RNA-dependent RNA polymerases from cowpea mosaic virus-infected cowpea leaves

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Printed by Modern, Bennekom

Aan mijn ouders

voor Ans en Rie

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LAMBERT DORSSERS

RNA-dependent RNA polymerases from cowpea mosaic virus-infected cowpea leaves

Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
hoogleraar in de veeteeltwetenschap,
in het openbaar te verdedigen
op woensdag 5 oktober 1983
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen.

STELLINGEN

1. Association of genes with the nuclear matrix for expression is very unlikely, since it would require enormous DNA rearrangements during cellular differentiation processes.

   Robinson *et al.* (1982), Cell 28, 99-106.

2. The observed 3.3kb Sst I restriction fragment of the integrated metallothionein-growth hormone fusion gene can more easily be explained by insertion of tandem copies in the mouse genome rather than by circularization of the gene.

   Palmiter *et al.* (1982), Nature 300, 611-615.

3. Purification and characterization of molecules regulating growth and development of cells *in vitro*, requires concomittant development of culture systems devoid of unknown variables.

   Bazill *et al.* (1983), Biochem.J. 210, 747-759.
   Burgess *et al.* (1982), Blood 60, 1219-1223.
   Whetton and Dexter (1983), Nature 303, 629-631.

4. The proposed connection between acquired immune deficiency syndrome (AIDS) and human T-cell leukemia virus (HTLV) is highly speculative.

   Essex *et al.* (1983), Science 220, 859-862.
   Gelmann *et al.* (1983), Science 220, 862-865.
   Gallo *et al.* (1983), Science 220, 865-867.
   Barré-Sinoussi *et al.* (1983), Science 220, 868-871.

5. Het overbrengen van het departement van Milieuhygiëne van Volksgezondheid naar Ruimtelijke Ordening gaat voorbij aan de samenhang tussen milieu en volksgezondheid en komt een "gezond" milieu beleid niet ten goede.

6. Adequate verwerking van chemisch afval is te belangrijk voor mens en milieu om aan het particuliere initiatief over te laten.

7. Het opnemen van wetenschappelijke (hoofd-)medewerkers in de promotie-kommissie, zal het nivo van de diskussie tijdens de promotie gunstig beïnvloeden.

8. Bij het voorzien in vakatures is het zonder meer geven van een voorkeursbehandeling aan met ontslag bedreigde ambtenaren strijdig, zowel naar letter als geest, met verdragen en wet en dient de overheid zich hiervan te onthouden.

   De Universele verklaring van de rechten van de mens. art: 1, 2^2, 7, 23^1, 28, 29^2.
   Internationaal verdrag inzake economische, sociale en culturele rechten. art: 2^2.
   Grondwet voor Koninkrijk der Nederlanden van 24 augustus 1815, S.45. art: 1.

9. De methode om uitkeringsgerechtigden, jongeren, ambtenaren en trendvolgers tot bezuinigingssluitposten van de nationale begroting te maken, heeft niets te maken met "no nonsense politiek" maar wel met asociaal handelen.

10. Omdat ieder mens er één is en niet de helft van een stel, dient het individu de hoeksteen van de samenleving te zijn.

Proefschrift van Lambert Dorssers,
RNA-dependent RNA polymerases from cowpea mosaic virus-infected cowpea leaves.
Wageningen, 5 oktober 1983.

CONTENTS

VOORWOORD

Voor de totstandkoming van dit proefschrift ben ik vele mensen dank verschuldigd.
Allereerst mijn ouders, die mij in staat gesteld hebben deze opleiding te volgen.
Ab van Kammen, die als promoter zijn stempel op dit proefschrift drukte. Dank voor de wetenschappelijke vrijheid die je me toevertrouwde, je kritische kanttekeningen ten aanzien van het onderzoek en het naar bovenhalen van de positieve resultaten.
Pim Zabel, die mij de eerste stapjes leerde in het moeizaam begaanbare replicase veld en vervolgens een belangrijk deel van de dagelijkse begeleiding voor zijn rekening nam. Zijn aandeel in de uiteindelijke versies van de verschillende publikaties is zeer groot, met name daar waar het er om ging "goed" engels in "beter" engels om te zetten.
Ine Jongen-Neven, die als analiste de eerste 2 jaren in dit onderzoek betrokken was en zorgde voor een gedegen praktische scholing.
Jos van der Meer, die als jonge analist in 1980 zijn intrede deed in de replicase groep en zich vervolgens zeer snel ontwikkelde tot een zelfstandig onderzoeker. Zijn inzet en kwaliteiten zijn terug te vinden in een aanzienlijk deel van dit proefschrift. Mijn hartelijke dank voor deze plezierige periode van samenwerking.
Robert van Gorcom, Marja Kuyvenhoven, Karel Wernars, Roeland Hanemaaijer en Sander van der Krol, die in het kader van hun doktoraalstudie betrokken waren bij dit onderzoek.
Marie-José van Neerven, voor haar onvermoeibare inzet om de geschreven teksten om te zetten in leesbaar machineschrift en voor de tot standkoming van dit proefschrift.
Ab Hoogeveen en Piet Madern, voor het teken- en fotografisch werk en hun inventieve inbreng bij het oplossen van praktische problemen.
Wil Landeweerd en Annelies Bruins, voor hun aandeel in het secretariaatswerk.
De heer Beekman en zijn collega's voor het oppotten van de Vigna bonen.
De leden van de Stralingsbeschermingsdienst van het I.T.A.L. voor het oplossen van onze radioaktieve problemen.
Alle leden van de CPMV-werkgroep, Henk Franssen, Rob Goldbach, Geertje Rezelman, Peter Rottier, Eugene Stuik, Jan Verver, Pieter Vos, Peter van Wezenbeek voor hun konstruktieve inbreng (in woord en daad) in dit onderzoek.
Alle leden van de vakgroep Moleculaire Biologie voor de uitstekende sfeer,

ondanks het gemopper over de klompen, die deze periode onvergetelijk maakt. Dit onderzoek werd verricht onder auspiciën van de Stichting voor Scheikundig Onderzoek in Nederland met financiële steun van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek.

Lambert Dorssers

LIST OF ABBREVIATIONS

ABM	aminobenzyloxymethyl
AlMV	alfalfa mosaic virus
ALPA	antibody-linked polymerase assay
AMP	adenosine 5'-monophosphate
APT	2-aminophenylthioether
ASV	avocado sunblotch viroid
ATP	adenosine 5'-triphosphate
BBV	black beetle virus
BMV	brome mosaic virus
BSA	bovine serum albumin
CCMV	cowpea chlorotic mottle virus
Ci	Curie
CMP	cytidine 5'-monophosphate
CMV	cucumber mosaic virus
cpm	counts per minute
CPMV	cowpea mosaic virus
CPSMV	cowpea severe mosaic virus
CTAB	cetyltrimethylammonium bromide
CTP	cytidine 5'-triphosphate
DBM	diazobenzyloxymethyl
DEAE-	diethylaminoethyl-
DNA	deoxyribonucleic acid
DPT	diazophenylthioether
DTE	dithioerythritol
EDTA	ethylenediaminetetra acetic acid
EMC	encephalomyocarditis virus
FMDV	foot-and- mouth disease virus
g	accelaration of gravity
GTP	guanosine 5'-triphosphate
K	kilo
M	molar
mol.wt.	molecular weight
mRNA	messenger ribonucleic acid
OAc	acetate
oligo(U)	oligoribouridylic acid
PMSF	phenylmethylsulphonyl fluoride
poly(A)	polyriboadenylic acid

poly(C)	polyribocytidylic acid
poly(U)	polyribouridylic acid
PSTV	potato spindle tuber viroid
RaMv	radish mosaic virus
RdRp	RNA-dependent RNA polymerase
RF	replicative form
RI	replicative intermediate
RNA	ribonucleic acid
RNase	ribonuclease
S	Svedberg (unit of sedimentation)
SBMV	southern bean mosaic virus
SDS	sodium dodecyl sulphate
SFV	semliki forest virus
SNMV	solanum nodiflorum mottle virus
SON	stichting voor scheikundig onderzoek in Nederland
SqMV	squash mosaic virus
SV	sindbis virus
TBRV	tomato black ring virus
TCA	trichloroacetic acid
TMV	tobacco mosaic virus
TNV	tobacco necrosis virus
TobRV	tobacco ringspot virus
Tris	Tris(hydroxyl)aminomethane
TRosV	turnip rosette virus
ts	temparature-sensitive
UMP	uridine 5'-monophosphate
UTP	uridine 5'-triphosphate
v	volume
VPg	viral protein genome-linked
VTMoV	velvet tobacco mottle virus
w	weight
Z.W.O.	Nederlandse organisatie voor zuiver wetenschappelijk onderzoek

I. SCOPE OF THE INVESTIGATIONS

A variety of plant species contains an RNA-dependent RNA polymerase, the activity of which is greatly enhanced upon virus infection. Since attempts to purify plant viral RNA replicases have been notoriously unsuccessfull up to the present time, this remarkable increase has led many investigators to believe that a host-encoded RNA-dependent RNA polymerase is involved in plant viral RNA replication.

The research described in this thesis deals with the replication of the plant virus cowpea mosaic virus (CPMV) in its natural host *Vigna unguiculata* (cowpea). Since cowpea leaves contain a host-encoded RNA-dependent RNA polymerase, which increases at least twenty-fold upon infection with CPMV, a possible role of the host-encoded enzyme in CPMV RNA replication had seriously to be considered. In this thesis we report how we have succeeded in demonstrating that this host-encoded enzyme is not involved in CPMV RNA replication. Instead we present evidence for a virus-encoded protein as the core polymerase of the CPMV RNA replicase.

In chapter II we review the current knowledge on the replication mechanisms of animal and plant viruses with a single-stranded (+)-type RNA genome.

In chapter III we describe the extensive purification of the host-encoded RNA-dependent RNA polymerase from cowpea leaves. Using an antiserum raised against the purified host enzyme we developed (chapter IV) a new antibody-linked polymerase assay (ALPA), which enabled us to identify the host-encoded RNA-dependent RNA polymerase as a 130,000 (130K) dalton protein.

Chapter V describes how two functionally different RNA-dependent RNA polymerase activities can be distinguished in CPMV-infected cowpea leaves on basis of the RNA products made by the respective enzymes. One RNA-dependent RNA polymerase represents the host-encoded enzyme, the other RNA polymerase is associated with the CPMV RNA replication complex. It appeared possible to isolate and purify functionally intact CPMV RNA replication complex devoid of host-encoded RNA-dependent RNA polymerase activity.
Using the antiserum against the host-encoded 130K RNA-dependent RNA polymerase, it is shown in chapter VI that this 130K host-specific enzyme does not occur in a functionally active CPMV RNA replication complex. It is further shown that the amount of host-encoded enzyme is strongly increased in cowpea leaves upon infection with CPMV, but no increase of host-encoded RNA-dependent RNA polymerase is found in isolated mesophyll protoplasts after infection with CPMV. These results lead to the conclusion that the host-en-

coded RNA-dependent RNA polymerase is not involved in CPMV RNA synthesis.

Finally, in chapter VII, we describe our attempts to identify the RNA polymerase in the CPMV RNA replication complex. Using antibodies against CPMV-specific proteins, we show that the major polypeptide associated with a highly purified replication complex is a virus-encoded protein with a molecular weight of 110,000 (110K) dalton. It appears plausible that this 110K virus-encoded polypeptide is the core polymerase engaged in synthesizing CPMV RNAs in the viral RNA replication complex.

The work described in chapters III and V has been published by "Virology". Chapter IV has appeared in EMBO Journal. Chapters VI and VII are to be submitted for publication. Therefore, some overlap in the Introduction sections is inevitable.

In this thesis, the paraphrase "RNA polymerase" is ofter used for the complete description "RNA-dependent RNA polymerase".

II. REPLICATION STRATEGIES OF EUKARYOTIC RNA VIRUSES

1. INTRODUCTION

Replication of a single-stranded (+)-type virus RNA genome proceeds through the formation of a complementary (-)-strand to be used as template for the synthesis of progeny viral RNAs. Such a replication mechanism requires an RNA-dependent RNA polymerase, termed an RNA replicase by Spiegelman and Hayashi (1963), for the transcription of both the (+)-strand and (-)-strand RNA into a polyribonucleotide chain. Initiation (i.e. the specific recognition of the RNA template) should precede the elongation step and take place on both (+)-strand and (-)-strand RNAs, which possess different 3'-termini. The complexity of this replication mechanism suggests that the RNA-dependent RNA polymerase requires other polypeptides for assistance. In view of the limited genetic information available on the viral genome, host-cell proteins are likely to participate in viral replication. Indeed, the bacteriophage Qβ replicase turned out to be composed of a virus-encoded core polymerase and three host-cell proteins, which are all required for Qβ RNA replication *in vitro* (see below).

Replication of eukaryotic RNA viruses resembles in broad outline the replication mechanism of RNA phages, but is only partly established yet. Animal RNA viruses also encode the core polymerase and depend on host proteins for transcription activity *in vitro*. With respect to plant virus replication, confusion appears to prevail. Plants contain an endogenous host-encoded RNA-dependent RNA polymerase, which is strongly stimulated upon virus infection. Although it has been suggested that the host enzyme might participate in viral RNA replication, the identity of the plant virus RNA replicase is still unknown.

In this chapter, I shall briefly review the replication mechanism of RNA phages (section 2) and then discuss in more detail the replication of eukaryotic viruses containing a single-stranded (+)-type RNA genome. The discussion of animal RNA viruses will be limited to the picornaviruses, togaviruses and nodaviruses (section 3). These (+)-type animal RNA viruses have structural features in common with some plant viruses and their replication might, therefore, have some analogy with plant virus RNA replication. Different plant viruses, of which the mechanism of replication is under study, are dealt with in section 4. Studies on the replication of plant viruses have been hampered by the occurrence of host-encoded RNA-dependent RNA polymerases. The possible role of these host-enzymes in plant virus replication are reviewed in section 5. The replication strategy of viroids, viroid-like

RNAs (virusoids) and RNA satellites is also included in this summary. Finally, differences and analogies in the replication mechanism of the various eukaryotic RNA pathogens will be discussed in section 7.

2. REPLICATION STRATEGY OF BACTERIAL RNA VIRUSES.

The replication mechanism of RNA phages, bacteriophage Qβ in particular, has been studied since 1963 and is now well established. The results of these studies have been reviewed by several authors (Kamen, 1975; Zabel, 1978; Blumenthal and Carmichael, 1979; Fiers, 1979) and only a recapitulation will be presented here.

Qβ replicase is composed of four subunits, referred to as I to IV, of which subunit II (65K) is virus-encoded and the other three subunits are host-encoded proteins. Subunit I is the ribosomal protein S1 (70K) and subunits III and IV are the protein synthesis elongation factors EF-Tu (45K) and EF-Ts (35K), respectively. The virus-encoded subunit II is an RNA-dependent RNA polymerase, which constitutes the core polymerase. The other three subunits are required for specific recognition of the viral template RNA and for initiation of transcription. Transcription of (+)-strand viral RNA requires all four subunits and, in addition, a host factor. This host factor consists of a hexamer of identical (12K) polypeptides, the authentic function of which in the host cell is yet unknown. Subunit I and the host factor are not required for the transcription of (-)-strands into progeny (+)-strand viral RNAs.

Transcription starts at the 3'-terminus of both (+)-strand and (-)-strand templates, but specific binding sites for Qβ replicase have not been found in the 3'-terminal region on either strand. Two internal domains of Qβ (+)-strand RNA interact strongly with the replicase to bring the 3'-terminus of the template in the proper position for initiation (Meyer *et al.*, 1981). Subunit II is also involved in specific template selection besides its function as core polymerase. Replicases of related phages, such as MS2, contain identical host-encoded subunits, but display a template-specificity differing from that of Qβ replicase. *In vitro* transcription of heterologous RNAs is possible if a template-primer complex is used and transcription can proceed from the primer sequence (Feix and Hake, 1975).

3. ANIMAL RNA VIRUSES.

The animal (+)-type RNA viruses of which the replication strategy is explored most intensively are the picornaviruses, togaviruses and nodaviruses.

These (+)-type animal RNA viruses resemble structurally some plant RNA viruses and the replication of the RNA of these viruses may be comparable with the plant virus RNA replication. The picornaviruses and togaviruses have a single-stranded RNA genome, whereas for nodaviruses the genetic information for virus multiplication has shown to be distributed among two single-stranded RNA molecules.

3.1. PICORNAVIRUSES.

All picornaviruses contain a single-stranded (+)-type RNA genome, approximately 7500 nucleotides in length. The picornavirus family includes four genera, the enteroviruses (poliovirus), cardioviruses (EMC, Mengo), rhinoviruses and aphtoviruses (FMDV). The genomic RNA is encapsidated in a protein shell, built up of 60 copies of each of four capsid proteins. The nucleoprotein particle is 25-30 nm in diameter, possesses icosahedral symmetry and is non-enveloped.

A common feature of picornaviruses is the occurrence of a small protein, referred to as VPg (viral protein genome-linked), covalently linked to the 5'-end of the viral RNA. The viral RNA is translated into a large polyprotein, which is processed into functional viral proteins by specific proteolytic cleavages. The protease involved in these cleavages is encoded by the viral RNA. Replication of picornavirus RNA is associated with cytoplasmic membranes. The mechanism of viral RNA replication is not yet fully understood but involves a viral-encoded core polymerase and possibly a host factor (Rueckert, 1971; Felner, 1979; Matthews, 1979; Putnak and Phillips, 1981; Wimmer, 1982).

3.1.1. POLIOVIRUS

3.1.1.1. *Genome structure and expression.*

The complete nucleotide sequence of the RNA of poliovirus type 1 has recently been determined (Mahoney strain: Kitamura *et al.*, 1981; Racaniello and Baltimore, 1981; Sabin strain: Nomoto *et al.*, 1982). The RNA genome consists of a strand of 7441 nucleotides (Mahoney strain, Dorner *et al.*, 1982) and, in addition, a poly(A)-track at the 3'-end (Armstrong *et al.*, 1972). A small protein (VPg) is covalently linked to the 5'-terminal UMP residue (Lee *et al.*, 1977; Flanegan *et al.*, 1977; Nomoto *et al.*, 1977). VPg is not required for infectivity of poliovirus RNA (Flanegan *et al.*, 1977) nor essential for translation, as it is missing in poliovirus mRNA as isolated from polysomes (Nomoto *et al.*, 1976; Hewlett *et al.*, 1976; Fernandez-Munoz and Darnell, 1976; Nomoto *et al.*, 1977).

Poliovirus proteins are synthesized from a single reading frame (Fig. 1),

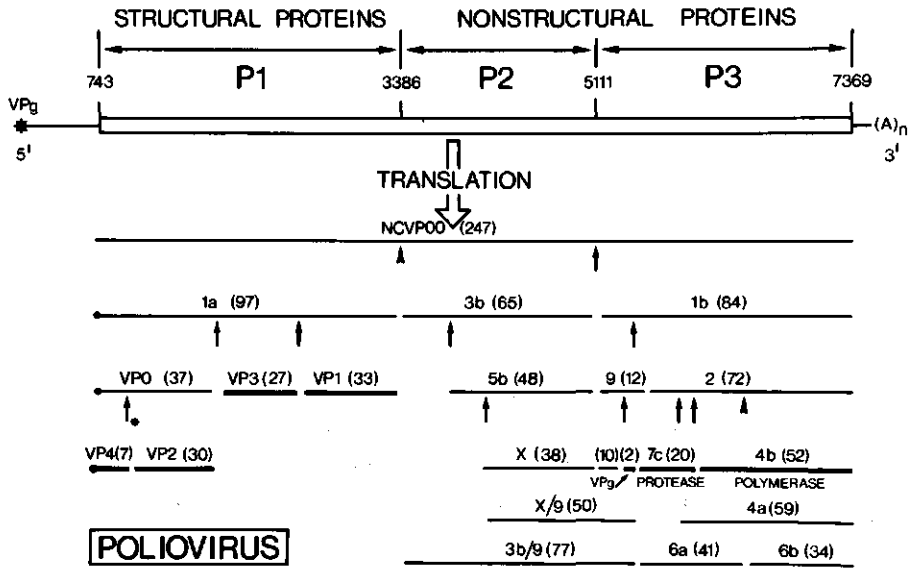

Fig. 1. Translation and processing map of poliovirus.
The coding region of the RNA is indicated by an open bar. * = genome-linked protein, VPg; $(A)_n$ = 3'-terminal poly(A)-tail; ● = blocked NH_2-terminus; ↑ cleavage site at gln/gly amino acid pairs; ↑* = cleavage site at asn/ser pair; ▲ = cleavage site at tyr/gly pair; VP = viral protein; NCVP = non-capsid viral protein. Molecular weights ($x10^{-3}$) of the polypeptides are denoted between brackets. Polypeptides involved in a known function are shown in dense lines. Polypeptides arising from alternative cleavages in the P3 region (Hanecack *et al.*, 1982) have been omitted. References and other abbreviations are included in the text.

starting at an AUG codon at position 743 and terminating at position 7369, close to the poly(A)-tail. The initiation codon at position 743 is preceeded by a long, apparently untranslated region, which harbors eight additional AUG codons. Some of these are followed by an open reading frame which may encode small proteins not yet identified *in vivo* (Dorner *et al.*, 1982). Translation of poliovirus RNA *in vivo* and *in vitro* results in a long polyprotein NCVPOO (mol.wt. 247K), which is subsequently cleaved into three polypeptides (P1-1a, P2-3b and P3-1b), corresponding to the three domains P1, P2 and P3 of the viral RNA (Fig. 1, Kitamura *et al.*, 1981).

