 to
15% gradient gels as described.
A: glycerol gradient-purified replicase (approximately 10 µg of protein)
B: unbound replicase fraction from Cibacron Blue-Sephadex column (approximately
   10 µg of protein).
C: bound replicase fraction from Cibacron Blue-Sephadex column (approximately
   20 µg of protein).
D: bound 'healthy' fraction from Cibacron Blue-Sephadex column (amount of pro-
   tein unknown).

6.4 DISCUSSION

At the onset we hoped to be able to achieve the complete purification of CPMV replicase and to provide information about the polypeptide composition. However, one of the major conclusions to be drawn from the results presented in this chapter, is that the path to a homogeneous enzyme is long and is only partially paved by the purification steps achieved. This conclusion stems from the fact that at the different purification steps, important changes in the overall polypeptide pattern have been observed, concomitant with an apparently increasing number of polypeptides but without a considerable enrichment of particular polypeptides. This must mean that considerable amounts of contaminating proteins predominate the gel pattern and thus prevent the replicase to come into the picture. In general, it appeared to be quite easy to detect polypeptide bands which were only present in replicase preparations and not in the corresponding protein preparations from healthy leaves. However, our results about the fate of specific polypeptides at the different purification steps, clearly demonstrate that the presence *per se* of polypeptide bands characteristic for replicase preparations does not suffice to ascribe these polypeptides to the replicase.

In view of these findings, it is evident that suggestions made by Hariharasubramanian *et al.* (4) and Fraenkel-Conrat (3) about the possible identification of a polypeptide chain as a component of respectively the BMV and TNV replicase, have to be considered as very sceptical, due to the very crude nature of the replicase preparations. It is significant that up to the present for only one plant virus replicase polyacrylamide gel patterns have been published (2). Having purified CMV replicase about 100-fold, Symons and coworkers (2) had to admit that the SDS-gel patterns of replicase and healthy material are very similar and that their replicase was a long way from homogeneity. A major drawback in the purification of CMV replicase proved to be the instability of the enzyme. In this respect, the remarkable stability of the glycerol gradient-purified CPMV replicase offers good prospects on the complete purification.

In preliminary experiments attempting to devise additional purification steps, we have employed chromatography on either phosphocellulose, heparine-Sepharose, Cibacron Blue F3GA-Sephadex G-100, aminoalkyl-Sepharose, alkyl-Sepharose or CPMV RNA-Sepharose columns. As an representative example, we have presented the results obtained with the Cibacron Blue-Sephadex G-100

column which show that the majority of the proteins runs through the column and only a small proportion is bound. Despite precautions taken not to overload the column, the RNA polymerase activity was distributed among the flow-through and bound material, the latter showing the highest specific activity. The separation of RNA polymerase activity into two peaks, did not only occur with the Cibacron Blue-Sephadex G-100 column, but was repeatedly observed with all types of columns described above. A possible explanation might be, that the binding of the replicase to the columns is very weak and incomplete so that the enzyme is only partially retained. The rather low salt concentration of about 0.1 M KCl required to elute the bound replicase is consistent with this idea. Alternatively, it may be assumed that the replicase is an enzyme complex consisting of several subunits of which only a part binds to the column whereupon the other components dissociate from the complex. Thus, the RNA polymerase activities present in the unbound and bound fraction represent two incomplete forms of the replicase complex.

Taking into account these considerations and the binding experiments described in the previous chapter, which showed that CPMV RNA is bound by the replicase preparation but not by the 'healthy protein', the following experiment seems to be very promising to obtain a purified replicase. The replicase is bound to template RNA but also allowed to initiate and synthesize a short nascent chain in order to stabilize the enzyme-template complex. The resulting RNA·replicase· RNA complex is separated from other proteins by gel filtration or, in the case where Sepharose-bound template RNA is used, the contaminating proteins are removed by washing. This approach should bypass the use of cascades of chromatographic techniques.