The 5'-terminal part of the genome (P1) encodes the structural proteins. P1-1a is the precursor of the four capsid proteins VP1-4 (Dorner *et al.*, 1982). P2-3b represents the central part of the coding region and is cleaved to generate P2-5b and P2-X (Semler *et al*, 1981). No function has yet been assigned to the polypeptides originating from this region. P3 represents the 3'-terminal region of the genome (Fig. 1) and encodes several nonstructural proteins including a protease (P3-7c, Hanecak *et al.*, 1982), an RNA-dependent RNA polymerase (P3-4b, Van Dijke and Flanegan, 1980) and VPg (Kitamura

et al., 1980). VPg has not been observed in free form (Wimmer, 1982) and thus is either contained in its precursor or covalently linked to viral RNA. In addition to P3-1b, three VPg-containing polypeptides (P3-9, 3b/9 and X/9) have been identified using antisera directed against synthetic VPg (Semler *et al.*, 1982; Baron and Baltimore, 1982a; Adler *et al.*, 1983; Takegami *et al.*, 1983). P3-9 is generated from P3-1b, whereas X/9 and 3b/9 result from an alternative cleavage pathway in which P3-9 remains linked to polypeptides of the P2 region (Takegami *et al.*, 1983). The protease P3-7c is involved in generating capsid proteins, VPg, the polymerase and P3-7c itself (Fig. 1; Hanecak *et al.*, 1982). In addition, a proteolytic activity of the host cell brings about several intermediate cleavages as indicated in Fig. 1 and can also produce cleavage products such as P3-6a and 6b with yet unknown functions (Hanecak *et al.*, 1982).

3.1.1.2. *Replication*

During virus multiplication in poliovirus-infected cells (partially) double-stranded poliovirus RNAs, to be regarded as *in vivo* replication intermediates accumulate (for review: Baltimore, 1969). The full-length (+)-strands, (-)-strands (Nomoto *et al.*, 1977) as well as short nascent chains of the replication intermediates contain VPg at their 5'-termini (Pettersson *et al.*, 1978). These findings suggest that VPg may be involved in initiation of RNA synthesis (Nomoto *et al.*, 1977; Wimmer, 1982).

Replication of poliovirus RNA *in vitro* was initially studied by isolating a viral RNA replication complex (Baltimore, 1964) consisting of RNA-dependent RNA polymerase molecules bound to endogenous (-)-strand templates, and capable of elongating nascent chains. The viral replication complex is associated with cytoplasmic membranes (Girard *et al.*, 1967; Caliguiri and Tamm, 1970) and has been characterized in detail with respect to its ability to elongate nascent chains *in vitro* (Etchison and Ehrenfeld, 1981). No evidence for *de novo* initiation of RNA synthesis has been obtained. The major viral protein associated with the replication complex was found to be P3-4b (Lundquist *et al.*, 1974; Butterworth *et al.*, 1976; Flanegan and Baltimore, 1979; Ethison and Ehrenfeld, 1980) and this protein might thus represent the core polymerase responsible for elongation.

In order to gain conclusive evidence for the origin of the replicase Flanegan and Baltimore (1977) attempted an alternative approach for purifying viral replicase. They reasoned that (-)-strand synthesis might involve a poly(U)-polymerase for the transcription of the poly(A)-tail at the 3'-end of the viral RNA and that the initiation step might be bypassed by using a primer complementary to the 3'-end of poliovirus RNA. The use of a poly(A)/oligo(U) template-primer complex in the polymerase assay resulted indeed in the detection of poly(A)/oligo(U)-dependent poly(U)-polymerase activity

(hereafter referred to as poly(U)-polymerase) (Flanegan and Baltimore, 1977). This activity was associated with cytoplasmic membranes but was also found in the soluble fraction (Flanegan and Baltimore, 1979). The poly(U)-polymerase could also copy poliovirus RNA in the presence of all four ribonucleoside triphosphates and oligo(U) as a primer (referred to as primer-dependent replicase) (Dasgupta *et al.*, 1979).

P3-4b was the major viral protein found associated with purified poly(U)-polymerase activity as well as the poliovirus replication complex (Flanegan and Baltimore, 1979). P3-2, the precursor of P3-4b, occurred in minor amounts in these preparations but was totally inactive as an RNA-dependent RNA polymerase (Van Dijke and Flanegan, 1980). Furthermore, antibodies raised against either a synthetic carboxy-terminal peptide of P3-4b (Baron and Baltimore, 1982c) or polyacrylamide gel-purified P3-2 (Semler *et al.*, 1983) inhibited poly(U)-polymerase and primer-dependent replicase activity (Table 1). These results demonstrate that P3-4b is the virus-encoded core polymerase of both the poly(U)-polymerase, the primer-dependent replicase and the replication complex.

Partially purified poly(U)-polymerase was capable of transcribing poliovirus RNA in the absence of primer (primer-independent replicase) (Dasgupta *et al.*, 1979), but this ability was readily lost upon further purification without affecting the poly(U)-polymerase activity. This suggested that some protein essential for initiation had been lost and led to the identification of a host-factor. Thus, the purified poly(U)-polymerase is capable of transcribing poliovirus RNA in the presence of either oligo(U) as a primer or host factor (referred to as primer-dependent- and host factor-dependent replicase, respectively) (Dasgupta *et al.*, 1980). Antibodies raised against the host-factor inhibited primer-independent replicase and host factor-dependent replicase (Table 1). Poly(U)-polymerase activity and primer-dependent replicase activity were unaffected by this antiserum, indicating that the host factor is involved in initiation and not elongation (Dasgupta *et al.*, 1982). The host factor, which is not engaged in host-cell protein synthesis, like the host-encoded subunits of Qβ replicase, has recently been purified and identified as a monomeric 67K polypeptide (Baron and Baltimore, 1982b).

TABLE 1

ANTIBODY INHIBITION OF *IN VITRO* RNA-DEPENDENT RNA POLYMERASE ACTIVITY OF POLIOVIRUS.

Antibodies raised against:	poly(U)-polymerase	replicase, primer-independent	replicase dependent on primer	replicase dependent on host factor
P3-4b [a]	+	ND	+	+
P3-2 [b]	+	ND	ND	ND
VPg [c]	-	ND	-	+
Host factor [d]	-	+	ND	+
Assay [e]	poly(A)/oligo(U)	poliovirus RNA	poliovirus RNA/oligo(U)	poliovirus RNA, host factor

a) Baron and Baltimore, 1982c
b) Semler *et al.*, 1983
c) Baron and Baltimore, 1982d
d) Dasgupta *et al.*, 1982
e) Typical components of the assay are indicated.

+ = inhibition
- = no inhibition
ND = not determined

Viral RNA synthesis *in vitro* by the primer-dependent replicase has been studied in detail by Van Dijke *et al.* (1982) and Baron and Baltimore (1982e). In the presence of oligo(U), full-length viral (-)-strand RNA covalently linked to the primer, was produced. The primer-dependent replicase did not display template specificity. All four homopolyribonucleotides were copied when only the appropriate primer was added. Some heterologous viral RNAs containing 3' poly(A)-tails (tobacco etch virus, CPSMV and SqMV) were copied with an efficiency similar to poliovirus RNA, using oligo(U) as a primer (Tuschall *et al.*, 1982). Without primer, transcription by the primer-depen-

dent replicase did not occur. Using a less purified primer-independent replicase preparation, both BMV- and TMV-RNA were transcribed in the absence of primer. In these experiments RNAs of double-unit length were produced, arisen from self-priming at the 3'-terminus of the template and consequent joining of the newly synthesized RNA to the 3'-end of the template (Tuschall *et al.*, 1982). This self-priming could have been facilitated by the occurrence of a tRNA-like structure at the 3'-ends of both BMV-RNA and TMV-RNA. Synthesis of double-unit length RNAs has also been observed with poliovirus RNA using either partially purified, primer-independent replicase or host factor-dependent replicase (J.B. Flanegan, personal communication).

To resolve a possible role of VPg in initiation of RNA synthesis, as suggested by its occurrence on all nascent viral RNA chains *in vivo*, antibodies directed against synthetic VPg have been prepared (Baron and Baltimore, 1982d). Such anti-VPg serum was unable to inhibit the elongation reaction by either poly(U)-polymerase or primer-dependent replicase (Table 1). On the other hand, incorporation of nucleotides by host factor-dependent replicase was completely abolished by anti-VPg serum (Baron and Baltimore, 1982d), suggesting that VPg indeed is involved in initiation of viral RNA synthesis. A small part of the RNA product of the host factor-dependent replicase was immunoprecipitable with anti-VPg serum, although no VPg-related protein could be identified in the enzyme preparation used (Baron and Baltimore 1982d). The polypeptide linked to the *in vitro* synthesized RNAs appeared larger than VPg and might be a precursor of VPg (P3-9?) attached via a UMP residue to the 5'-end of a poly(U)-track. The authors suppose that probably all newly started chains contained the VPg-related protein but failed to precipitate because they occurred in complex with template RNA. This might explain the difference between the small amount of immunoprecipitable RNA product and the complete inhibition of the replicase activity by the anti-VPg serum (Baron and Baltimore, 1982d).

In summary, the replication cycle of poliovirus may involve the following steps: P3-1b, associated with cytoplasmic membranes and bound to template RNA, is processed proteolytically to generate P3-9. This VPg-precursor is uridylated and then capable of functioning as primer in viral RNA synthesis. The active polymerase (P3-4b) is generated by removal of the protease (P3-7c), possibly by an intramolecular cleaving mechanism, whereupon elongation can take place. An additional proteolytic cleavage removes the amino-terminal part of P3-9 to produce mature VPg. Such a model accounts for the observations that P3-9 is strictly membrane-bound (Semler *et al.*, 1982; Adler *et al.*, 1983; Takegami *et al.*, 1983) and covalently linked to the nascent RNA chain at the onset of RNA synthesis, and that only P3-4b is active as an RNA-dependent RNA polymerase. The first step in the initiation of RNA synthesis would then involve the linkage of a UMP residue to P3-9, analogous

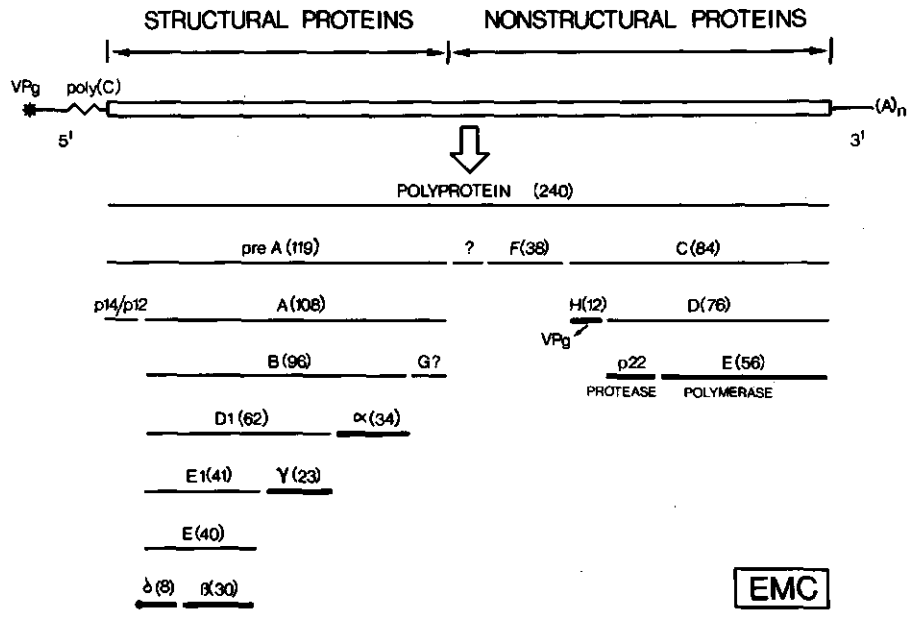

Fig. 2. Translation and processing map of encephalomyocarditis virus. ∿∿∿internal poly(C)-track; other symboles as described in Fig. 1. References are included in the text.

to the initiation step in adenovirus DNA replication (Wimmer, 1982; Lichy *et al.*, 1981; Ikeda *et al.*, 1982; Challberg *et al.*, 1982; Tamanoi *et al.*, 1982; Nagata *et al.*, 1982). *In vivo*, host factor and other polypeptides besides P3-1b (and its processing products) are probably essential for poliovirus RNA replication to provide specific selection of viral RNA templates, since *in vitro* the replicase lacks this specificity.

3.1.2. ENCEPHALOMYOCARDITIS VIRUS (EMC).

3.1.2.1. *Genome structure and expression.*

EMC RNA contains a 3'-terminal poly(A)-tail and a small protein (VPg) covalently linked to its 5'-terminus and a poly(C)-track located near the 5'-end (Sangar, 1979; Putnak and Phillips, 1981). Although poliovirus and EMC have many features in common, little nucleotide sequence homology has been found in their 3'-terminal RNA sequence (Drake *et al.*, 1982).

Translation of EMC RNA starts at an AUG codon located after the poly(C)-track and is terminated near the poly(A)-tail (Drake *et al.*, 1982). Translation results in a large polyprotein (240K), only detectable under conditions which prevent processing (Rueckert *et al.*, 1980). Both *in vivo* and *in vitro*, precursor polypeptides pre A, F and C (Fig. 2) arise by cleavage of the polyprotein. Pre A is the precursor of the four capsid proteins (α, β, γ and

δ) and contains a leader polypeptide (p12/p14) at its amino-terminal end (Kazachkov *et al.*, 1982; Campbell and Jackson, 1983). The capsid proteins are released from polypeptide A by proteolytic cleavage and can assemble *in vitro* into shell-like structures (Palmenberg, 1982). The protease (p22) responsible for generating the capsid proteins and several other viral polypeptides, is contained in polypeptide C (Fig. 2; Palmenberg *et al.*, 1979; Svitkin *et al.*, 1979) which is also the precursor of H (containing VPg), D and E (Pallansch *et al.*, 1980), to be generated by the proteolytic activity in p22 via an intramolecular cleaving mechanism (Palmenberg and Rueckert, 1982; Rueckert *et al.*, 1980).

3.1.2.2. *Replication.*

The RNA replication complex of EMC is associated with the smooth cytoplasmic membrane fracion of infected cells (Rosenberg *et al.*, 1979). In order to obtain a soluble and template-dependent enzyme, the replication complex has been solubilized from the membranes and freed of its endogenous template (Traub *et al.*, 1976; Rosenberg *et al.*, 1979). However, the partially purified replicase did not display template-specificity and its labile nature hampered further purification. The polypeptides D and E (Fig. 2) were the major viral proteins copurifying with the replicase (Rosenberg *et al.*, 1979).

Further information on the mechanism of EMC RNA replication was obtained by an alternative approach based on coupled translation/replication studies *in vitro* (Palmenberg *et al.*, 1981). EMC RNA is efficiently translated *in vitro* in reticulocyte lysates to produce considerable amounts of precursor polypeptides which are rapidly processed to mature proteins. When following translation the salt conditions were adjusted, *in vitro* transcription of endogenous EMC RNA (replicase activity) or added poly(A)/oligo(U) template-primer complex (poly(U)-polymerase activity) was observed. Poly(U)-polymerase activity was strongly stimulated by translation of EMC RNA but not BMV RNA4. Addition of Zn^{2+} to the translation mixture to inhibit processing of the EMC primary translation products did not affect the poly(U)-polymerase activity. Accumulation of pre A, A, F and C viral precursors (Fig. 2) clearly enhanced replicase activity on the endogenous EMC RNA template. These results suggest that precursor polypeptides play a role in specific initiation of EMC RNA replication. In analogy to poliovirus replication, polypeptide C (VPg-containing precursor) might thus be involved in initiation and, following proteolytic cleavage, the generated polypeptide E might function as the core polymerase in EMC RNA synthesis (Palmenberg *et al.*, 1981; Van Dijke and Flanegan, 1980; Baron and Baltimore, 1982d).

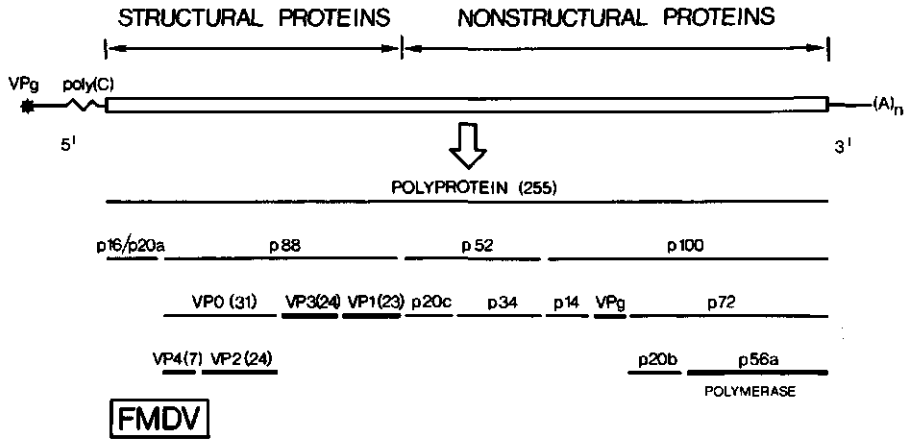

Fig. 3. Translation and processing map of foot-and-mouth disease virus. ∿∿∿ internal poly(C)-track; other symboles as described in Fig. 1. References are included in the text.

3.1.3. FOOT-AND-MOUTH DISEASE VIRUS (FMDV).

3.1.3.1. *Genome structure and expression.*

FMDV is the prototype of the aphtoviruses, which can be distinguished from the entero- and cardioviruses by their low acid stability and their high density in CsCl. Like cardioviruses, aphtoviruses contain an internal poly(C)-track located approximately 400 nucleotides from the 5'-terminal VPg (Sangar 1979; Putnak and Phillips, 1981).

FMDV RNA is translated *in vitro* into four polypeptides (p88, p52, p100 and p16/p20a), which apparently are cleaved from a nascent polyprotein (Fig. 3). A leader polypeptide (p16/p20a) precedes the precursor (p88) of the FMDV capsid proteins (VP1-4) (Lowe and Brown, 1981; Grubman and Baxt, 1982). P88 is processed into the capsid proteins (Boothroyd *et al.*, 1981, 1982; Kurz *et al.*, 1981; Küpper *et al.*, 1981) by a virus-encoded protease, probably located in the p100 region (Grubman and Baxt, 1982). Recent RNA sequencing studies have revealed, that FMDV RNA contains three closely related VPg-genes located in tandem in the p100 region (Forss and Schaller, 1982). The presence of three genes for VPg agrees with earlier observations showing heterogeneity in VPg preparations from FMDV RNA as revealed by isoelectric focussing (King *et al.*, 1980). In this respect, the aphtoviruses differ from poliovirus encoding only one VPg sequence.

3.1.3.3. *Replication.*

A cytoplasmic membrane fraction, has been shown to contain the FMDV RNA replication complex (Arlinghaus and Polatnick, 1969; Lazarus and Barzilai, 1974). After solubilization and partial purification, the major viral poly-

peptide associated with the replication complex was p56a (Newman *et al.*, 1979). Besides the membrane-bound replication complex, infected cells harbor a cytoplasmic poly(A)/oligo(U)-dependent poly(U)-polymerase (Polatnick, 1980; Polatnick and Wool, 1981; Lowe and Brown, 1981). The primer-dependent poly(U)-polymerase forms part of a 70S complex composed of p56a and four host-specified polypeptides (Polatnick and Wool, 1981). By using an alternative purification procedure, Lowe and Brown (1981) obtained a poly(U)-polymerase preparation capable of transcribing FMDV RNA in the absence of primer. Upon further purification, the ability to transcribe FMDV RNA was lost, but p56a was recovered in a monomeric form which still displayed poly(U)-polymerase activity. The precursor of p56a, p72, did not display polymerase activity *in vitro*, in analogy to the precursor polypeptide P3-2 of poliovirus (Lowe and Brown, 1981; Van Dijke and Flanegan, 1980). In addition, partially purified enzymes prepared from cells that have been infected with temperature-sensitive mutants defective in FMDV RNA synthesis and producing an aberrant p56a polypeptide, exhibited a temperature-sensitive poly(U)-polymerase activity *in vitro* (Lowe *et al.*, 1981). From the available information, it can be concluded that p56a is the viral core polymerase, the amino acid sequence of which has recently been determined (Robertson *et al.*, 1983), and that other, not yet identified proteins are involved in initiation of FMDV RNA synthesis.

3.2. NODAVIRUSES

3.2.1. *Genome structure and expression.*

The nodaviruses are the only animal viruses with a segmented (+)-type RNA genome (Friesen and Rueckert, 1981). In contrast to picornaviruses, they lack a 3' poly(A)-tail; the nature of the 5'-end has not yet been established (Clewley *et al.*, 1982). Their best studied members are nodamuravirus and black beetle virus (BBV). A single isometric virus particle of 30 nm diameter contains two single-stranded RNAs (Longworth and Carey, 1976; Newman and Brown, 1977), referred to as vRNA1 (mol.wt. 1.12×10^6) and vRNA2 (mol.wt. 0.46×10^6). Both RNAs are required for infectivity (Friesen and Rueckert, 1982) and have no base sequences in common (Clewly *et al.*, 1982). In addition to these genomic RNAs, a subgenomic mRNA (RNA3, 9S) has been detected in infected *Drosophila* cells (Friesen and Rueckert, 1982).

BBV RNAs can direct protein synthesis in cell-free translation systems to produce polypeptides of 104K (A) and 47K (α), encoded by vRNA1 and vRNA2, respectively (Fig. 4; Guarino *et al.*, 1981; Crump and Moore, 1981; Friesen and Rueckert, 1981). Polypeptide α is the precursor of the BBV coat proteins β (43K) and γ (5K). BBV-infected *Drosophila* cells contain in addition to the viral polypeptides described above, a fifth polypeptide (B, 10K) which

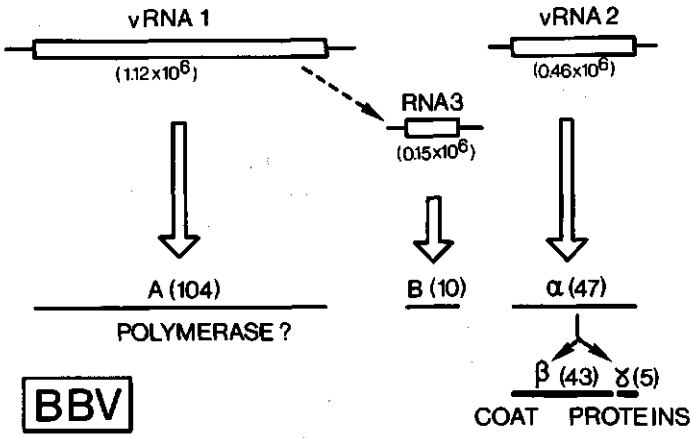

Fig. 4. Translation strategy of black beetle virus.
Molecular weights of RNAs are denoted just below the RNA molecules; other symboles as described in Fig. 1. References are included in the text.

has never been found in any *in vitro* system programmed with vRNA1 and/or vRNA2. Recently, it was shown that protein B is the translation product of the subgenomic messenger RNA3 (Friesen and Rueckert, 1982). *In vivo*, polypeptide A is only produced early in infection, although there is no significant difference between the ratios of vRNA1 and vRNA2 early and late in the infection cycle. This result suggests that production of polypeptide A is *in vivo* regulated at the level of translation. No such regulation has been observed in an *in vitro* protein synthesizing system (Friesen and Rueckert, 1982).

3.2.2. *Replication.*

Multiplication of BBV in cultured *Drosophila* cells appears to be very abundant as the amount of virus can run up to 20% of the cell mass (Friesen *et al.*, 1980). Synthesis of viral RNAs *in vivo* is not affected by actinomycin D, suggesting the involvement of an RNA-dependent RNA polymerase in BBV replication (Friesen and Rueckert, 1982). A BBV RNA replication complex (~ 120S) capable of elongating nascent chains *in vitro* has been solubilized from membranes by Mg^{2+}-depletion. The labeled RNA product was recovered in a double-stranded form and represented (-)-type BBV RNA (Guarino and Kaesberg, 1981). Recently, however, it was shown that in the presence of RNase inhibitors mainly (+)-type BBV RNA, both in single-stranded and partially double-stranded form, was synthesized by the replication complex (Linda Guarino, personal communication). The size of the replication complex (120S) suggests the presence of residual membrane structures. The replicase has not been further identified sofar.

Recently, BBV vRNA1 was shown to be capable of self-replication in

Drosophila cells, a property not associated with vRNA2 (Friesen and Rueckert, 1982; Gallagher *et al.*, 1983). Replication and expression of vRNA1 in the absence of vRNA2 is accompanied by the synthesis of large amounts of the subgenomic RNA3 and its translation product (B). These results indicate that vRNA1 carries genes involved in BBV RNA replication and that RNA3 is derived from vRNA1 (Fig. 4). A similar distribution of functions between two genomic RNAs is encountered among plant viruses containing a bipartite genome (section 4).

3.3. ALPHAVIRUSES.

The alphaviruses of which sindbis virus (SV) and semliki forest virus (SFV) have been studied in detail, belong to the togavirus family. Recently, several authors have extensively reviewed the properties of these viruses (Kääriäinen and Söderlund, 1978; Schlesinger, 1980; Strauss and Strauss, 1982) and, therefore, only the essential features of these viruses will be recapitulated here.

3.3.1. *Genome structure and expression.*

The single-stranded RNAs (42S) of SV and SFV, approximately 12,000 nucleotides in length, are packaged in capsids (~ 150S) composed of 200-300 copies of the capsid protein and the nucleocapsids are surrounded by an envelope consisting of a lipid bilayer and glycoproteins. In the infected cell, a 26S subgenomic mRNA is present in addition to the genomic RNA. Both RNAs possess a cap structure (m^7GpppN) at their 5'-termini and a 3'-terminal poly(A)-tail. The 26S subgenomic mRNA, representing the 3'-terminal one-third of the genomic RNA (Fig. 5), has recently been sequenced (Garoff *et al.*, 1980a, b; Rice and Strauss, 1981; Ou *et al.*, 1982).

The 26S mRNA is translated into a polyprotein (NVP130, Fig. 5) which is the precursor of the capsid protein and three glycoproteins (E1, E2 and E3) found in the envelope. The maturation of these structural polypeptides probably requires two virus-encoded and two host proteases. One of the viral proteases, which seems to be contained in the capsid protein might be responsible for the intramolecular cleavage in NVP130 (see Rice and Strauss, 1981).

Three stable nonstructural SV proteins (ns89, ns76 and ns60, Fig. 5) have been detected *in vivo* (Kääriäinen and Söderlund, 1978) and are also found after *in vitro* translation of SV 42S RNA (Collins *et al.*, 1982). These polypeptides appear to be derived from three precursors (p250, p205 and p145), which share the same amino-terminus (Collins *et al.*, 1982). However, temperature-sensitive (ts) mutants have been divided into four complementation groups (Strauss and Strauss, 1980), indicating the presence of a fourth

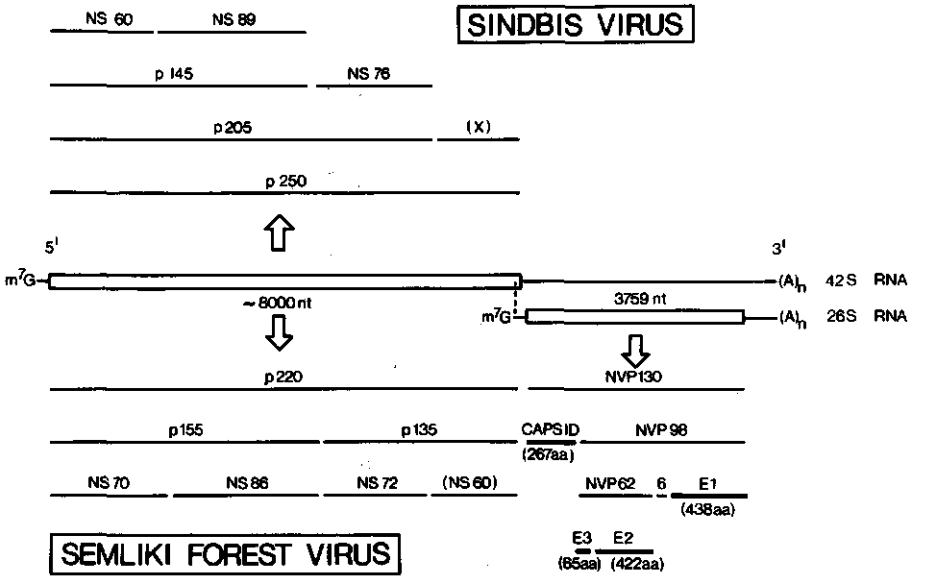

Fig. 5. Translation and processing map of sindbis virus (SV) and semliki forest virus (SFV).
The structural proteins of SV have been omitted, since they are very similar to the SFV structural proteins. nt= number of nucleotides; m^7G = 5'-terminal cap structure; aa = number of amino acids; other symboles as in Fig. 1. References and abbreviations are included in the text.

stable protein (X) which might be located in the carboxy-terminus of polypeptide p250 (Fig. 5). A similar processing map has been obtained for SFV nonstructural proteins (Fig. 5; Schlesinger and Kääriäinen, 1980).

3.3.2. *Replication.*

Actinomycin D-resistent RNA polymerase activity has been demonstrated in subcellular fractions derived from alphavirus-infected cells. This RNA polymerase activity was associated with a viral RNA replication complex bound to cellular membranes. The viral RNA replication complex was capable of producing single-stranded viral RNAs *in vitro* (reviewed by Kääriäinen and Söderlund, 1978). Further purification of the replication complex caused the immediate loss of the ability to synthesize single-stranded RNAs, suggesting that essential factors for the release of single-stranded RNAs from the replication complex were removed (Clewly and Kennedy, 1976; Gomatos *et al.*, 1980). The SFV replication complex purified by affinity chromatography contained two viral polypeptides, probably corresponding to ns70 and ns86 (Clewly and Kennedy, 1976). Of these, only polypeptide ns70 was found associated with the SFV replication complex prepared by an alternative procedure (Ranki and Kääriäinen, 1979; Gomatos *et al.*, 1980). These

results suggest that polypeptide ns70 might be responsible for polymerase activity in the SFV RNA replication complex.

The involvement of three other viral polypeptides in alphavirus RNA replication is suggested by the fact, that ts-mutants of SV defective in viral RNA synthesis can be divided into four complementation groups, A, B, F and G. One mutant of complementation group B failed to produce (-)-strand RNA at the nonpermissive temperature (Sawicki *et al.*, 1981a), whereas another mutant of group A was unable to turn off (-)-strand sythesis, normally occurring 3-4 hr after infection (Sawicki and Sawicki, 1980; Sawicki *et al.*, 1981b). Another ts-mutant, belonging to complementation group F, was defective in synthesizing both (+)-strand and (-)-strand viral RNAs at the nonpermissive temperature (Sawicki *et al.*, 1981a). These results obtained with ts-mutants of SV indicate that group F may represent the core polymerase, whereas the product of group B seems to be involved in specific initiation of (-)-strand RNA synthesis. Complementation group A could be required for regulation and termination of (-)-strand synthesis. Synthesis of subgenomic RNA appears to be controlled by both complementation groups A and G (Strauss and Strauss, 1982).

In summary, alphavirus replication may involve four viral polypeptides required for viral RNA polymerase activity, specific initiation of (+)- and (-)-strand and subgenomic RNA synthesis, and regulation of the synthesis of the three viral RNA species, respectively.

4. PLANT RNA VIRUSES

Among the plant viruses containing a single-stranded (+)-type RNA genome are viruses with a monopartite genome, like TMV and TYMV, a bipartite genome, like CPMV and TBRV, and a tripartite genome, like BMV, CMV and AlMV. Monopartite viruses carry the information for virus multiplication contained within a single RNA molecule, whereas with bipartite- and tripartite genome viruses this information is divided among two and three single-stranded RNA molecules, respectively. With bipartite- and tripartite genome viruses, the different pieces of the genome are separately encapsidated.

A variety of structures are found at the 5'- and 3'-termini of plant viral RNAs. With respect to the 3'-terminus, a poly(A)-tail (CPMV, TBRV), or a tRNA-like structure which can be aminoacylated *in vitro* (TMV, TYMV, BMV and CMV) has been found. Some viral RNAs have neither a poly(A)-tail nor a tRNA-like structure at their 3'-end (AlMV). The 5'-ends of the genomic RNAs of a variety of viruses (TMV, TYMV, BMV, CMV and AlMV) carry a cap (= m^7GpppN similar to the structure found on eukaryotic mRNAs, but the genomic RNAs of other viruses contain a protein (VPg) covalently linked to the 5'-end (CPMV, TBRV and SBMV).

Two strategies for the expression of the genetic information encoded on the genome RNAs are to be distinguished: (i) Each genomic RNA is used as mRNA to direct the synthesis of one type of viral protein, even if the genomic RNA carries more than one gene. The expression of a second (or third) gene on the RNA molecule is regulated *in vivo* by the generation of a subgenomic mRNA, which only contains a part of the nucleotide sequence of the genomic RNA. This is found for example for TMV, BMV, CMV and AlMV. (ii) The genome RNAs are translated into high molecular weight, so-called, polyproteins, which comprise the complete coding information of the RNAs and are subsequently processed into smaller sized, presumably functional proteins. This mechanism has been described for CPMV and TBRV. A combination of these two strategies appears to be used by TYMV and TRosV.

In the following, plant viruses will be discussed for which RNA replication has been studied in some detail. It should be kept in mind, however, that uninfected plants contain RNA-dependent RNA polymerases, the activity of which is greatly enhanced upon virus infection. In several cases, this has led to confusing results. The RNA-dependent RNA polymerase from (healthy) plants will be considered in a separate section.

4.1. TOBACCO MOSAIC VIRUS (TMV)

4.1.1. *Genome structure and expression.*

TMV is the type member of tobamo viruses carrying a monopartite single-stranded RNA genome packaged in rod shaped particles with a length of about 300 nm (for review see Shikata, 1977). The viral RNA is capped at its 5'-terminal end, whereas the 3'-terminal sequence of the RNA can fold into a tRNA-like structure, to be aminoacylated *in vitro* with histidine and valine in the case of the common strain and the cowpea strain of TMV, respectively (Haenni *et al.*, 1982). TMV RNA has recently been completely sequenced and shown to consist of a strand of 6395 nucleotides (Goelet *et al.*, 1982). The 5'-proximal half of the TMV RNA genome encodes a 126K polypeptide, which is occasionally extended to a 183K polypeptide by read-through of a leaky amber termination codon (Fig. 6; Goelet *et al.*, 1982). Two other cistrons in TMV RNA encoding a 30K polypeptide, possibly involved in cell to cell spreading of the virus (Leonard and Zaitlin, 1982), and the 17.5K coat protein, respectively, are expressed by the generation of subgenomic mRNAs (Fig. 6) (Goelet and Karn, 1982; Hunter *et al.*, 1983).

4.1.2. *Replication.*

Replication of TMV RNA appears to be associated with membrane structures in TMV-infected tobacco leaves. The particulate fraction of TMV-infected leaves contains RNA-dependent RNA polymerase activity capable of synthesizing

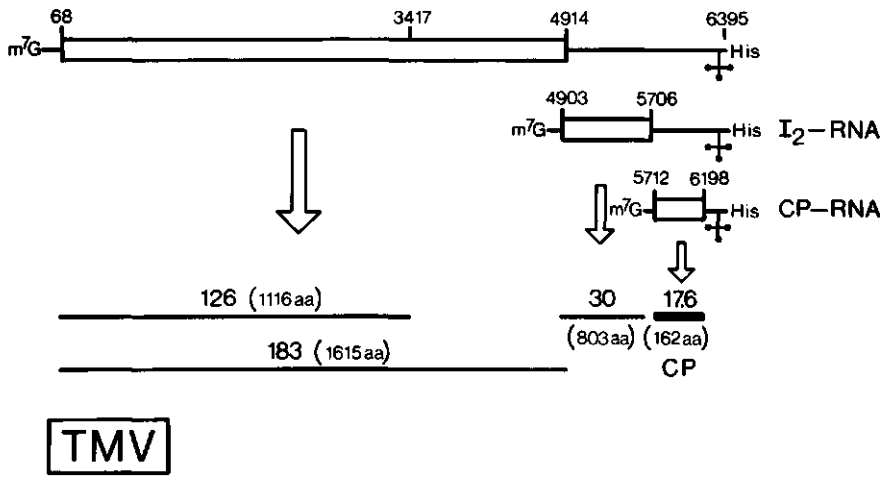

Fig. 6. Translation strategy of tobacco mosaic virus.
The coding region of the RNA is indicated by an open bar. m^7G = 5'-terminal cap structure; ⊣ = tRNA-like structure at the 3'-end; His = histidine; polypeptides are indicated by their molecular weights (x 10^{-3}); aa = number of amino acids; CP = coat protein. References and other abbreviations are included in the text.

in vitro double-stranded TMV RNA on endogenous viral RNA templates (Bradley and Zaitlin, 1971). The RNA-dependent RNA polymerase activity could be solubilized from the membranes using a nonionic detergent (NP-40). Upon further purification, an enzyme was obtained which was almost completely dependent on the addition of RNA for activity, suggesting that the endogenous template of the viral replication complex had been removed. This solubilized enzyme, however, did not display template-specificity (Zaitlin *et al.*, 1973) and turned out to be indistinguisable from an RNA-dependent RNA polymerase activity occurring in the soluble (cytoplasmic) fraction of TMV-infected (Brishammer and Junti, 1974) as well as uninfected tobacco leaves (Romaine and Zaitlin, 1978). It, thus, seems likely that the RNA-dependent RNA polymerase solubilized from the membranes is a host-encoded enzyme. When on the other hand, the RNA polymerase activity in the particulate fraction of TMV-infected tobacco callus cultures was solubilized by extraction with a Mg^{2+}-deficient buffer, a completely template-dependent enzyme was obtained, which, upon further purification, showed a remarkable preference for TMV RNA as a template (White and Murakishi, 1977). The enzyme transcribed added TMV RNA into complementary RNA, some of which was recovered in long double-stranded forms (RF). These results suggest that factors required for specific initiation of TMV (-)-strand RNA synthesis were present in the enzyme preparation of TMV-infected callus cells. Unfortunately, this approach has not been pursued for identifying the core polymerase and factors involved in initia-

tion of TMV RNA synthesis.

Evidence for the involvement of viral proteins in TMV RNA synthesis has been obtained from studies using ts-mutants of TMV (Dawson and White, 1978, 1979). One ts-mutant was unable to produce viral RNA at the nonpermissive temperature; another mutant did not synthesize single-stranded RNA, whereas production of double-stranded RNA was unaffected. These results suggest that functions for both (-)-strand and (+)-strand RNA synthesis are located on the viral genome.

4.2. TURNIP YELLOW MOSAIC VIRUS (TYMV)

4.2.1. *Genome structure and expression.*

TYMV is the type member of the tymoviruses, a group of viruses composed of icosahedral particles about 30 nm in diameter and capsids made up of 180 coat protein subunits (Matthews, 1977). The genomic RNA, approximately 6500 nucleotides in length, is capped (m^7GpppN) at the 5'-terminus (Davies and Hull, 1982) and contains a tRNA-like structure at the 3'-end, which can be aminoacylated *in vitro* with valine (Haenni *et al.*, 1982). Recently, it has been reported that a small portion of the TYMV RNA molecules in TYMV-infected chinese cabbage leaves occurs also charged with valine (Joshi *et* ., 1982). This aminoacylation of TYMV RNA must have been established *in vivo*, since virion RNA does not carry a valine residue at its 3'-terminus (Joshi *et al.*, 1982). TYMV coat protein is expressed by a subgenomic mRNA derived from the 3'-terminal region of the genome (Fig. 7; Guilley and Briand, 1978).