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SUMMARY

This thesis concerns the partial purification and properties of an RNA-dependent RNA polymerase (RNA replicase) produced upon infection of *Vigna unguiculata* plants with Cowpea Mosaic Virus (CPMV). The enzyme is believed to be coded, at least in part, by the virus genome and to be responsible for the replication of the virus RNA.

In chapter 1 we describe the scope of the investigations and the motives underlying this thesis.

In chapter 2 a literature review is presented of the RNA replicases of viruses containing a single-stranded RNA genome of the plus type. With respect to the prokaryote virus RNA replicases, studies are described on the structure and properties of Qβ replicase, with special emphasis on the role the individual subunits of the enzyme are playing in the different stages of RNA synthesis. Reviewing the research on animal and plant virus RNA replicases had to be limited necessarily to a description of the isolation and properties of several crude enzyme preparations, since no purified replicases have been obtained and little progress is made with their purification.

In chapter 3 we describe the detection of an RNA-dependent RNA polymerase activity which is present in *Vigna unguiculata* leaves infected with CPMV but not in uninfected leaves. It is shown, that this RNA polymerase activity, which is designated as CPMV replicase and is associated with a membrane fraction, becomes detectable one day after infection and then continues to increase until the fourth day. This membrane-bound replicase activity was found to require Mg^{2+}-ions and all four ribonucleoside triphosphates and to be resistant to DNase and actinomycin D. Analysis of the *in vitro* synthesized RNA products by sucrose gradient centrifugation and treatment with RNases revealed, that the majority consisted of double-stranded RNA species sedimenting at 17S and 20S, probably representing the replicative forms of both virus RNAs. A minor part consists of two single-stranded RNA species, similar in sedimentation rate (26S and 34S) to the virion RNAs. From these results we concluded, that we were dealing with a bound replicase complex most likely representing the

replicase involved in virus replication *in vivo*. Having the final purification of CPMV replicase in view, we were then faced with the solubilization of the enzyme required to continue the purification.

In chapter 4 we describe a very gentle and easy method to release the replicase from the membranes without employing detergent. The method consists of a washing procedure involving a Mg^{2+}-deficient buffer, and provides several advantages in comparison with other solubilization procedures. Firstly, the solubilized replicase is highly stable, thus facilitating the further purification. Secondly, the release of the replicase from the membranes is rather selective. The majority of proteins is retained in the membrane pellet and the specific activity of the solubilized replicase is increased about 2-3 fold with respect to the membrane-bound replicase. Thirdly, more than 80% of the replicase activity is detached from the membranes. The solubilized replicase can be further purified and freed of endogenous template RNA by DEAE-BioGel column chromatography to provide a highly stable enzyme dependent on template.

In chapter 5 we describe several properties of the DEAE-purified replicase preparation. Replicase activity is not inhibited by α-amanitin, rifampicin, cordycepin, actinomycin D, DNase and orthophosphate but is completely suppressed by pyrophosphate and RNase A plus RNase T_1. The *in vitro* RNA synthesis is shown to proceed for at least 15 hours under the following optimal conditions: 8 mM $Mg(OAc)_2$ or 12 mM $MgCl_2$; 60 mM $(NH_4)_2SO_4$, up to 100 mM K(OAc), but KCl as low as possible; pH 8.2; 30 to 34^oC; all four ribonucleoside triphosphates present and 5-10 μg of CPMV RNA as template per 15 μg of protein.

Having established the optimal conditions for RNA synthesis, we have studied the template specificity using a variety of viral, nonviral and synthetic template RNAs. It is shown that the replicase readily accepts natural RNAs as templates but is unable to efficiently synthesize RNA complementary to the synthetic ribopolymers poly(C), poly(G) and poly(U); poly(A) is able to direct the incorporation of ^{3}H-UMP, but only at a high concentration (400 μg/ml) and inefficiently with respect to CPMV RNA. Several possibilities to account for the lack of template specificity displayed by CPMV replicase and many other eukaryote replicases, are discussed. It is argued that template specificity does not have to be an intrinsic property of, and a prerequisite for, eukaryote virus RNA replicases to function properly *in vivo*, taking into account the specific location of the replication process in the cell and the occurrence of host RNA molecules as ribonucleoprotein particles. Moreover, the loss of essential protein factor(s), the possible requirement for primer(s) and the use of non-

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specific reaction conditions are considered.