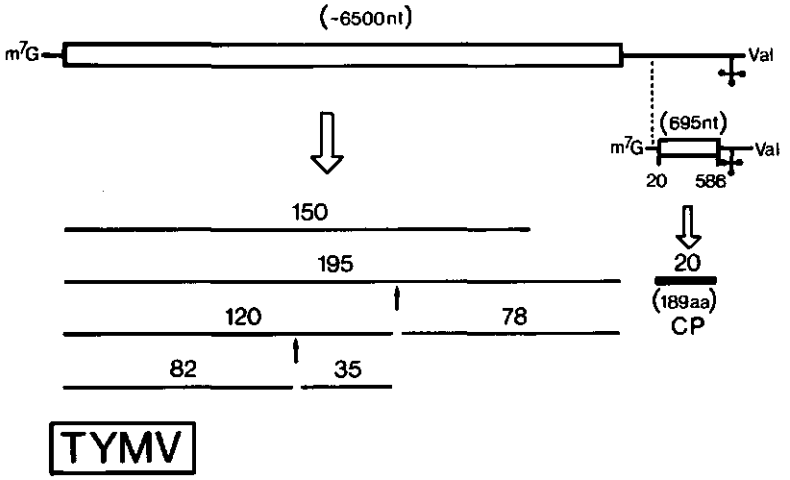

Fig. 7. Translation and processing map of turnip yellow mosaic virus. Val = valine; ↑ = cleavage site; other symboles as in Fig. 6. References are included in the text.

In vitro translation of the genomic RNA results in two primary overlapping translation products of 150K and 195K, the latter of which arises by readthrough (Morch and Benicourt, 1980) and is subsequently processed *in vitro* into 120K and 78K polypeptides (Morch *et al.*, 1982). The 120K polypeptide, which might be a component of TYMV replicase (see below), is further cleaved *in vitro* into 82K and 35K polypeptides (Fig. 7). In contrast to the 195K polypeptide, the 150K primary translation product appears to be stable *in vitro* (Morch and Benicourt, 1980). *In vivo*, a large number of polypeptides with molecular weights varying between 12K and 190K have recently been detected in Chinese cabbage protoplasts infected with TYMV (Sugimura *et al.*, 1981), but it remains to be established whether these proteins are actually virus-encoded.

4.2.2. *Replication.*

TYMV RNA replication *in vivo* is associated with the chloroplast outer membrane (Lafleche *et al.*, 1972). TYMV replicase has been extensively purified, starting with the particulate fraction isolated from TYMV-infected chinese cabbage cells (Lafleche *et al.*, 1972). This fraction contained a replication complex capable of synthesizing *in vitro* (+)-strand viral RNA occurring in a double-stranded form (Mouches *et al.*, 1974). Solubilization of the membrane-bound replication complex using the nonionic detergent Lubrol and removal of the endogenous template, resulted in a partially template-specific RNA-dependent RNA polymerase sedimenting with a sedimentation coefficient of 15S and capable of synthesizing (-)-type viral RNA, which remained associated with the added (+)-type template (Mouches *et al.*, 1976, 1981). After further purification, the polymerase appeared to be composed of two major polypeptides of 120K and 45K. Antibodies raised against the purified enzyme were able to neutralize polymerase activity for 40-60%. These antibodies, moreover, immunoprecipitated the viral 120K polypeptide produced upon *in vitro* translation of TYMV RNA (Mouches *et al.*, 1981). In addition, a host-encoded RNA-dependent RNA polymerase was identified in the soluble (cytoplasmic) fraction of both TYMV-infected and uninfected chinese cabbage, which sedimented at 6S and did not display template-specificity (Astier-Manifacier and Cornuet, 1978; Mouches *et al.*, 1981).

In conjunction, these results indicate that TYMV replicase as characterized by Mouches *et al.*, (1981), differs from the host-encoded RNA-dependent RNA polymerase and that the virus-encoded 120K protein may be a functional component of the TYMV replicase.

4.3. COWPEA MOSAIC VIRUS (CPMV).

4.3.1. *Genome structure and expression.*

CPMV is the type member of the comoviruses. These bipartite viruses contain a single-stranded RNA genome divided among two RNA molecules (B- and M-RNA), which are separately encapsidated. The capsids of CPMV are about 24 nm in diameter and consist of two proteins (VP37 and VP23) present in equimolar amounts (Van Kammen and Mellema, 1977). Both RNA molecules contain a 3'-terminal poly(A)-tail and a small protein (VPg) covalently linked to the 5'-terminal UMP residue (Daubert *et al.*, 1978; Stanley *et al.*, 1978).

Translation of M-RNA *in vitro* results in two primary translation products (105K and 95K) which overlap at their carboxy-terminal ends (Fig. 8) (Pelham, 1979; Goldbach *et al.*, 1981). The carboxy-terminal parts of these precursors represent the direct precursor (60K) of the two capsid proteins VP37 and VP23. The 60K capsid-precursor is cleaved from the 105K and 95K primary translation products by a B-RNA encoded protease (Franssen *et al.*, 1982). Generation of the mature capsid proteins from the 60K precursor has not yet been achieved *in vitro*. Indeed, the occurrence of two primary translation products *in vitro* can be derived from the complete M-RNA nucleotide sequence (Van Wezenbeek *et al.*, 1983), showing a single reading frame with two possible initiation codons in phase (Fig. 8) and with the proper distance between them to account for the difference in size of the 105K and

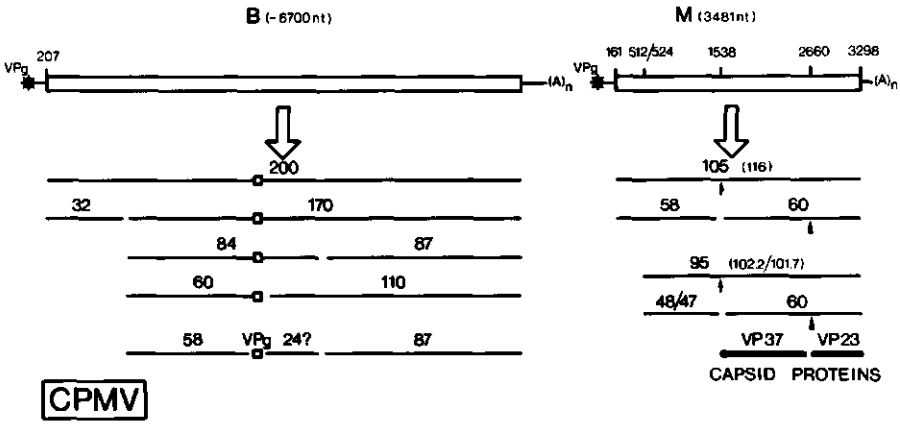

Fig. 8. Translation and processing map of cowpea mosaic virus.
The position of the AUG codon of B-RNA is taken from Lomonossoff *et al.*, (1982). M-RNA sequence data are from Van Wezenbeek *et al.* (1983).
* = VPg; $(A)_n$ = 3'-terminal poly(A) tail; ● = blocked NH_2-terminus; ↑ and ▲ are cleavage sites. Molecular weights ($x10^{-3}$) of polypeptides calculated from the M-RNA sequence data are shown between brackets. Other symboles as in Fig. 6. References are included in the text.

95K polypeptides. Sofar, it has not yet been shown whether M-RNA *in vivo* is also translated into two polypeptides.

Although both B- and M-RNA are required for multiplication of CPMV in intact leaves, B-RNA is capable of self-replication and expression in cowpea mesophyll protoplasts (Goldbach *et al.*, 1980; Rezelman *et al.*, 1982). Therefore the translation strategy of B-RNA has been studied both *in vivo* and *in vitro*. B-RNA is translated into a 200K polyprotein, which is subsequently cleaved into 32K and 170K polypeptides (Fig. 8). The 170K protein is further processed into 110K, 87K, 84K, 60K, 58K, and possibly 24K polypeptides (Rezelman *et al.*, 1980; Goldbach *et al.*, 1982; Goldbach and Rezelman, 1983).

The genome-linked protein (VPg) is encoded on B-RNA (Stanley *et al.*, 1980) and the membrane-bound, 60K polypeptide was demonstrated to be its direct precursor. Free VPg, which would be generated by processing of 60K into 58K, has never been detected in the infected cell. VPg thus occurs either contained in its precursor or linked to the 5'-ends of viral RNAs and is thought to be involved in initiation of viral RNA synthesis (Zabel *et al.*, 1982; Goldbach *et al.*, 1982; Dorssers *et al.*, 1982).

The proteolytic activity responsible for cleaving the precursor of the capsid proteins appears to reside in the 32K polypeptide as inferred from the ability of antiserum raised against 32K polypeptide to inhibite the *in vitro* processing of the 105K and 95K polypeptides (Henk Franssen, personal communication).

One or more B-RNA-specific polypeptides appears to be involved in viral RNA replication, since B-RNA is replicated in cowpea protoplasts independently of M-RNA expression (Goldbach *et al.*, 1980). Infection of isolated cowpea protoplasts with B components alone results in the induction of large vesicular membrane structures (Rezelman *et al.*, 1982), in agreement with earlier findings (De Zoeten *et al.*, 1974) showing that CPMV RNA replication is associated with such membranes. When purified B component was inoculated to primary cowpea leaves, symptoms were not produced and B-RNA was incapable of spreading to surrounding cells. These observations suggest that M-RNA-encoded functions may be involved in spreading throughout leaves (Rezelman *et al.*, 1982).

4.3.2. *Replication.*

Replication of CPMV RNA *in vivo* is associated with vesicular membranes of a cytopathic structure (Assink *et al.*, 1973; De Zoeten *et al.*, 1974). The particulate fraction of infected cells was shown to contain an RNA replication complex, capable of synthesizing double-stranded and possibly some single-stranded viral RNAs *in vitro* (Zabel *et al.*, 1974; Stuik, 1979). This particulate fraction has been used as starting material for purification of the putative CPMV RNA replicase (Zabel *et al.*, 1976; 1979). The

essential first step in this purification procedure was the solubilization of the bulk of RNA-dependent RNA polymerase activity by extracting the membranes with a Mg^{2+}-deficient buffer. The solubilized enzyme was subsequently freed of endogenous template by salt-gradient elution from a DEAE-Biogel A column. Glycerol gradient centrifugation, although a very efficient and gentle step for the further purification of the CPMV replicase, did not allow yet the RNA polymerase to be identified (Zabel, 1978; Zabel *et al.*, 1979).

The completely template-dependent, DEAE-Biogel A column-purified enzyme displayed all properties of an RNA-dependent RNA polymerase (Zabel *et al.*, 1979). However, the enzyme copied all added templates with comparable efficiency. To determine whether specific initiation did occur (i.e. specific recognition of the template as the first step in RNA replication), Zabel *et al.* (1979) used a nitrocellulose filter assay to monitor RNA-protein complexes, which are retained by nitrocellulose. The DEAE-Biogel A column-purified enzyme appeared to bind ^{32}P-labeled CPMV-RNA very efficiently. No such binding activity was observed in a comparable fraction from uninfected tissue. The binding of labeled CPMV RNA was specific, as only unlabeled RNA from CPMV and RaMV (another comovirus) was able to compete with labeled CPMV RNA in the binding assay. It was thus suggested that the DEAE-Biogel-purified enzyme contains some protein (possibly the CPMV RNA replicase) interacting specifically with CPMV RNA. This protein might be virus-encoded as inferred from its absence in preparations from uninfected cowpea leaves (Zabel, 1978; Zabel *et al.*, 1979). Other investigators were unable to identify a specific CPMV RNA-binding activity in preparations from CPMV-infected cowpeas and therefore concluded that a host-encoded RNA-dependent RNA polymerase was probably involved in CPMV RNA replication (Ikegami and Fraenkel-Conrat, 1980).

In this thesis, data will be presented showing that the polymerase solubilized from the membranes by a Mg^{2+}-deficient buffer is identical to an RNA-dependent RNA polymerase occurring in low amounts in uninfected cowpeas (Dorssers *et al.*, 1982; Chapter III). Using antiserum raised against the purified RNA-dependent RNA polymerase and an antibody-linked polymerase assay (ALPA), the host-encoded RNA polymerase was shown to be composed of a 130K polypeptide (Van der Meer *et al.*, 1983; chapter IV). In order to identify the core polymerase actually involved in viral RNA replication, we again focussed our attention on the membrane-bound CPMV RNA replication complex. By characterization of RNAs synthesized *in vitro* on the endogenous template, it was found that the viral replication complex remained strictly membrane-bound under conditions allowing the bulk of host enzyme to be released (Dorssers *et al.*, 1983; Chapter V). Following solubilization with Triton X-100 and partial purification on a Sepharose 2B column, the repli-

cation complex remained capable of synthesizing full-length viral RNAs, without being contaminated by the host-encoded RNA-dependent RNA polymerase. In stead, a virus-specific B-RNA-encoded polypeptide was found associated with the replication complex and may constitute the core polymerase (Chapters VI and VII).

4.4. BROME MOSAIC VIRUS (BMV).

4.4.1. *Genome structure and expression.*

BMV is the type member of the bromoviruses containing a tripartite genome encapsidated in icosahedral virion particles. The three single-stranded genomic RNAs 1, 2 and 3 possess lenghts of 3300, 2865 and 2114 nucleotides, respectively (Ahlquist *et al.*, 1981a; P. Ahlquist, personal communication). The 5'-termini of the genomic RNAs have a cap structure, whereas the 3'-terminus can be folded into a tRNA-like structure (Haenni *et al.*, 1982; Ahlquist *et al.*, 1981b). Aminoacylation occurs at the 3'-ends of BMV RNAs both *in vivo* and *in vitro*, but no viral RNAs charged with tyrosine are found in virions (Loesch-Fries and Hall, 1982).

Translation of the genomic RNAs 1, 2 and 3 *in vivo* and *in vitro* results in polypeptides of 110K, 105K and 35K, respectively (Fig. 9; Kiberstis *et al.*, 1981). RNA 3 contains a second cistron located in the 3'-terminal half of the molecule, which is only expressed via a subgenomic mRNA (RNA 4, Dasgupta and Kaesberg, 1982) to encode the coat protein.

Production of new virus particles in barley protoplasts requires all three genomic RNAs. Omitting RNA 3 from the inoculation mixture does not prevent the synthesis of viral proteins encoded by RNA 1 and 2. Apparently, RNA 1 and 2 are replicated and expressed in the absence of RNA 3 and thus may encode proteins involved in viral RNA replication (Kiberstis *et al.*, 1981).

4.4.2. *Replication.*

A BMV RNA replication complex capable of producing double-stranded and single-stranded BMV RNAs *in vitro* is associated with membranes in the particulate fraction of BMV-infected barley (Semal and Kummert, 1971). Solubilization with a nonionic detergent (NP-40) resulted in an enzyme preparation comprising both an RNA replication complex synthesizing (+)-strands on endogenous RNA template molecules and an RNA-dependent RNA polymerase activity synthesizing (-)-strands on added RNA templates (Kummert and Semal, 1977). The template-dependent enzyme showed some template-specificity, but did not discriminate between BMV RNA and RNA from CCMV, another bromovirus (Hadidi and Fraenkel-Conrat, 1973). Further purification led to stable enzyme preparations showing higher levels of template-dependence and template-specificity (CCMV RNA being five-fold less active than BMV RNA) (Hardy *et al.*,

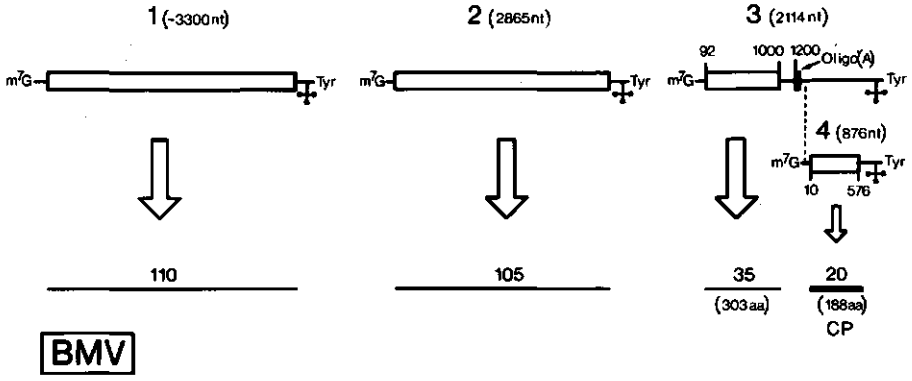

Fig. 9. Translation strategy of brome mosaic virus.
RNA 3 contains an internal oligo(A)-track. Tyr = tyrosine; other symboles as in Fig. 6. References are included in the text.

1979). In addition, the purified enzyme appeared to initiate correctly, as full-length double-stranded RNA was synthesized on exogenous RNA4. By using dodecyl-maltoside as detergent instead of NP-40, an enzyme preparation was obtained which was more stable and displayed almost complete template-specificity (Bujarski *et al.*, 1982).

A complete template-dependent RNA-dependent RNA polymerase from BMV-infected barley was recently obtained by digesting the endogenous template RNA with micrococcal nuclease (Miller and Hall, 1983; Hall *et al.*, 1982), without affecting the template-specificity. A comparable enzyme prepared from CCMV-infected cowpeas, catalyzed incorporation of nucleotides into RNA complementary to either CPMV, CCMV or BMV RNA. However, product analysis demonstrated that only CCMV and BMV RNA were transcribed into full-length copies. These results suggest that the enzyme from CCMV-infected cowpeas shows some template-specificity but does not discriminate between CCMV and BMV RNA (W.A. Miller, personal communication). The transcription of added BMV RNA into full-size copies indicates that the isolated enzyme from BMV-infected barley might constitute the BMV replicase.

The partially purified enzyme from barley contained, among many other polypeptides, a 110K polypeptide, which was shown to be identical to the *in vitro* translation product of BMV RNA1 (Bujarski *et al.*, 1982). Functional involvement of this 110K polypeptide in viral RNA replication, remains to be demonstrated.

4.5. CUCUMBER MOSAIC VIRUS (CMV)

4.5.1. *Genomic structure and expression.*

CMV, the type member of the cucumoviruses, contains a tripartite genome.

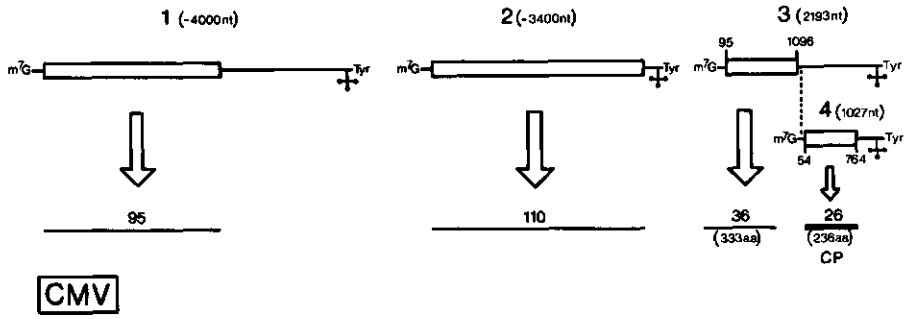

Fig. 10. Translation strategy of cucumber mosaic virus.
Tyr = tyrosine; other symboles as in Fig. 6. References are included in the text.

The three single-stranded genomic RNAs 1, 2 and 3 are separately encapsidated in icosahedral particles and possess lengths of about 4000, 3400 and 2193 nucleotides, respectively (Gould and Symons, 1982; Symons *et al.*, 1983). The 5'-termini of the genomic RNAs have a cap structure (m^7GpppN), whereas the 3'-end can be folded into a tRNA-like structure to be charged with tyrosine *in vitro* (Haenni *et al.*, 1982). Translation of the genomic RNAs 1 and 2 in cell-free translation systems results in polypeptides of 95K and 110K, respectively (Fig. 10). RNA 3 is translated *in vitro* in a 36K polypeptide, but also carries the cistron for coat protein in its 3'-terminal part. The coat protein, which is the only CMV-specific protein identified sofar *in vivo*, is expressed via a subgenomic mRNA (RNA 4) (Symons *et al.*, 1983).

4.5.2 *Replication.*

The particulate fraction of CMV-infected cucumber contains a CMV RNA replication complex capable of elongating CMV RNAs *in vitro* on endogenous template RNA. (May *et al.*, 1970; Symons *et al.*, 1983). Both the particulate fraction and the soluble fraction of CMV-infected cucumbers have been used for the purification of template-dependent RNA-dependent RNA polymerases (Kumurasamy and Symons, 1979; Gill *et al.*, 1981). The purified polymerase preparations were shown to be composed of a 100K polypeptide and variable amounts of 110K and 35K polypeptides. Although none of these polypeptides were present in preparations of uninfected cucumbers virtually devoid of polymerase activity, no homology with the *in vitro* translation products of CMV RNAs 1, 2 and 3 could be detected (Gordon *et al.*, 1982). Furthermore, RNA-dependent RNA polymerase purified from CMV-infected tobacco and cucumbers are composed of different polypeptides, indicating the absence of a CMV-specific polymerase (Takanami and Fraenkel-Conrat, 1982). These results, which suggest that the 100K polypeptide is the cucumber RNA-dependent RNA polymerase, strongly stimulated upon CMV infection, have been interpreted

in favour of a role for the host-enzyme as being the core polymerase in CMV RNA replication (Symons *et al.*, 1983; Fraenkel-Conrat, 1983).

4.6. ALFALFA MOSAIC VIRUS (AlMV)

4.6.1. *Genome structure and expression.*

AlMV, the type member of the tricornaviruses (Van Vloten-Doting *et al.*, 1981), has an RNA genome consisting of three single-stranded RNA molecules, RNA 1 (3644 nucleotides, Cornelissen *et al.*, 1983a), RNA 2 (2593 nucleotides, Cornelissen *et al.*, 1983b) and RNA 3 (2037 nucleotides, Barker *et al.*, 1983), which are separately encapsidated in three bacilli-form particles of different size(B, M and Tb components, respectively).Virus preparations contain a fourth component (Ta) encapsidating two copies of an RNA 4 molecule, 881 nucleotides in length (Brederode *et al.*, 1980), and not required for virus infectivity. All four viral RNAs possess a 5'-terminal cap structure and have an almost identical sequence at their 3'-ends (Koper-Zwarthoff *et al.*, 1979; Cornelissen *et al.*, 1983a). For infectivity, three genomic RNAs (1-3) and either a few coat protein molecules or the coat protein mRNA (RNA 4) are required (Smit *et al.*, 1981).

Translation of RNA 1 *in vitro* results in a 115K polypeptide (Fig. 11). At high messenger concentrations, two amino-terminal overlapping polypeptides (58K and 62K) are produced for reasons which are not yet understood (Van Tol *et al.*, 1980; Cornelissen *et al.*, 1983a). RNA 2 encodes a 100K polypeptide and RNA 3 is translated into a 35K polypeptide (Van Vloten-Doting and Jaspars, 1977). In addition, RNA 3 carries the coat protein cistron located at its 3'-end. *In vivo*, RNA 4 is generated from RNA 3 as a subgenomic mRNA to produce coat protein. Remarkably, no viral polypeptides besides the

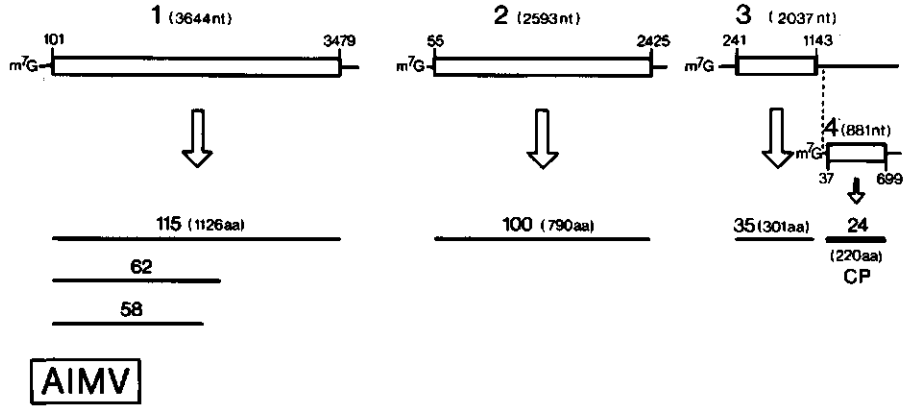

Fig. 11. Translation strategy of alfalfa mosaic virus.
Symboles as in Fig. 6. References are included in the text.

coat protein have been identified sofar *in vivo*.

4.6.2. *Replication.*

AlMV multiplication in cowpea protoplasts requires inoculation with all three encapsidated genomic RNAs (B, M and Tb component) (Nassuth *et al.*, 1981). Inoculation of protoplasts with B + M component alone resulted in reduced synthesis (about 40%) of RNAs 1 and 2, whereas no viral RNA synthesis occurred upon inoculation with either B + Tb, or M + Tb components. In addition, no viral RNAs were produced upon inoculation with naked RNAs 1, 2 and 3 in the absence of either RNA 4 or coat protein (Nassuth *et al.*, 1981). Protoplasts inoculated with B + M + Ta component did synthesize RNA 1 and 2, but failed to produce RNA 4 or coat protein (Nassuth *et al.*, 1981, 1983b), indicating that RNA 4 is not replicated autonomously. This was in agreement with both the failure to rescue a coat protein mutant with wild-type RNA 4 (Smit and Jaspars, 1982) and the absence of (-)-strand RNA 4 in infected protoplasts (Nassuth and Bol, 1983). In contrast to (+)-strand RNA synthesis, the production of (-)-strand RNA increased when Tb component was omitted from the inoculation mixture and protoplasts were inoculated with B- and M-particles (Nassuth and Bol, 1983). These results suggest that RNA 1 and 2 encode proteins required for viral RNA synthesis and that RNA 3 encodes some protein, probably coat protein, which regulates the balance of (+)-strand and (-)-strand RNA production. Analysis of temperature-sensitive mutants of AlMV supported these conclusions (Sarachu *et al.*, 1983; Roosien, 1983).

Since naked AlMV RNAs do not replicate in protoplasts nor in inoculated leaves, it is postulated that the coat protein is involved in initiation of viral RNA synthesis (Houwing and Jaspars, 1978; Nassuth *et al.*, 1981). Specific binding sites for the coat protein have been identified on all viral RNAs both at internal positions and near the 3'-terminus (Koper-Zwarthoff and Bol, 1979; Zuidema *et al.*, 1983). In addition to viral proteins, host-encoded polypeptides appear to be required for viral RNA synthesis since actinomycin D, administrated early in infection, inhibited the production of viral RNAs (Nassuth *et al.*, 1983a).

In vitro, AlMV RNA synthesis was demonstrated to occur in the particulate fractions of broad bean (Weening and Bol, 1975) and tobacco (Bol *et al.*, 1976). In this fraction, both full-size viral RNAs of positive polarity and small, RNase-sensitive RNA molecules were produced by enzymes associated with endogenous templates. The small RNA products were also synthesized by enzyme preparations from uninfected leaves, apparently representing *in vitro* products of a host-encoded RNA-dependent RNA polymerase (Bol *et al.*, 1976). After removal of this host-encoded enzyme by extraction with a nonionic detergent, the virus-specific RNA-synthesizing activity was solubilzed from

the membranes with a Mg^{2+}-deficient buffer. The solubilized enzyme from infected leaves, however, sedimented with a similar rate as an enzyme prepared from uninfected tobacco leaves (Clerx and Bol, 1978). A comparable enzyme has been prepared from the soluble fraction of AlMV-infected and uninfected tobacco leaves (Le Roy *et al.*, 1977; Chifflot *et al.*, 1980; Linthorst, 1982). Each of these enzyme preparations did not display template-specificity and produced small, RNA fragments complementary to added template RNAs. It thus can be concluded, that all attempts to purify the AlMV replicase have sofar resulted in enzyme preparations indistinguishable from RNA-dependent RNA polymerase preparations from uninfected leaves (Linthorst, 1982; Sommer *et al.*, 1981).

Recently, the partially purified template-dependent enzymes from the soluble fraction of AlMV-infected and uninfected tobacco leaves were both shown to be capable of copying AlMV RNA 4. Small amounts of these transcripts appeared to be full-length (Linthorst, 1982). These results suggest, that the host-encoded RNA-dependent RNA polymerase might be the core polymerase responsible for viral RNA synthesis and that viral proteins are necessary for specific initiation of viral RNA replication. In support of this hypothesis is adduced, that partially purified polymerases from tobacco leaves infected with ts-mutants of AlMV, failed to show temperature-sensitivity when tested *in vitro* (Linthorst *et al.*, 1980).

5. PLANT RNA-DEPENDENT RNA POLYMERASES

Since the discovery of an RNA-dependent RNA polymerase in uninfected chinese cabbage (Astier-Manifacier and Cornuet, 1971), the occurrence of similar enzymes in tobacco (Duda *et al.*, 1973; Bol *et al.*, 1976; Le Roy *et al.*, 1977; Clerx and Bol, 1978; Ikegami and Fraenkel-Conrat, 1978a, 1979a; Romaine and Zaitlin, 1978; Duda, 1979; Chifflot *et al.*, 1980; Sommer *et al.*, 1981), cowpea (Ikegami and Fraenkel-Conrat, 1978b; White and Dawson, 1978; Dorssers *et al.*, 1982), tomato (Boege and Sänger, 1980), cauliflower (Astier-Manifacier and Cornuet, 1978), *Phaseolus* (Lazar *et al.*, 1979), broad bean (Weening and Bol, 1975), barley (Hardy *et al.*, 1979) and cucumber (Linthorst, 1982; Takanami and Fraenkel-Conrat, 1982) has been reported. Rabbit reticulocyte lysates also seem to contain and RNA-dependent RNA polymerase (Downey *et al.*, 1973) but this enzyme activity has not been further explored sofar.

RNA-dependent RNA polymerases are usually purified from the soluble fraction of leaf homogenates but also appear to occur in the particulate fractions of the same homogenates. The enzyme purified from cauliflowers was found to be composed of a single polypeptide with a molecular weight of approximately 140K (Astier-Manifacier and Cornuet, 1978). Similar molecular weights have been reported for (less) purified enzymes from other hosts (see

Fraenkel-Conrat, 1983; Dorssers *et al.*, 1982). Antibodies raised against the purified RNA-dependent RNA polymerase from cauliflower cross-reacted with enzymes from chinese cabbage and tobacco. In contrast, no homology was observed with DNA-dependent RNA polymerase II (Astier-Manifacier and Cornuet, 1981).

Although the host-encoded RNA-dependent RNA polymerase has not been implicated in a specific function in uninfected cells, it appears to be engaged in transcribing plant RNA templates. (i) Small double-stranded RNAs were found in tobacco leaves independent of the presence of actinomycin D (Ikegami and Fraenkel-Conrat, 1979b). (ii) The host-encoded RNA polymerase of cowpea was found associated with endogenous plant RNA templates to transcribe these into short complementary RNAs (Dorssers *et al.*, 1983). (iii) Furthermore, partially purified RNA-dependent RNA polymerases from uninfected plants have recently been shown to be capable of transcribing *in vitro* small circular RNAs, like PSTV RNA, ASV RNA and SNMV RNA 2 (Boege *et al.*, 1982; Symons *et al.*, 1983). These results strongly suggest that the RNA-dependent RNA polymerase from healthy plants indeed functions as an RNA polymerase and is not an artefact of the *in vitro* assay.

The occurrence of RNA-dependent RNA polymerases in uninfected plants, which are strongly stimulated upon virus infection and, at the other hand, the failure to identify a virus-specific polymerase has led some investigators to postulating a role for this host-encoded enzyme in viral RNA replication (Fraenkel-Conrat, 1976; Romaine and Zaitlin, 1978; Clerx and Bol, 1978; Ikegami and Fraenkel-Conrat, 1978a/b, 1979a; Hall *et al.*, 1982; Fraenkel-Conrat, 1983; Symons *et al.*, 1983). In that concept, the host enzyme might function as the core polymerase for viral RNA synthesis, with virus-encoded proteins conferring specific initiation on viral RNA templates. Any experimental evidence for this model is missing sofar. In chapters V and VI of this thesis, this model is refuted for the replication of CPMV RNA.

6. OTHER PLANT PATHOGENS.

6.1. VIROIDS.

Viroids are the smallest plant pathogens currently known. They are composed of a naked, circular single-stranded RNA, 250-400 nucleotides in size, which display the features of a double-stranded RNA because of excessive intramolecular base pairing (for review: Sänger, 1982). Viroids apparently do not code for proteins: no viroid-specific proteins have been detected in the infected cells nor in *in vitro* translation systems programmed with purified viroid RNA. Indeed, sequence analysis of a large number of viroids failed to reveal an open reading frame encoding a viroid specific protein

(Gross *et al.*, 1978; Haseloff *et al.*, 1982; Van Wezenbeek *et al.*, 1982).

As viroid replication is apparently not mediated by a viroid-encoded polymerase, host enzymes should be involved in viroid replication. DNA sequences complementary to viroid RNA are absent in infected plants implying that viroid replication proceeds via complementary RNA (Zaitlin *et al.*, 1980; Branch and Dickson, 1980). Since plants contain RNA-dependent RNA polymerases, these enzymes might be involved in viroid replication. On the other hand, since viroid replication takes place in the nucleus and viroid RNAs resemble double-stranded nucleic acids, DNA-dependent RNA polymerases have been implicated. In agreement with this hypothesis is that the synthesis of viroid RNA in protoplasts is sensitive to both actinomycin D and α-amanitin (Mühlbach and Sänger, 1979). In particular, the sensitivity towards α-amanitin was suggestive for the involvement of DNA-dependent RNA polymerase II. This model was supported by the demonstration of *in vitro* transcription of viroid RNA by purified DNA-dependent RNA polymerase II from tomato and wheat germ (Rackwitz *et al.*, 1981). Furthermore, isolated nuclei were able to synthesize linear and circular viroid RNA *in vitro* and this activity was not inhibited by the presence of actinomycin D, whereas addition of α-amanitin inhibited viroid replication (Flores and Semancik, 1982). These data suggest indeed the involvement of DNA-dependent RNA polymerase II in viroid RNA replication. However, the α-aminitin sensitivity of viroid RNA replication can not be regarded as conclusive evidence for the possible role of DNA-dependent RNA polymerase II. The observation that plant RNA-dependent RNA polymerases can also transcribe viroid plant RNA *in vitro* (Boege *et al.*, 1982; Symons *et al.*, 1983), even though it probably does not reflect the *in vivo* situation, further shows the relativity of the results obtained with isolated DNA-dependent RNA polymerase II.

Recent reports have shown that in viroid-infected cells multimeric viroid (-)-strands and (+)-strands are present which might represent intermediates of viroid RNA replication (Branch *et al.*, 1981; Rohde and Sänger, 1981; Owens and Diener, 1982). Such large viroid RNAs may result from a rolling circle replication mechanism which would involve specific cutting of the multimeric precursors into unit-length viroid molecules and subsequent circularization (Branch *et al.*, 1981). A specific RNA ligase from wheat germ (Konarska *et al.*, 1981; 1982) was indeed able to convert *in vitro* linear PSTV RNA into circular forms (Branch *et al.*, 1982). It seems therefore plausible that replication of viroid RNA proceeds by a rolling circle mechanism, involving host-specific DNA-depenent RNA polymerase II, specific endonucleolytic scissions and finally circularization of linear viroid RNA molecules. Further experiments are required to confirm this model.

6.2. VIROID-LIKE RNAs OR VIRUSOIDS.

Recently, a new group of plant viruses has been discovered, including velvet tobacco mottle virus (VTMoV) and solanum nodiflorum mottle virus (SNMV). Virus particles contain a single-stranded RNA 1 (mol.wt. 1.5×10^6) and a small RNA (± 370 nucleotides) in either linear (RNA 3) or circular (RNA 2) form (Gould, 1981; Randles *et al.*, 1981; Gould and Hatta, 1981; Tien *et al.*, 1981). Both RNA 1 and RNA 2/3 are encapsidated and required for infectivity (Gould *et al.*, 1981). Because of its small size and circular form, RNA 2 has been termed a viroid-like RNA or virusoid. The RNA 2 of SNMV and VTMoV showed an extensive homology in nucleotide sequences (Haseloff and Symons, 1982), but did not form infectious combinations with heterologous RNA 1 (Gould *et al.*, 1981). The dependence of RNA 1 on RNA 2 for infectivity seems to exclude that RNA 2 is a satellite of RNA 1. The basis of the mutual dependence of the two RNAs remains to be established.

RNA 2 appears not to code for a protein and thus resembles the viroids in encoding only its own structure (Haseloff and Symons, 1982; Haseloff *et al.*, 1982). Replication of RNA 2 involves long double-stranded RNAs which consist of small, heterogenous (+)-strand RNA molecules and a long, multimeric (-)-strand (Chu *et al.*, 1983). On the other hand, for RNA 1 only double-stranded RNAs with a length corresponding to monomeric genomic RNAs were detected in the infected cell, indicating that RNA 1 may be replicated by a mechanism different from that for RNA 2.

6.3. SATELLITES.

The term satellite refers to a virus or nucleic acid that (i) is only able to multiply in cells with the assistance of a specific helper virus, (ii) is not necessary for the multiplication of the helper virus and (iii) has no appreciable sequence homology with the helper virus genome (Murant and Mayo, 1982). Satellite viruses contain linear single-stranded RNA molecules encapsidated in a protein shell. Type A satellites (e.g. satellite virus of TNV) encode their own coat protein (21K), whereas group B and C satellites are packaged in capsids of the helper virus. Type B satellites (e.g. TBRV satellite RNA) consist of RNA molecules with a molecular weight of approximately 5.10^5 dalton, encoding a protein of approximately 48K, while type C satellites (e.g. satellite RNAs of CMV and TobRV) are RNAs of approximately 1.10^5 dalton containing small open reading frames for yet hypothetical polypeptides (Richards *et al.*, 1978; Gordon and Symons, 1983). Type B and C satellites interfere with symptom expression and multiplication of the helper virus.

Different strains of satellite RNAs can change completely the symptoms

produced upon infection with the helper virus. With satellite RNAs of CMV, interference of symptom expression is accompanied by accumulation of large amounts of double-stranded satellite RNA (Kaper, 1982; Piazolla *et al.*, 1982) and replication of the helper virus RNA appears to be outcompeted by that of satellite RNA, suggesting that they share the same machinery for RNA replication. The RNA satellites of CMV show very little sequence homology with the CMV RNAs, but yet they can be folded in such a way that their 3'-ends form a tRNA-like structure analogous to the genomic CMV RNAs (Gordon and Symons, 1983). Whether this structure has a role in RNA replication is unknown.

With TBRV (a nepovirus), the satellite-helper specificity is encoded on RNA 1 which carries information for TBRV RNA replication (Robinson *et al.*, 1980; Murant and Mayo, 1982), whereas RNA 2 of TBRV encodes coat protein required for encapsidation of both TBRV RNA and satellite RNA, suggesting that satellite RNA utilizes the replication strategy of the helper virus. A similar conclusion may be derived from the multiplication of the satellite virus of TNV, the RNA of which is only replicated in the presence of multiplying TNV (Kassanis, 1962). Replication of satellite RNA of TobRV (another nepovirus) was shown to involve long multimeric (+)-strand and (-)strand intermediates (Kiefer *et al.*, 1982; Sogo and Schneider, 1982). Furthermore, satellite RNA is lacking the 5'-terminal VPg and the 3'-terminal poly(A), characteristic for the genomes of nepoviruses (Kiefer *et al.*, 1982). These results indicate that the mechanism of TobRV satellite RNA replication differs from that of its helper virus.

7. SUMMARY.

7.1. INITIATION OF RNA REPLICATION.

Viral RNA replication starts with specific recognition and binding of viral template RNA by the viral RNA replicase. Binding of RNA templates, being either (+)-strand or (-)-strand, should occur in such a way that the 3'-end of the RNA is placed in the proper position for transcription to start. With bacteriophage Qβ, the replicase does not bind to the 3'-end itself, but recognizes two internal domains of the viral template RNA (Meyer *et al.*, 1981). Thus, template-specificity is apparently not contained in the initiation site of RNA synthesis. A similar strategy may apply to the initiation of RNA replication of eukaryotic RNA viruses. The subgenomic mRNAs found for TMV, TYMV, BMV, CMV, AlMV and alphaviruses are not replicated autonomously although they are identical to the 3'-terminal region of the genomic RNA (Zelcer *et al.*, 1981; Miller and Hall, 1983; Gonda and Symons, 1979; Smit and Jaspars, 1982; Strauss and Strauss, 1982), in-

dicating that essential sequences for binding of replicase are located more upstream from the 3'-terminus of the viral RNAs. The inability of the subgenomic RNAs to replicate autonomously might however also be explained if the structure of the 3'-end of the complementary (-)-strand of the subgenomic mRNA precludes initiation of transcription. However, no (-)-strand of the subgenomic AlMV RNA 4 was detected *in vivo* (Nassuth and Bol, 1983; Nassuth *et al.*, 1983b), suggesting that transcription of AlMV RNA 4 did indeed not occur.