Initial studies have been carried out on the binding of CPMV replicase to ^{32}P-CPMV RNA and, in addition on the size and nature of the *in vitro* synthesized RNA products. The binding experiments using a nitrocellulose filter technique to detect RNA-protein complexes, demonstrate that the DEAE-purified replicase, but not a corresponding protein preparation isolated from healthy leaves, binds to CPMV RNA. This binding can be abolished by synthetic poly(A) and poly(U) but not by poly(C), suggesting that the poly(A) on the CPMV RNA genome comprises a potential part of the replicase binding site. However, further experiments are needed to substantiate this hypothesis.

The bulk of the *in vitro* synthesized RNA was found to consist of 16S RNA and a rather small amount of faster sedimenting RNA (20S-38S), the latter representing single-stranded RNA molecules still attached to their parental template strand. Although about 60% of the RNA products appears to be sensitive to treatment with RNase A plus RNase T_1, no free, full-length size virus RNA molecules were formed, due to the presence of RNase(s) contaminating the replicase preparation.

In chapter 6 we show that the DEAE-purified replicase can be purified further by glycerol gradient centrifugation. This step affords the removal of some proteins predominating in all earlier stages. A final purification of about 150-200 fold relative to the crude extract is achieved. From analysis by polyacrylamide gel electrophoresis of the replicase purified by glycerol gradient centrifugation and of a corresponding protein preparation from mock-infected leaves, we conclude that the replicase still needs additional purification steps to allow its identification. However, the stability of the enzyme seems to offer good prospects to achieve this aim.

SAMENVATTING

In tegenstelling tot de zeer gedetailleerde kennis van het replicatiemechanisme van RNA bacteriofagen en met name van het enzym dat verantwoordelijk is voor de replicatie van de bacteriofaag Qβ, is van de replicatie van eukaryotische virussen met een enkelstrengs RNA genoom van het (+) type nog weinig bekend. Tot op heden is men er niet in geslaagd om een eukaryotisch virus RNA replicase te zuiveren en daarvan de structuur en het werkingsmechanisme op te helderen.

Het in dit proefschrift beschreven onderzoek had tot doel de isolatie en karakterisering van Cowpea Mosaic Virus (CPMV) RNA replicase, een RNA-afhankelijk RNA polymerase dat na infectie van *Vigna unguiculata* planten met CPMV gevormd wordt en zeer waarschijnlijk gecodeerd wordt door en verantwoordelijk is voor de replicatie van het virus genoom.

In hoofdstuk 1 wordt nader ingegaan op het belang van het onderzoek en op de motieven die eraan ten grondslag liggen.

In hoofdstuk 2 wordt een literatuur overzicht gegeven van de RNA replicases van virussen met een enkelstrengs RNA genoom van het (+) type. Eerst worden de algemene eigenschappen en de structuur van Qβ replicase beschreven waarbij speciale aandacht wordt besteed aan de rol die de afzonderlijke "subunits" spelen bij de verschillende stappen van de virus RNA synthese. Daarna wordt een overzicht gegeven van de isolatie en eigenschappen van dier- en plantevirus RNA replicases.

Hoofdstuk 3 beschrijft de eerste fase van het onderzoek. Daarin was het doel een RNA-afhankelijke RNA polymerase activiteit, aangeduid als replicase, op te sporen, die aanwezig moest zijn in CPMV-geïnfecteerde *Vigna* bladeren maar niet in ongeïnfecteerde bladeren. De resultaten laten zien dat een dergelijke enzymactiviteit verschijnt op de eerste dag na infectie in een celfractie die sedimenteert bij 31.000 xg. Daarna neemt de activiteit toe en bereikt haar maximum op de vierde dag. De replicase activiteit is afhankelijk van Mg^{2+}-ionen en alle vier de ribonucleoside trifosfaten en wordt niet geremd door actinomycine D en DNase. De *in vitro* RNA synthese verloopt met een constante snelheid gedurende ongeveer 20 à 30 minuten maar stopt daarna vrij snel. Analyse van het *in vitro*

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gesynthetiseerde RNA door middel van sucrose gradient centrifugatie en behande-
ling met RNases in hoog en laag zout toonde aan, dat ongeveer 70% bestaat uit
dubbelstrengs RNA, waarvan het grootste gedeelte een sedimentatiesnelheid heeft
van ongeveer 17 S en 20 S. De produkten representeren waarschijnlijk de repli-
catieve vormen van respectievelijk CPMV M-RNA en B-RNA. Het *in vitro* gesyntheti-
seerde enkelstrengs RNA bleek dezelfde sedimentatiesnelheid te bezitten als de
beide virion RNAs, nl. 26 S en 34 S. Uit deze resultaten werd geconcludeerd dat
we een membraan-gebonden replicase complex in handen hadden dat *in vivo* verant-
woordelijk is voor de replicatie van CPMV.

Voor de verdere zuivering was het echter noodzakelijk om het replicase los te
maken uit de membranen. Deze fase van het onderzoek wordt beschreven in hoofd-
stuk 4. In eerste instantie is nagegaan of met de toen gangbare methoden voor
het oplosbaar maken van membraan-gebonden replicases, nl. met behulp van (non)-
ionogene detergentia en/of hoogzout, goede resultaten verkregen konden worden
voor CPMV replicase. Hoewel het inderdaad mogelijk bleek om onder bepaalde con-
dities CPMV replicase vrij te maken uit de membranen met behulp van een detergens
behandeling, was de labiliteit van het oplosbaar gemaakte enzym een groot nadeel.
In hoofdstuk 4 wordt echter aangetoond dat CPMV replicase zeer eenvoudig losge-
weekt kan worden uit de membranen door deze te onderwerpen aan een wasprocedure
met een Mg^{2+}-deficiënte buffer. Deze methode biedt verschillende voordelen ten
opzichte van het gebruik van detergentia. In de eerste plaats is het oplosbaar
gemaakte replicase zeer stabiel; het kan dagenlang bij $0-4^{o}C$ bewaard worden
zonder verlies van activiteit. In de tweede plaats gaat het losweken van het
replicase vrij selectief; het grootste gedeelte van de eiwitten blijft achter
in het membraanpellet en de specifieke activiteit van het oplosbaar gemaakte
enzym is 2 à 3 maal hoger dan die van het membraan-gebonden enzym. In de derde
plaats wordt meer dan 80% van de replicase activiteit oplosbaar gemaakt. Verdere
zuivering van het oplosbaar gemaakte replicase werd bereikt door DEAE-BioGel
kolom chromatografie. Deze stap bleek tevens zeer geschikt te zijn om nucleïne-
zuren die als verontreiniging in het oplosbaar gemaakte enzympreparaat aanwezig
zijn te verwijderen en daardoor een matrijs-afhankelijk replicase te verkrijgen.
Na deze kolom chromatografie stap is het replicase in staat om gedurende ten-
minste 9 uur RNA te synthetiseren met een constante snelheid. De matrijs-afhanke-
lijkheid en de grote stabiliteit openden zo de mogelijkheid voor een bestudering
van de matrijsspecificiteit van CPMV replicase.