Correct initiation of Qβ RNA synthesis requires a host factor in addition to each of the four subunits of the Qβ replicase. The extreme template-specificity can be overcome by using a primer-template complex, which then eliminates the detailed structural requirements of the initiation step and allows Qβ replicase to transcribe even heterologous templates (Blumenthal and Carmichael, 1979). Although in broad outline the mechanism of replication of bacteriophage RNA does apply to eukaryotic RNA viruses, there are certainly important differences concerning various viruses.

For poliovirus, wich has a protein covalently linked to the 5'-end of its RNA, a more complicated initiation mechanism has been proposed (Wimmer, 1982). The genome-linked protein (VPg), found on all replicating poliovirus RNAs, is supposed to act as primer of (-)-strand and (+)-strand RNA synthesis (Wimmer, 1982; Baron and Baltimore, 1982d). A similar replication strategy may apply to plant viruses, which carry a genome-linked protein (comoviruses, sobemoviruses, potyviruses and nepoviruses; Davies and Hull, 1982). However, no evidence has been presented for any of these plant viruses, showing that VPg is required for initiation of RNA replication.

Different modes of template recognition and initiation of RNA synthesis are expected for other viruses. For alfalfa mosaic virus (AlMV), binding of the coat protein to the 3'-ends of the viral RNA is a prerequisite for virus multiplication, and it has been suggested that coat protein plays an important role in initiation of RNA replication (Smit and Jaspars, 1982; Houwing and Jaspars, 1978; Zuidema *et al.*, 1983). Several plant viruses contain a tRNA-like structure at their 3'-terminus (tymoviruses, tobamoviruses, bromoviruses and cucumoviruses), which can be aminoacylated *in vitro* and *in vivo* (Haenni *et al.*, 1982). The significance, if any, of this particular structure in the life cycle of these viruses is unknown, but may lie in the initiation event of RNA synthesis.

7.2. RNA STRAND ELONGATION.

The mechanism by which the single-stranded (+)-type RNA genome of viruses is replicated includes transcription of the infecting RNA into a complementary (-)-strand. The (-)-strand is then copied into (+)-strands, the produc-

tion of which exceeds the synthesis of (-)-strands manifold. This mechanism has been established for the replication of bacteriophage Qβ RNA and has appeared to be similar for animal-and plant viruses with a (+)-type single-stranded RNA genome. With Qβ bacteriophage, the infecting viral RNA first directs the synthesis of the viral polymerase which, together with host components, uses the infecting RNA as template for (-)-strand-synthesis and then the (-)strand for the synthesis of progeny (+)-type viral RNA. Also with picornaviruses, the infecting viral RNA is first translated into protein to produce the viral polymerase used for viral RNA replication.

For plant viruses, there is not yet conclusive evidence as to the origin of the core polymerase destined to play the prominent role in plant viral RNA replication. Attempts to purify the viral replicase of plant RNA viruses, notably with TMV, AlMV and CMV, resulted in the isolation of a host-encoded RNA-dependent RNA polymerase, the activity of which was strongly stimulated by virus infection. Because of this remarkable increase and the failure to identify a virus-encoded RNA-dependent RNA polymerase, involvement of this host-encoded RNA-dependent RNA polymerase in plant viral RNA replication has been proposed.

There is however, evidence that also plant viral RNA encodes information essential to virus RNA synthesis, as can be inferred from the following observations: (i) temperature-sensitive mutants with a defect in viral RNA synthesis have been isolated for TMV and AlMV. (ii) For viruses with a bipartite and a tripartite genome, like CPMV, TBRV, BMV and AlMV, it has been demonstrated that replication of an individual genomic RNA depends on the replication and expression of other genomic RNAs. (iii) Satellite viral RNAs and satellite RNAs are only replicated in the presence of a replicating helper virus. Since no viral proteins with RNA polymerase activity have been identified, these viral proteins are assumed to confer template-specificity to the host-encoded RNA-dependent RNA polymerase, which lacks such specificity completely. Such a mechanism has been proposed for the RNA replication of AlMV, CMV and TMV, without any support by sound experimental data.

Partially purified RNA-dependent RNA polymerase from barley infected with BMV, which did display considerable template-specificiy, was similarly supposed to be composed of a host-encoded core polymerase completed with a virus-encoded subunit for template-specificity and association of the native viral replication complex with membranes. Also in that case, it has not yet been possible to determine the polypeptide composition of the replicase and the function of different subunits, as the preparation contained too many contaminating proteins.

In view of the lack of solid experimental data, it seems premature to set much value on the proposal that the host-encoded RNA-dependent RNA polymerase is indeed responsible for the synthesis of plant viral RNAs. It should

be taken into account that the host-encoded enzyme might overshadow the true viral replicase, because it is present in large amounts. For TYMV, results were reported at the 5th International Congress of Virolgoy (Mouches *et al.*, 1981) showing that TYMV replicase purified from TYMV-infected chinese cabbage leaves, differed from the host-encoded RNA-dependent RNA polymerase. The purified TYMV replicase displayed, to a certain extent, template-specificity for TYMV RNA and contained a viral polypeptide as the putative core polymerase.

We have shown (Dorssers *et al.*, 1983, chapter V of this thesis) that the crude membrane fraction of CPMV-infected cowpea leaves harbors two functionally different RNA synthesizing activities, one of which is host-encoded and the other virus-specific. In this thesis we shall present further evidence that the host-encoded RNA-dependent RNA polymerase of cowpea leaves is not involved in viral RNA replication. Our results argue in favour of a virus-encoded RNA polymerase for plant virus RNA replication.

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VIROLOGY 116, 236-249 (1982)

III. Purification of a Host-Encoded RNA-Dependent RNA Polymerase from Cowpea Mosaic Virus-Infected Cowpea Leaves

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Received June 11, 1981; accepted September 15, 1981

A membrane-bound RNA-dependent RNA polymerase from cowpea mosaic virus (CPMV)-infected cowpea leaves (*Vigna unguiculata*) has been purified 15,000-fold by DEAE-Sepharose CL-6B chromatography, affinity chromatography on poly(U)-Sepharose 4B, and glycerol gradient centrifugation. Particularly, poly(U)-Sepharose 4B chromatography was a very efficient purification step and, in addition, achieved the separation of a host-encoded terminal uridylyl transferase activity from the RNA polymerase activity. On glycerol gradient centrifugation, the polymerase activity sedimented as a homogeneous peak with a rate corresponding to a molecular weight of 120,000. Analysis of the protein composition of the gradient fractions revealed that only one polypeptide with a molecular weight of 130,000 cosedimented with the polymerase activity, suggesting a monomeric enzyme. The most purified enzyme preparations from CPMV infected leaves did not contain polypeptides encoded by RNA from CPMV B-component which presumably carries functions essential for CPMV replication. Using the same purification procedure, an RNA-dependent RNA polymerase has also been purified from mock-inoculated leaves, which appeared to be identical to the RNA polymerase from infected leaves. This host enzyme was strongly stimulated in cowpea leaves infected with CPMV. The role, if any, of the RNA-dependent RNA polymerase from cowpea leaves in CPMV-RNA replication is discussed in view of the recent evidence for virus encoded functions involved in CPMV multiplication.

INTRODUCTION

Replication of plant RNA viruses with a single-stranded, (+) type RNA genome proceeds via the synthesis of a complementary (−) strand, that is copied into progeny (+) strands (Baltimore, 1971). The key role in this process is played by an RNA-dependent RNA polymerase (RNA replicase). Although such an enzyme activity has been investigated over 15 years, until recently complete purification has not yet been achieved and thus no detailed knowledge is available on the structure of the replicase and on the mechanism of plant viral RNA replication. Furthermore, no conclusive evidence has been obtained demonstrating a viral origin of the polymerizing activity of the plant viral RNA replicase, as has been shown for bacteriophage (Kamen, 1975) and animal viral RNA replicases (Flanegan and Baltimore, 1979). Upon the discovery of RNA-dependent RNA polymerases in noninfected plants (Astier-Manifacier and Cornuet, 1971; Duda *et al.*, 1973; Bol *et al.*, 1976; Romaine and Zaitlin, 1978; White and Dawson, 1978; Ikegami and Fraenkel-Conrat, 1978; Clerx and Bol, 1978; Astier-Manifacier and Cornuet, 1978; Ikegami and Fraenkel-Conrat 1979; Chifflot *et al.*, 1980), involvement of such enzymes in viral RNA replication has been proposed (Ikegami and Fraenkel-Conrat, 1978, 1979; Chifflot *et al.*, 1980; Linthorst *et al.*, 1980). Recently, Rackwitz *et al.* (1981) have found that the RNA genome of a viroid can be copied *in vitro* by DNA-dependent RNA polymerase

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II, suggesting that specific exogenous RNA may be replicated *in vivo* by host enzymes. On the other hand, as far as plant RNA viruses are concerned, there is also an increasing amount of evidence suggesting that viral encoded function(s) play a crucial role in viral replication (Mouchès *et al.*, 1976; Hardy *et al.*, 1979; Goldbach *et al.*, 1980; Robinson *et al.*, 1980; Nassuth *et al.*, 1981). To elucidate whether or not the plant virus codes for its own replicase, extensive purification, that allows for structural identification of the replicase evidently is one of the major aims to be achieved.

The most purified plant viral replicase is the replicase for cucumber mosaic virus (CMV) RNA. For that enzyme it is suggested that among several polypeptides occurring in the purified replicase preparation a 100,000 molecular weight polypeptide is encoded by the virus genome, because this polypeptide was clearly absent in a corresponding fraction from uninfected cucumber plants (Kumurasamy and Symons, 1979). Previously, Zabel *et al.* (1974) demonstrated that a cowpea mosaic virus (CPMV) RNA replicase was present in the membrane fraction of CPMV-infected cowpea leaves. The RNA-dependent RNA polymerase activity could be solubilized from the membranes by washing with a Mg^{2+}-deficient buffer (Zabel *et al.*, 1976) and further purified by DEAE-Bio-Gel A chromatography and glycerol gradient centrifugation (Zabel *et al.*, 1979). The DEAE-Bio-Gel A-purified enzyme was shown to possess no template specificity in a replicase assay, but was able to specifically immobilize CPMV-RNA on nitrocellulose filters in a template binding assay (Zabel *et al.*, 1979).

The genome of CPMV consists of two RNA molecules, denoted B- and M-RNA, respectively (Van Kammen, 1968; Bruening, 1977; Van Kammen and De Jager, 1978), which are encapsidated in separate nucleoprotein particles (B- and M-component, respectively). Both RNAs have a poly(A) track at their 3'-end and a protein (VPg; ~5K) covalently linked to their 5'-end (Daubert *et al.*, 1978; Stanley *et al.*, 1978). Goldbach *et al.* (1980) have recently shown by inoculating cowpea protoplasts with either purified B- or M-component that *in vivo* B-RNA was replicated and expressed, but not encapsidated in the absence of M-RNA. On the other hand, replication and expression of M-RNA was completely dependent on the expression of B-RNA, thus suggesting that an essential part of CPMV replicase is encoded by B-RNA (Goldbach *et al.*, 1980). This implies that one or more of the known B-RNA-encoded polypeptides (molecular weights 170,000, 110,00, 87,000, 84,000, 60,000, 32,000, and VPg) found in protoplasts after infection with B-component (Rezelman *et al.*, 1980; Stanley *et al.*, 1980), might be a component of the RNA replicase.

In this paper we report the purification of an RNA-dependent RNA polymerase from CPMV-infected cowpea leaves to near homogeneity. Using the same purification procedure, an RNA-dependent RNA polymerase has been purified from mock-inoculated leaves, which appears to be identical to the RNA polymerase from infected leaves. We conclude that a host-encoded RNA-dependent RNA polymerase is strongly stimulated upon CPMV infection of cowpea leaves. The possible role of this enzyme in viral RNA replication is discussed in view of the recent evidence for viral-encoded functions involved in CPMV replication.

MATERIALS AND METHODS

Materials. DEAE-Sepharose CL-6B (Batch 9298) and poly(U)-Sepharose 4B (Batch DH 7886) were purchased from Pharmacia. Oligo$(rU)_{10-20}$ (Batch 951-50) was from Collaborative Research Inc. and DE-81 filter disks were from Whatman. $Na^{125}I$ was obtained from the Radiochemical Centre, Amersham, England. SDS-polyacrylamide gel electrophoresis marker proteins were purchased from Bio-Rad Laboratories [high-molecular-weight kit containing myosin (210,000), β-galactosidase (116,200), phosphorylase *b* (92,500), bovine serum albumin (68,000), and ovalbumin (46,000)] and from Pharmacia [low-molecular-weight kit containing phosphorylase *b*, bovine serum albumin, oval-

bumin, carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400)], respectively. *Escherichia coli* RNA polymerase (165,000 and 155,000) and aprotinin (6500) were obtained from Boehringer-Mannheim GmbH. Human γ-globulin (Cohn fraction II) was from Sigma Chemical Company and bovine catalase, rabbit aldolase, and bovine serum albumin were from Serva Feinbiochemica, Heidelberg. The source of all other materials was as previously described by Zabel *et al.* (1979).

Virus and plants. Cowpea plants (*Vigna unguiculata* L., Walp. var. "California Blackeye") were grown as described (Zabel *et al.*, 1979). Nine days after sowing, the primary leaves were either inoculated with a crude homogenate of leaves infected with a SB isolate of CPMV in 0.1 *M* sodium phosphate buffer, pH 7.0, or mock-inoculated with buffer alone. Four days later, the inoculated leaves were harvested and immediately used for polymerase preparation.

Terminal uridylyl transferase (TUT) assay (Zabel et al., 1981). The TUT assay mixture contained in a total volume of 60 μl: 40 m*M* Tris/acetic acid, pH 8.2; 6% glycerol; 5 m*M* Mg-acetate; 1m*M* EDTA; 1.25 μg of actinomycin D; 20 μ*M* UTP; 2.5 μCi [5-^{3}H]UTP (specific activity 11.8 Ci/mmol); 0.05 A_{260} unit of oligo(rU)$_{10\text{-}20}$, and 10-μl aliquots of the enzyme preparation. The assay mixture was incubated for 1 hr at 30° and then transferred onto 23-mm Whatman DE-81 filter disks. The filters were washed batchwise: five times with 5% Na_2HPO_4 (10 ml/filter), twice with water, twice with ethanol, and once with ether (Rose and Jacob, 1976; Coleman, 1977). Each filter was treated with 0.3 ml Soluene 350 tissue solubilizer for 30 min at 50°, to solubilize the radioactivity from the filter, and subsequently counted with 7 ml Insta-Fluor in a Packard scintillation counter.

RNA-dependent RNA polymerase assay. The polymerase assay contained: 40–50 m*M* Tris/acetic acid, pH 8.2; 6–10% glycerol; 8 m*M* Mg-acetate; 1 m*M* EDTA; 1.25 μg of actinomycin D; 1 m*M* each of ATP, GTP, and UTP; 20 μ*M* CTP; 2.5 μCi [5-^{3}H]CTP (specific activity 17.4 Ci/mmol); 3 μg of unfractionated CPMV-RNA; and 5–20 μl of the enzyme preparation in a total volume of 60 μl. In some experiments 60 m*M* ammonium sulfate was added for optimal salt conditions (Zabel *et al.*, 1979). Incubation was at 30° for 1 hr and the assay mixtures were then transferred onto Whatman DE-81 filter disks and further processed as described above. A unit of polymerase activity is defined as that amount of enzyme which incorporated 1 nmol of CMP in 1 hr at 30°.

RNA-dependent RNA polymerase purification. The standard buffer used throughout the purification was TGEDP (0.05 *M* Tris/acetic acid, pH 8.2, 25% (v/v) glycerol, 1 m*M* EDTA, 10 m*M* DTE (dithioerythritol), 0.5 m*M* PMSF (phenylmethylsulfonyl fluoride), containing K-acetate as indicated. PMSF and DTE were added and dissolved just before use of the buffer. PMSF was stored as a 0.2 *M* stock solution in 96% ethanol at −20°. The whole procedure was carried out at 4°.

(i) Membrane-bound replicase was prepared from 100–200 g of CPMV-infected cowpea leaves as described by Zabel *et al.* (1974) and RNA-dependent RNA polymerase activity was solubilized by Mg^{2+} depletion (Zabel *et al.*, 1976), except that KCl was replaced by K-acetate.

(ii) About 200 ml of solubilized polymerase was applied to a DEAE-Sepharose CL-6B column (2.6 × 28 cm), equilibrated with 5-10 bed vol of TGEDP + 0.05 *M* K-acetate using a peristaltic pump at a flow rate of 100 ml/hr. After washing the column with about 200 ml of the same buffer, polymerase was eluted with 0.35 *M* K-acetate. Fractions of 4 ml were collected and 10-μl aliquots were assayed for polymerase activity. Fractions containing the bulk of polymerase activity were pooled and dialyzed against TGEDP + 0.25 *M* K-acetate. If required, the polymerase could be stored at −80° at this stage.

(iii) A poly(U)-Sepharose 4B column (0.8 × 2 cm), which had been washed with buffer containing 2.0 *M* K-acetate and equilibrated with TGEDP + 0.25 *M* K-acetate, was loaded with about 35 ml of dialyzed DEAE-Sepharose-purified poly-

merase solution at a flow rate of 10–20 ml/hr using a peristaltic pump. After extensive washing with 25 ml of buffer containing 0.25 *M* K-acetate, the polymerase was eluted with a 25-ml linear 0.25–1.25 *M* K-acetate gradient in TGED (PMSF was often omitted in this step). Fractions of 0.8 ml were collected and 5-μl aliquots were tested for terminal transferase and polymerase activity. Alternatively, after removal of TUT by stepwise elution with 10 ml of buffer containing 0.55 *M* K-acetate, the polymerase could be recovered by stepwise elution with 1.25 *M* K-acetate in TGED. Fractions containing the bulk of polymerase activity were pooled and concentrated by dialysis against saturated ammonium sulfate solution in TGEDP. Sometimes, concentration was also carried out by adding 1.66 vol of saturated ammonium sulfate in buffer without glycerol. The precipitate was collected by centrifugation (26,000 *g*, 30 min, J-13 swing out rotor in a Beckman J2-21 centrifuge) and dissolved in about 250 μl of TGEDP + 0.25 *M* K-acetate.

(iv) The concentrated poly(U)-Sepharose-purified polymerase solution was dialyzed against TEDP containing 0.25 *M* K-acetate and 10% glycerol and subsequently loaded onto a 10.5-ml linear 15–30% (v/v) glycerol gradient in the same buffer. The enzyme was centrifuged in a Beckman SW-41 rotor at 38,500 rpm for 41 hr at 4°. Gradients were pumped out from the bottom and collected in 0.5-ml fractions, using a glass capillary. Ten or twenty-microliter aliquots were tested in a polymerase assay containing 60 m*M* ammonium sulfate. Sedimentation marker proteins (catalase, aldolase, and bovine serum albumin, with approximate molecular weights of 240,000, 147,000, and 68,000, respectively) were run in a parallel gradient and used for molecular weight estimation according to Martin and Ames (1961).

SDS-polyacrylamide gel electrophoresis. Aliquots of the poly(U)-Sepharose column fractions and glycerol gradient fractions were precipitated with 20% trichloroacetic acid (TCA) in the presence of 0.5 μg of aprotinin as carrier protein. The precipitates were collected by centrifugation, washed once with ice-cold 20% TCA, twice with acetone (−20°), dried under reduced pressure, and dissolved in 8 μl of buffer containing 25 m*M* Tris/acetic acid, pH 8.2, 0.5 m*M* EDTA and 2% SDS. Iodination was essentially according to Greenwood *et al.* (1963), using 5 μCi $Na^{125}I$, 5 μg of chloramine-T, and incubation for 1 min at room temperature. The reaction was stopped by the addition of 1 vol of twice-concentrated sample buffer for electrophoresis (10 m*M* Tris/HCl, pH 8.0, 1 m*M* EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.001% (w/v) Bromophenol blue), containing 1 mg/ml NaI. Marker proteins were precipitated and iodinated in the same way. Proteins were heated for 5 min at 100° and analyzed by electrophoresis on polyacrylamide slab gels (12 × 13 × 0.15 cm) containing 12.5% acrylamide, 0.09% methylenebisacrylamide, 0.375 *M* Tris/HCl, pH 8.8, and 0.1% SDS. The stacking gel contained 4% acrylamide, 0.1% methylenebisacrylamide, 0.125 *M* Tris/HCl, pH 6.8, and 0.1% SDS. Electrophoresis was performed at 150 V for 3.5 hr or overnight at 40 V in 0.05 *M* Tris, 0.384 *M* glycine, pH 8.6, and 0.1% SDS. To remove the free iodine and most of the iodinated aprotinin, the bromophenol blue dye was allowed to migrate off the gel. Gels were fixed for 30 min in 25% isopropanol–10% glacial acetic acid, stained in 0.25% Coomassie brilliant blue (42.7% methanol and 8.3% acetic acid), and destained in 5% ethanol–7.5% acetic acid. The gel was dried onto Whatman 3MM paper and autoradiography was for 1–7 days at room temperature using Kodak safety film XRP.