In hoofdstuk 5 worden eerst de condities beschreven die optimaal zijn voor de
incorporatie van ^{3}H-UMP. *In vitro* RNA synthese blijkt tenminste 15 uur te ver-

lopen onder de volgende condities: 8 mM Mg-acetaat of 12 mM $MgCl_2$; 60 mM $(NH_4)_2SO_4$; tot 100 mM K-acetaat, een zo laag mogelijke KCl concentratie; pH 8.2; 30 tot 34°C; en 5 tot 10 µg CPMV RNA per 15 µg eiwit. Onder deze optimale condities is vervolgens de matrijsspecificiteit onderzocht. De experimenten tonen aan dat CPMV replicase een grote verscheidenheid aan virus en niet-virus RNAs als matrijs accepteert. Daarentegen zijn de synthetische ribopolymeren poly(C), poly(G) en poly(U) niet of nauwelijks actief als matrijs; poly(A) fungeerde alleen bij hoge concentraties (400 µg/ml) en ten opzichte van CPMV RNA inefficiënt. Ter verklaring van het ontbreken van matrijsspecificiteit van CPMV replicase, en trouwens van vele andere eukaryotische virus replicases, worden een aantal mogelijkheden ter discussie gesteld. In de eerste plaats behoeft matrijsspecificiteit geen intrinsieke eigenschap van, noch een noodzakelijke voorwaarde voor een eukaryotisch RNA replicase te zijn om *in vivo* optimaal te functioneren, als we de specifieke locatie van het replicatieproces in de cel in aanmerking nemen en het feit dat de RNA moleculen van de gastheer vrijwel altijd als ribonucleoproteïne deeltjes voorkomen. Als andere mogelijke verklaringen voor het ontbreken van matrijsspecificiteit worden geopperd het verlies van essentiële eiwitfactoren, het gebruik van aspecifieke reactiecondities en een "primer"-afhankelijke replicatie.

In hoofdstuk 5 worden een aantal voorlopige resultaten gepresenteerd over de binding van het replicase aan ^{32}P-gemerkt CPMV RNA en over de grootte en aard van de *in vitro* gesynthetiseerde produkten. De bindingsexperimenten, waarbij gebruik is gemaakt van nitrocellulose membraanfilters om RNA-eiwitcomplexen te isoleren, tonen aan dat het DEAE-gezuiverde replicase, maar niet een corresponderend eiwitpreparaat uit gezonde bladeren, in staat is om ^{32}P-gemerkt CPMV RNA te binden. Deze binding kan voorkomen worden door toevoeging van poly(A) of poly(U) maar nauwelijks door poly(C). Het merendeel van het *in vitro* gesynthetiseeerde RNA blijkt te bestaan uit 16 S RNA en voor ongeveer 25-30% uit sneller sedimenterend (20S-38S) RNA. Aangezien deze laatste klasse van RNA moleculen verdwijnt na een voorbehandeling met RNases, zijn dit waarschijnlijk nascente RNA ketens die nog geassocieerd zijn met het matrijs RNA in de vorm van een replicatieve intermediair.

De verdere zuivering en karakterisering van het replicase door middel van respectievelijk glycerol gradient centrifugatie en SDS-polyacrylamide gelelektroforese vormt het onderwerp van hoofdstuk 6. Tevens wordt daarin een methode beschreven om het CPMV replicase na glycerol gradient centrifugatie verder te zuiveren met behulp van Cibacron Blue F3GA-Sephadex chromatografie.

Uit de gelelektroforese patronen van het replicase tijdens verschillende fasen van haar zuivering en van de corresponderende fracties van niet-geïnfecteerde bladeren kan geconcludeerd worden dat extra zuiveringsstappen nodig zullen zijn om een opheldering van de structuur van CPMV replicase mogelijk te maken. Niettemin is nu reeds met het in dit proefschrift beschreven replicase preparaat een gedetailleerde bestudering van het replicatiemechanisme van CPMV RNA *in vitro* mogelijk geworden.

CURRICULUM VITAE

Pim Zabel is als Willem Jan Tony Zabel geboren op 29 april 1947 te Amsterdam.
In 1965 behaalde hij het gymnasium-β diploma aan het Christelijk Lyceum West
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