Protein determination. Protein was measured according to Bradford (1976), using the Bio-Rad protein assay dye reagent concentrate and human γ-globulins as a standard.

RESULTS

Purification of RNA-Dependent RNA Polymerase

A summary of the purification procedure is outlined in Table 1. An explanation of each step is given below.

TABLE 1

SUMMARY OF PURIFICATION OF RNA-DEPENDENT RNA POLYMERASE FROM CPMV-INFECTED COWPEA LEAVES

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (*n*-fold)	Yield (%)
Membrane extract[a]	190	336	58.4	0.17	1	100
DEAE-Sepharose	36	74.5	34.3	0.46	2.7	59
Poly(U)-Sepharose[b]	0.37	0.0925	7.56	81.7	480	13
Glycerol gradient peak fraction[c]	0.5	0.0055	1.90	345.5	2032	3.3

[a] Membrane-bound replicase was prepared from 152 g of CPMV-infected cowpea leaves and RNA polymerase activity was extracted by Mg^{2+} depletion. Compared to the crude homogenate the membrane extract was 7.8-fold purified (Zabel *et al.*, 1979).

[b] After elution of TUT with 0.55 *M* K-acetate, polymerase was eluted with 1.25 *M* K-acetate in TGED, pooled, concentrated by dialysis against TGEDP saturated with $(NH_4)_2SO_4$, and dialyzed against buffer containing 0.25 *M* K-acetate.

[c] Fraction 12 (Fig. 2) was used for protein and polymerase activity determination.

(i) RNA polymerase activity was extracted from a crude membrane fraction (Zabel *et al.*, 1976), bound to a DEAE-Sepharose CL-6B column at 0.05 *M* K-acetate, and then eluted with 0.35 *M* K-acetate in TGEDP. DEAE-Sepharose, an anion exchanger that allows for high flow rates, afforded rapid and complete removal of nucleic acids present in the membrane extract and resulted in an approximately 2.5-fold purification (Table 1). About 20–25% of the protein and 50–80% of the polymerase activity applied, were recovered in the 0.35 *M* K-acetate wash. Using DEAE-Sepharose CL-6B, step elution was a prerequisite for recovery of enzyme activity, since elution with a linear gradient of 0.05–0.50 *M* K-acetate in TGEDP led to a virtually complete loss of enzyme activity, a phenomenon not previously met with DEAE-Bio-Gel A (Zabel *et al.*, 1976, Zabel *et al.*, 1979).

(ii) The DEAE-Sepharose-purified polymerase was bound to a poly(U)-Sepharose 4B column at 0.25 *M* K-acetate, which resulted in an extensive purification (±150-fold) in one step (Fig. 1, Table 1). Over 100 mg of protein could be applied to a small (0.8 × 2 cm) poly(U)-Sepharose column at this salt concentration and more than 99% of the protein was removed with complete retention of the polymerase activity. This step also achieved the removal of an UMP-incorporating activity, that contaminated the DEAE-Sepharose-purified polymerase preparation (Fig. 1). This activity was noted when we investigated—in analogy to studies performed with poliovirus replicase (Flanegan and Baltimore 1977, Flanegan and Baltimore 1979, Dasgupta *et al.*, 1979, Dasgupta *et al.*, 1980)—the effect of an oligo(U) primer on the template activity of CPMV-RNA. Dasgupta *et al.* (1980) have shown that poliovirus replicase contains in addition to a protein factor from the host, a virus-encoded core polymerase activity that can be assayed for in the absence of host factor by measuring the elongation of an oligo(U) primer annealed to either poly(A) or poliovirus RNA as template. In our case the addition of oligo(U) to a polymerase assay with CPMV-RNA also led to an increase in UMP incorporation, but this stimulation appeared to be caused by a contaminating enzyme activity that effected elongation of the oligo(U) primer in the absence of template. Further investigation showed that the UMP-incorporating activity, hereafter referred to as terminal uridylyl transferase (TUT), was specific for UTP, did not require other nucleoside triphosphates,

CPMV-RNA did copurify with the polymerase activity on the particular batch of DEAE-Bio-Gel A used previously (Zabel *et al.*, 1979) but not on DEAE-Sepharose CL-6B. The nature of this binding activity, which is specific for CPMV-infected cowpea leaves, is currently under investigation.

(v) Enzyme preparations containing about 50 μg/ml protein were stable in 25% glycerol buffers in liquid nitrogen or at −80°. In liquid nitrogen no significant loss of activity (<5%) was observed for 6 months of storage. Very dilute polymerase preparations lost significant amounts of activity upon freezing and thawing. Stability was obtained by adding bovine serum albumin (100 μg/ml) in 25 or 50% glycerol buffers at −20° or −80°.

Protein Composition

Polymerase preparations at various stages of purification were analyzed by SDS-polyacrylamide slab gel electrophoresis.

(i) The DEAE-Sepharose-purified enzyme preparation from infected and mock-inoculated cowpea leaves revealed quite different polypeptide patterns (Fig. 3A, lanes DI, DM). Besides numerous host-specified polypeptides, three known virus-specific polypeptides, having approximate molecular weights of 170K, 110K, and 87K, were clearly visible in polymerase preparations from CPMV-infected leaves upon staining with Coomassie brilliant blue (Fig. 3A, lane DI, bands marked with a dot), whereas only a minor 87K host polypeptide was detectable in mock-inoculated preparations. Similar polypeptides (along with a 84K, 60K, and 32K, which are generally not detected in stained patterns) have been identified previously in cowpea protoplasts infected with B-component of CPMV by labeling with [^{35}S]methionine (Rezelman *et al.*, 1980). The synthesis of several host-coded polypeptides was stimulated upon virus infection, although the synthesis of one major polypeptide was inhibited (Fig. 3A, lanes DI,DM).

(ii) As the poly(U)-Sepharose column binds only a small portion of the DEAE-Sepharose-purified proteins (Table 1), the polypeptide patterns of the input and flowthrough of the poly(U)-Sepharose column are indistinguishable (Fig. 3A). The viral polypeptides 170K, 110K, and 87K are also present in the unbound fraction, thus suggesting that these polypeptides are not involved in viral RNA replication. The polypeptide pattern of the gradient fractions of the poly(U)-Sepharose-purified enzyme preparation from CPMV-infected cowpea leaves reveals several bands coeluting with the polymerase activity (Figs. 1 and 3B). Polypeptides with approximate molecular weights of 210K, 130K, 98K, 54K, and 24K, and several minor bands were detected after *in vitro* labeling with ^{125}I (Fig. 3B). The lower salt part of the gradient, containing the TUT activity, comprised polypeptides with estimated molecular weights of 111K, 72K, 68K, 62K, 32K, and 26K. Staining with Coomassie brilliant blue gave similar polypeptide patterns, but required half or more of each gradient fraction. The gel patterns from mock-inoculated leaves contained many polypeptides in common with infected leaves (Figs. 3B and 4). Polypeptides with molecular weights of 130K, 111K, 90K, 72K, 68K, 62K, 32K, and 26K were detected.

(iii) The polypeptide composition of the polymerase from infected leaves after glycerol gradient purification was analyzed by Coomassie brilliant blue staining (Fig. 5A) and *in vitro* labeling with ^{125}I (Fig. 5B). Staining revealed only a few bands (Fig. 5A), even though 80% of each of the glycerol gradient fractions was used. The 130K polypeptide was the only polypeptide that cosedimented with the polymerase activity profile (Fig. 2). To improve detection of proteins, iodination was performed on the gradient fractions except for fraction 12, which had been used for protein determination. Although more polypeptides were detected upon iodination, no polypeptide other than the 130K was found to cosediment with the polymerase activity (Figs. 2, 5B). The pooled, stepwise eluted poly(U)-Sepharose-purified enzyme revealed similar polypeptides

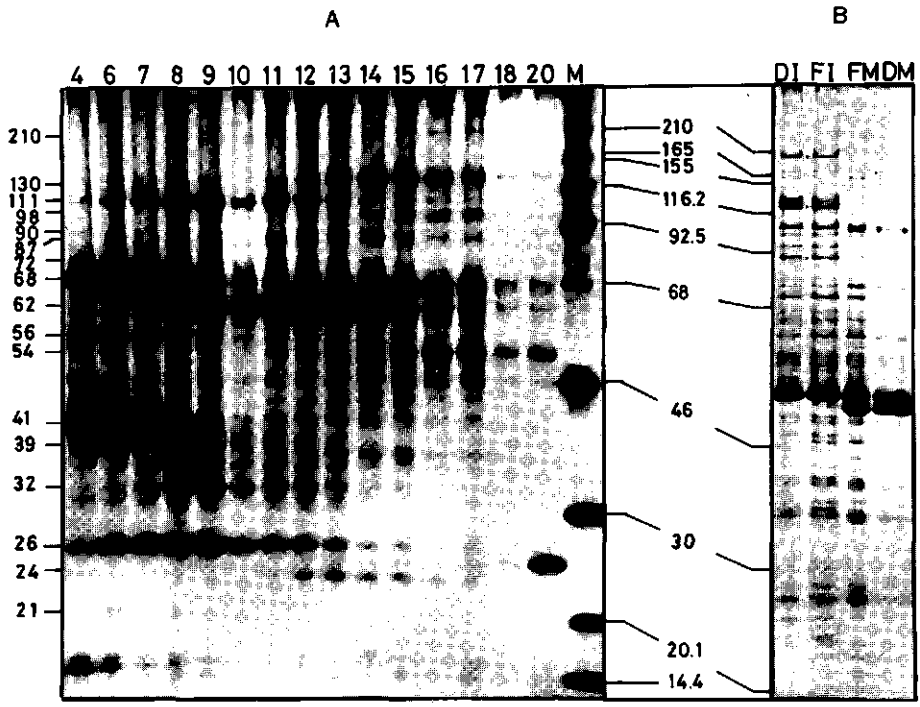

FIG. 3. SDS-polyacrylamide gel electrophoresis of proteins after poly(U)-Sepharose 4B chromatography. (A) Protein composition of DEAE-purified polymerase (D) and flowthrough fractions of poly(U)-Sepharose column (F) from CPMV-infected (I) and mock-inoculated (M) leaves after staining with Coomassie brilliant blue. All samples analyzed contained approximately 30–50 μg of protein. (B) Protein composition of poly(U)-Sepharose column fractions from CPMV-infected leaves after labeling with ^{125}I. Fifty-microliter samples from the column fractions (Fig. 1) as indicated were precipitated with TCA in the presence of aprotinin as carrier protein, washed with acetone, and labeled *in vitro* with ^{125}I. After SDS-polyacrylamide gel electrophoresis labeled proteins were detected by autoradiography. The molecular weights ($\times 10^{-3}$) of iodinated protein markers (M) are indicated between panels A and B. Molecular weights presented at the left are average values determined from several gelpatterns.

as observed in gradient eluted enzymes (Fig. 5B, lane In and Fig. 3B).

As the molecular weight of the native polymerase (approximately 120K) seems to correspond with the 130K molecular weight polypeptide present in enzyme preparations from both infected and mock-inoculated cowpea leaves, it is suggested that the 130K polypeptide is the only subunit of the purified polymerase. Attempts to obtain polypeptide patterns of the RNA-dependent RNA polymerase from mock-inoculated leaves after glycerol gradient centrifugation were unsuccessful. Because of the low concentration of enzyme a large amount of BSA had to be added to preserve a stable enzyme, which obscured many polypeptide bands after *in vitro* iodination.

DISCUSSION

Previously we reported the partial purification of an RNA-dependent RNA polymerase from the membrane fraction of CPMV-infected cowpea (*Vigna unguiculata*) leaves (Zabel *et al.*, 1979). This RNA polymerase activity has now been purified

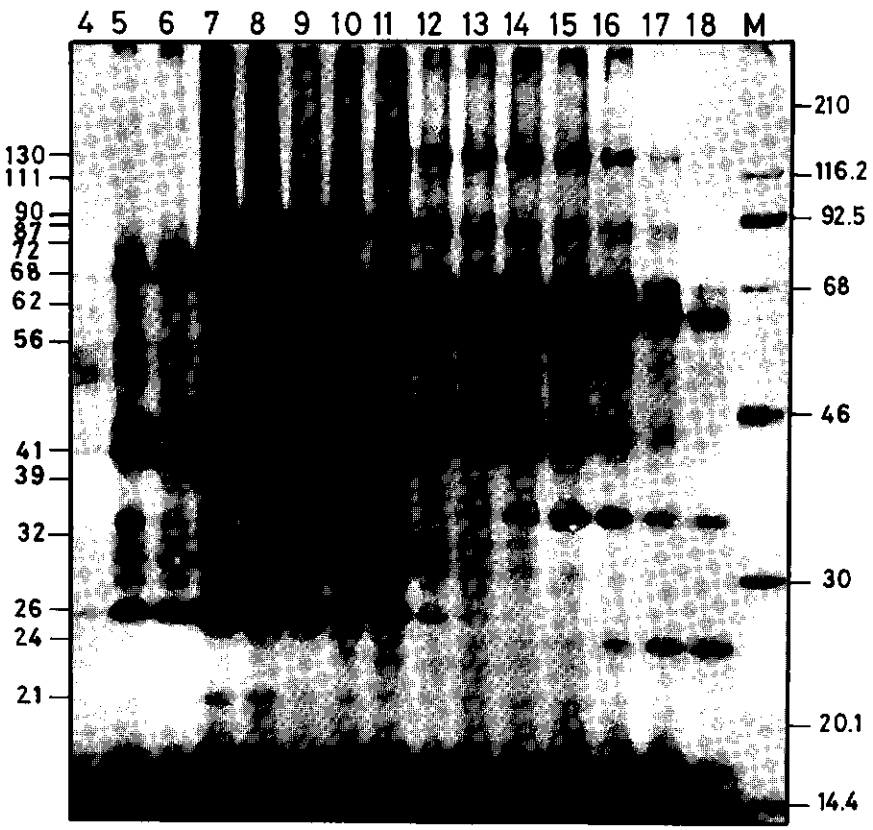

FIG. 4. SDS-polyacrylamide gel electrophoresis of poly(U)-Sepharose column fractions from mock-inoculated cowpea leaves. Fifty-microliter samples from the poly(U)-Sepharose column fractions (Fig. 1) were analyzed as described in Fig. 3B.

extensively and the purified enzyme was found to be similar to an enzyme from uninfected leaves and to consist of a 130K polypeptide. The purification procedure has been modified and extended by substituting DEAE-Sepharose CL-6B for DEAE-Bio-Gel A and including affinity chromatography on poly(U)-Sepharose 4B preceding glycerol gradient centrifugation. DEAE-Sepharose CL-6B was chosen as anion exchanger for its superior flow rate but first and foremost because the particular batch of DEAE-Bio-Gel A used previously, deteriorated upon aging with respect to its major function in the purification procedure and no longer produced template-dependent polymerase preparations. Poly(U)-Sepharose 4B chromatography turned out to play a key role in the purification procedure. In this step a 150-fold purification was accomplished and the occurrence of RNA-dependent RNA polymerase activity in mock-inoculated cowpea leaves was unambigiously established. In our previous studies such polymerase activity in uninfected leaves was not detected (Zabel *et al.*, 1974). This enzyme was overlooked as it occurs only in very low concentrations in mock-inoculated leaves and in crude preparations no template dependency can be measured. Our results indicate that the RNA-dependent RNA

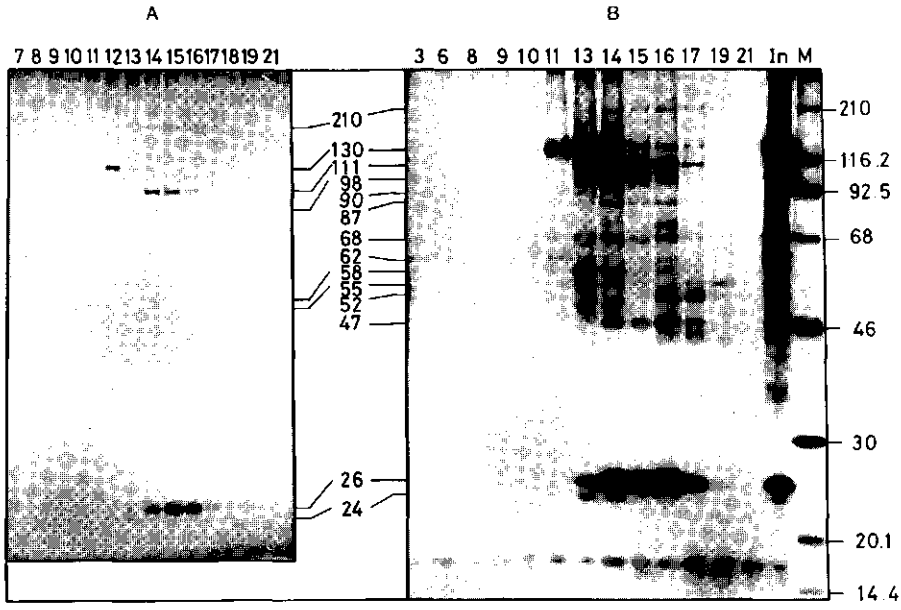

FIG. 5. Protein composition of RNA polymerase from CPMV-infected cowpea leaves after glycerol gradient centrifugation. (A) Samples (400 μl) of the glycerol gradient fractions (Fig. 2) as indicated, were precipitated in the absence of carrier protein. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by staining with Coomassie brilliant blue. (B) Samples (25 μl) of the glycerol gradient fractions (Fig. 2) as indicated, were analyzed as described in Fig. 3B. Lane In represents the concentrated input material loaded on glycerol gradient (Fig. 2).

polymerase from mock-inoculated leaves and the polymerase from CPMV-infected leaves are identical enzymes. Both enzymes are isolated from the membrane fraction of cowpea leaves and display similar properties upon purification. After infection with CPMV, the concentration of the enzyme in cowpea leaves increases at least 10-fold. This increase might be related to the increase of membrane structure in CPMV-infected cells (Assink *et al.*, 1973; Zabel *et al.*, 1974). An additional striking feature of the poly(U)-Sepharose chromatography step was that a number of virus-specific noncapsid proteins (170K, 110K, and 87K) known to be coded by B-RNA, appeared in the flowthrough and were not found to be constituents of the purified RNA polymerase. Since B-RNA can replicate independently and the replication of M-RNA depends on B-RNA (Goldbach *et al.*, 1980), it is obvious to consider B-RNA-encoded proteins as possible candidates for a role in viral RNA replication. Apparently, none of the three proteins mentioned above have polymerase activities by themselves. Another B-RNA encoded polypeptide, 60K (Goldbach *et al.*, 1980) was already lost prior to poly(U)-Sepharose chromatography as it remained bound to the DEAE-Sepharose column, upon elution of the polymerase with 0.35 *M* K-acetate (results not shown), possibly by virtue of its binding to RNA. The question arises whether we have isolated an incomplete RNA-dependent RNA polymerase which is coded by the host but requires a viral coded protein to function as a template-specific replicase (see also Romaine and Zaitlin, 1978). In this respect it is tempting to hypothesize that the B-RNA-encoded 60K polypeptide plays a crucial role in CPMV-RNA synthesis. The 60K protein was recently found to be the im-

mediate precursor of VPg, a small protein covalently linked to the 5'-ends of CPMV-RNAs (Zabel *et al.*, 1982). Therefore the 60K protein may function in specific initiation of viral RNA synthesis by supplying VPg as a primer (Flanegan *et al.*, 1977; Nomoto *et al.*, 1977; Pettersson *et al.*, 1978; Palmenberg *et al.*, 1979; Kitamura *et al.*, 1980; Pallansch *et al.*, 1980; Rueckert *et al.*, 1980). The essential feature of this model is that the viral genome does not encode the polymerase subunit of the replicase but only contributes protein factor(s) which associate with the host-coded core polymerase to constitute the holoenzyme. This view on plant viral RNA replication, which finds much favor, but is not supported yet by any experimental evidence (Romaine and Zaitlin, 1978; White and Dawson, 1978; Clerx and Bol, 1978; Ikegami and Fraenkel-Conrat, 1979; Chifflot *et al.*, 1980; Linthorst *et al.*, 1980), actually is the opposite of what is known about bacteriophage and animal viral RNA replicases (Kamen, 1975; Flanegan and Baltimore, 1979; Wimmer, 1979; Dasgupta *et al.*, 1980; Van Dijke and Flanegan, 1980). These enzymes have been shown to be composed of a viral-coded polypeptide responsible for the polymerizing activity of the enzyme, and of host-encoded subunit(s) fulfilling ancillary functions.

As discussed by Romaine and Zaitlin (1978) "an equally plausible possibility is that the host polymerase has no role in viral replication." We have therefore to face as well the possibility of having isolated the wrong RNA polymerase. The type of assay used to monitor RNA polymerase during purification may be blamed for failure in tracing the real viral RNA replicase. If CPMV follows the same replication strategy as encephalomyocarditis virus and poliovirus (Rueckert *et al.*, 1980; Wimmer, 1979) which seems attractive to assume considering the similarities between the animal picornaviruses and the plant comoviruses, the viral-coded RNA polymerase will be inactive in a polymerase assay devoid of host factor and/or a proper primer such as oligo(U) or VPg(pU) (Flanegan *et al.*, 1977; Flanegan and Baltimore, 1977; Flanegan and Baltimore, 1979; Dasgupta *et al.*, 1980). Unfortunately, the presence of a contaminating terminal uridylyl transferase activity in our crude polymerase preparations precluded the use of a template-primer to assay for virus-specific RNA polymerase similar to the assay used with polioviral RNA replicase. On the other hand, as mentioned above, the B-RNA-encoded 60K polypeptide contains VPg and might therefore be considered as replicase precursor according to the picornaviral model (Rueckert *et al.*, 1980; Pallansch *et al.*, 1980; Kitamura *et al.*, 1980). On that line of argument, purification of CPMV replicase should involve isolation of the 60K protein from CPMV-infected leaves, which as such may then be inactive as polymerase but should become coverted into an active polymerase upon generation of VPg by specific proteolytic cleavage.

Currently, we are applying antibodies raised against B-RNA-encoded polypeptides as specific probes for identifying viral proteins associated with CPMV-RNA replication in the membrane fraction of CPMV-infected cells and for elucidation of their possible function(s).

ACKNOWLEDGMENTS

We thank Ine Jongen-Neven for her technical assistence and Robert van Gorcom, Marja Kuyvenhoven, and Karel Wernars for their participation as students in this work. We are grateful to Annelies Bruins for typing the manuscript and Ab Hoogeveen for photography.

This research was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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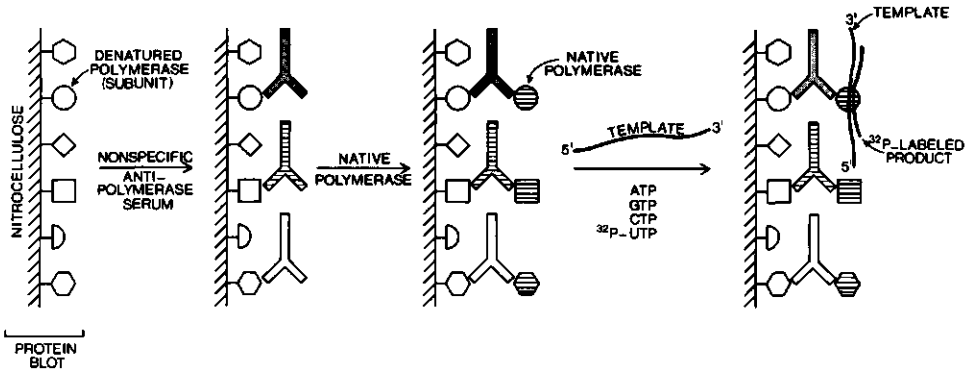


Fig. 1. Schematic representation of ALPA. Partially purified polymerase is subjected to SDS-PAGE and blotted onto nitrocellulose. After successive incubations with excess non-specific anti-polymerase serum and partially purified native polymerase, the protein blot is assayed for antibody-linked native polymerase activity using an appropriate template and ^{32}P-labeled nucleotide. The newly formed radioactive products, which are complexed to template and native polymerase, can be precipitated *in situ* on the nitrocellulose with TCA. Autoradiography of the protein blot reveals the reaction products at the position of the polymerase.

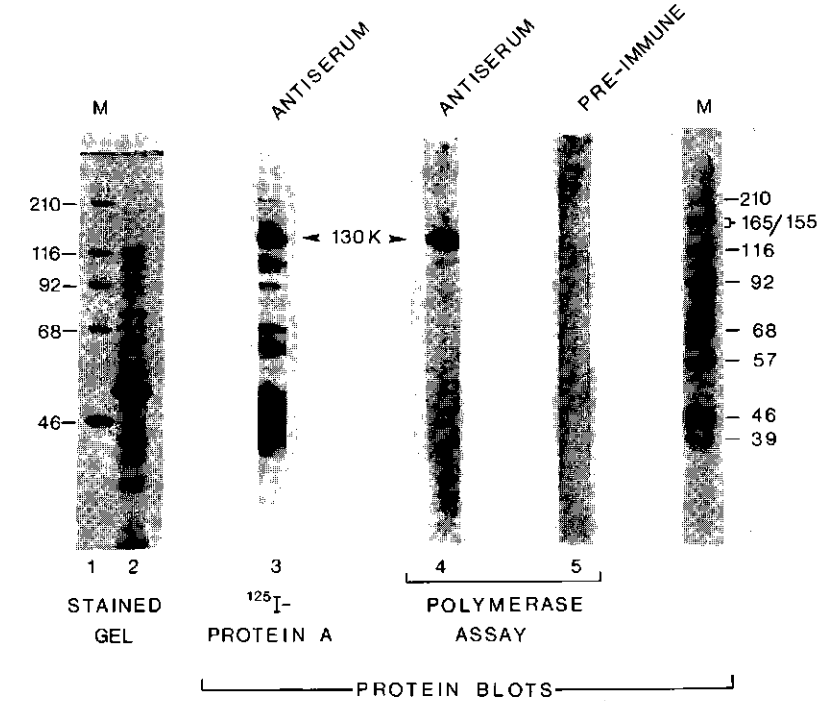
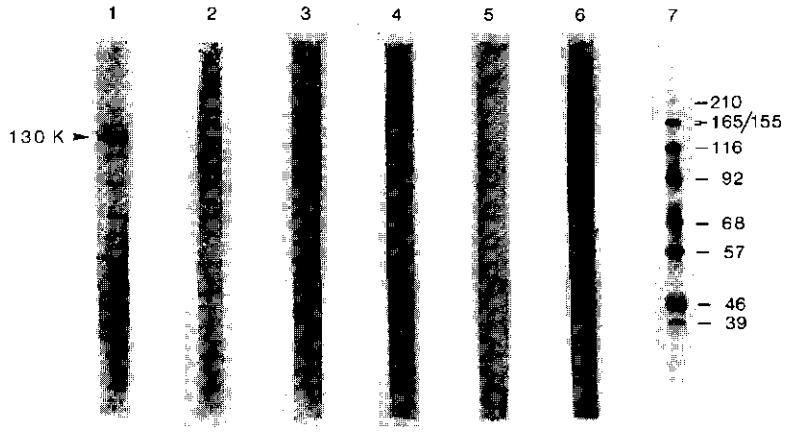


Fig. 2. Detection of RdRp by ALPA. A series of identical samples of partially purified RdRp containing ~40 μg of protein (0.064 enzyme units) was electrophoresed on a 12.5% SDS-polyacrylamide gel along with marker proteins (M). **Lanes 1** and **2** were excised and stained with Coomassie blue. Polypeptides on the other lanes were transferred to a nitrocellulose sheet which was then cut into individual strips. The strips were treated as indicated and autoradiographed. **Lane 3**: incubation with anti-RdRp serum and ^{125}I-labeled protein A. **Lane 4** and **5**: incubation with either anti-RdRp serum (**lane 4**) or preimmune serum (**lane 5**) followed by incubation with partially purified RdRp (0.16 units). These strips were then assayed for antibody-linked RdRp activity for 30 min as described in Materials and methods. **Lane 6**: ^{125}I-labeled marker proteins which were autoradiographed directly after protein transfer. Note that the RdRp-polypeptide (130 K) is not detectable by staining. Autoradiography of **lanes 4** and **5** was for 24 h without use of an intensifying screen. Numbers at the sides of the figure refer to the mol. wts. (x 10^{-3}) of the marker proteins.



ANTISERUM		+	+	+	+	−	−
PRE-IMMUNE SERUM		−	−	−	−	+	+
NATIVE POLYMERASE		+	+	+	−	+	−
ASSAY	RNA	+	−	+	+	+	+
	ATP, GTP, CTP	+	+	−	+	+	+
	^{32}P-UTP	+	+	+	+	+	+

Fig. 3. Requirements of the ALPA. Nitrocellulose strips from the protein blot shown in Figure 2 were treated with anti-RdRp serum (**lanes 1–4**) or preimmune serum (**lanes 5** and **6**) and subsequently incubated with partially purified native RdRp (**lanes 1–3** and **lane 5**) or polymerase buffer (**lanes 4** and **6**). The strips were then assayed for RdRp activity. **Lanes 1, 4–6**: complete reaction mixture. **Lane 2**: complete reaction mixture minus template RNA. **Lane 3**: complete reaction mixture minus unlabeled ATP, GTP and CTP. **Lane 7**: ^{125}I-labeled protein markers. Numbers at the right side of the figure refer to the mol. wts. (x 10^{-3}) of the marker proteins.

the labeled RNA products *in situ* with TCA, autoradiography revealed one major band at the position of the 130-K polypeptide (Figure 2, lane 4). This band was not detected on a strip which had been similarly treated with preimmune serum (Figure 2, lane 5). To rule out the possibility that the band at the position of the 130-K polypeptide merely reflected adventitious binding of [^{32}P]UTP to protein rather than incorporation into RNA, a series of control experiments was performed. As shown in Figure 3, the appearance of the 130-K band was dependent on incubating the protein blot both with anti-RdRp serum and native enzyme. Furthermore, the requirement for adding both template RNA and unlabeled ribonucleoside triphosphates (ATP, GTP and CTP) clearly showed that the 130-K polypeptide was a true RNA-dependent RNA polymerase. It should be noted that also on nitrocellulose blots encompassing the full length of the SDS-gel, with polypeptides as small as 10 K, the 130-K polypeptide was the only band detected by ALPA. Taking into acount that the 130-K polypeptide could not be detected by staining with Coomassie blue (Figure 2, lane 2) and only represented a very small fraction of the total amount of protein applied to the original gel, these results clearly show the feasibility and high sensitivity of ALPA. From calculations based on the specific activity of the most purified enzyme preparation, we estimate that <80 ng of 130-K polypeptide was present in 40 μg of partially purified enzyme, an amount that appeared to be readily detectable upon an exposure of 24 h (Figures 2 and 3).

In addition to the 130-K band, a minor band was occasionally observed just below the position of the 116-K marker protein (Figure 3). However, this band is apparently not related to polymerase activity since it occurred throughout the entire control series (Figure 3); it is likely to represent a [^{32}P]UTP-binding polypeptide on the nitrocellulose filter.

The amount of ^{32}P-labeled, TCA-precipitable product in the 130-K band was maximal after 15–20 min of RNA synthesis and accounted for ~20% of total RNA synthesis. The immobilized enzyme, however, continued to synthesize RNA for at least 60 min as determined by measuring the amount of labeled product appearing in the reaction mixture (results not shown). Apparently, growing nascent RNA chains were released form the antibody-linked enzyme upon prolonged incubation.

In the experiments described so far, UTP was used at a concentration of ~2 μM (sp. act. 31 Ci/mmol). Recently, we found that the sensitivity of the assay was further increased by using 0.6 μM of UTP (sp. act. 106 Ci/mmol). However, the use of undiluted [^{32}P]UTP (final concentration 0.12 μM; sp. act. 550 Ci/mmol) was detrimental.

Discussion

With the advent of methods to transfer proteins from SDS-

polyacrylamide gels onto a solid support (nitrocellulose or diazobenzyloxymethyl-paper) new approaches to identify specific proteins have become feasible. The immobilized proteins being readily accessible to probes can be assayed directly for their ability to bind specific ligands such as proteins, hormones or nucleic acids (Renart *et al.*, 1979; Towbin *et al.*, 1979; Bowen *et al.*, 1980). This approach has proved to be particularly fruitful for detecting and characterizing antigens by antibody binding using ^{125}I-labeled protein A from *Staphylococcus aureus* or a second antibody as a probe to reveal immune complexes (Renart *et al.*, 1979; Towbin *et al.*, 1979; Burnette, 1981; Symington *et al.*, 1981; Zabel *et al.*, 1982).

By taking advantage of the presence of two independent antigen-binding sites on a single antibody molecule, antibodies can also be used, however, to link an active (native) enzyme to its corresponding denatured polypeptide on a nitrocellulose filter. Thus, a specific solid-phase enzyme 'sandwich' is generated, the position of which can be revealed by allowing the antibody-linked native enzyme to display its characteristic activity in a subsequent assay (see Figure 1). This idea has recently been elaborated by Muilerman *et al.* (1982) to identify rat liver phosphodiesterase I using a chromogenic substrate for the antibody-linked native enzyme.

We have applied the same principle and modified the procedure of Muilerman *et al.* (1982) to allow detection of nucleic acid-synthesizing enzymes, among others, by using a radioactive substrate and TCA to precipitate *in situ* the radioactive reaction product. The essential feature of this novel and powerful enzyme-immunoassay is that – ironically enough – the use of non-specific antiserum as well as partially purified enzyme is sufficient to allow identification of a specific protein following SDS-PAGE, because only immune complexes containing active enzyme are being revealed. We have thus been able to correlate the activity of an RNA-dependent RNA polymerase from cowpea leaves (Dorssers *et al.*, 1982) with a 130-K polypeptide. In combination with our previous results indicating a native mol. wt. of ~120 K (Dorssers *et al.*, 1982), the results presented in this paper clearly demonstrate the monomeric nature of this enzyme.

Detection of the catalytic activity of polymerases following SDS-PAGE is scarcely documented and has to our knowledge only been described for DNA-dependent DNA polymerases (Spanos *et al.*, 1981; see also Chang *et al.*, 1982). The crucial point in the method of Spanos *et al.* (1981), however, is the ability of the polymerase to regain its catalytically active conformation within the gel matrix upon removal of SDS, a condition which, in our case, appeared to be difficult to fulfil (see also Muilerman *et al.*, 1982).

The procedure described here has been developed for detecting RdRp activity but should be similarly applicable to other polymerases and proteins interacting with nucleic acids. For example, by using radioactively labeled nucleic acids, this technique can be applied to detect nucleic acid-binding proteins under native conditions. More generally, it is our belief that this type of enzyme immunoassay is versatile and has potential as a tool for identifying any protein that is capable of retaining, at least temporarily, its substrate or product while being fixed to a solid support. We have found that the amount of radioactive product appearing in the complex with antibody-linked native enzyme on the nitrocellulose filter only partially reflects the activity of the enzyme. After 15 min of RNA synthesis ~20% of the labeled RNA products was maximally recovered on the nitrocellulose filter, the remainder being released in the reaction mixture. Upon longer incubation, the amount of product retained by the antibody-linked polymerase was found to decrease. Accordingly, in accommodating this enzyme immunoassay to the detection of other proteins, it would be advisable to establish reaction conditions (for example time, substrate concentration, ionic strength) which will provide maximal retention of product by the antibody-linked native enzyme.

Although not pertinent to the RdRp described in this paper, a major advantage of ALPA involves the possibility of detecting enzymes whose activities require the interaction of several different subunits. Assuming that all subunits of such an enzyme are immunogenic, a multimeric native enzyme will, in principle, bind to all its subunits on the nitrocellulose blot *via* their respective antibodies and thus give rise to a corresponding number of bands on the autoradiogram upon subsequent enzymatic assay. It will be of interest to elaborate this idea by using a model system, for example *Escherichia coli* DNA-dependent RNA polymerase or phage Qβ RNA replicase, the subunit composition of which has been firmly established. In this context, ALPA might then be used to follow the fate of a multimeric enzyme during purification and to reveal possible loss of subunits. Furthermore, ALPA does not seem to be restricted to detecting proteins which have been separated by SDS-polyacrylamide gels. Protein blots from isoelectric focusing gels or two-dimensional gels should be similarly amenable to this approach.

Finally, it should be emphasized that the anti-RdRp serum which was raised against native enzyme apparently was capable of recognizing denatured enzyme on the nitrocellulose filter. In those cases where a particular antiserum preparation displays diminished reactivity towards denatured protein, it would be advisable to use the protein blotting procedure of Bowen *et al.* (1980) which has been designed to allow (partial) renaturation of proteins prior to blotting.

Materials and methods

Materials

The following chemicals were used in addition to those mentioned previously (Dorssers *et al.*, 1982): Nonidet P40 (NP40 Bethesda Research Laboratories); Triton X-100, gelatin from calf skin type IV, ATP, GTP, CTP and UTP (*Sigma Chemical Company*); *bovine serum albumin* (BSA), crystallized (Schwarz/Mann); sodium deoxycholate (Merck); SDS, specially pure (British Drug House Chemicals Ltd.); nitrocellulose (0.45 mμ, BA85; Schleicher and Schuell); protein A from *S. aureus* (Pharmacia Fine Chemicals); [α-^{32}P]UTP, sp. act. 550 Ci/mmol (New England Nuclear). Protein A was labeled with ^{125}I to a specific activity of 4–8 μCi/μg using either the solid-phase lactoperoxidase/glucose oxidase system ('Enzymobeads', Bio-Rad Laboratories) or more recently 'IodoBeads' Pierce). A high mol. wt. protein standard kit (BioRad Laboratories) was supplemented with *E. coli* RNA polymerase (Boehringer) and pyruvate kinase (Boehringer) and labeled with ^{125}I as previously described (Dorssers *et al.*, 1982).

Purification of RdRp from cowpea and preparation of antiserum

The preparation of RdRp from cowpea plants *(Vigna unguiculata)* has been described previously (Dorssers *et al.*, 1982). In brief, RdRp was solubilized from a crude membrane fraction of cowpea leaves infected with CPMV and purified by DEAE-Sepharose CL-6B chromatography, poly(U)-Sepharose 4B chromatography and glycerol gradient centrifugation. More recently, the latter step was replaced by phosphocellulose column chromatography. For preparation of an anti-RdRp serum, the purified enzyme preparation was emulsified 1:1 with Freund's incomplete adjuvant and injected s.c. into New Zealand White rabbits using 5–15 μg of protein per injection at 2 month intervals. Blood was collected after the fifth injection and serum was used without further purification.

Preparation of partially purified RdRp for ALPA

RdRp was extracted from a crude membrane fraction of CPMV-infected cowpea leaves and taken through the DEAE-Sepharose CI-6B column chromatography step as previously described (Dorssers *et al.*, 1982). The resulting partially purified RdRp preparation is completely dependent upon the addition of template RNA and is stable upon storage (Zabel *et al.*, 1976). Enzyme activity was assayed according to Dorssers *et al.* (1982) using [^{3}H]CTP as substrate. One unit of enzyme activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of labeled CTP in 1 h at 30°C using 20 μM of CTP and 1 mM each of ATP, GTP and UTP.

SDS-PAGE

Partially purified RdRp (0.064 units, ~40 μg of protein) was adjusted to 1 x sample buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 0.001% (w/v) bromophenol blue), heated for 5 min at 100°C and subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel as previously described (Dorssers *et al.*, 1982). A series of identical samples was applied to a single slabgel alternated with ^{125}I-labeled marker proteins in every third lane.

Protein blotting

After electrophoresis, one track was excised and stained with Coomassie brilliant blue whereas the polypeptides in the other tracks were transferred electrophoretically (60V, 7 h at 4°C) to a sheet of nitrocellulose using the Bio-Rad Trans Blot electrophoretic blotting apparatus and 20 mM Tris, 150 mM glycine, 20% (v/v) methanol at pH 8.2 as transfer buffer.

Immunological detection of polypeptides on protein blots using ^{125}I-labeled protein A

After protein transfer, the nitrocellulose sheet was incubated successively with: (i) 1% (w/v) gelatin in 10 mM Tris/HCl pH 7.5, 350 mM NaCl; (ii) anti-RdRp serum or preimmune serum in RIA buffer (10 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS using 0.25 – 0.5 ml of serum per 25 ml of RIA buffer; and (iii) ^{125}I-labeled protein A in RIA buffer as described in detail elsewhere (Zabel *et al.*, 1982).

ALPA

After protein transfer, the nitrocellulose sheet was dried (1 h, 70°C) and autoradiographed to locate the position of the ^{125}I-labeled protein standards. These were then used as a guide to cut out strips corresponding to the individual lanes of the gel. The strips were each transferred into a 12 ml capped polypropylene tube where they remained stuck to the wall during subsequent incubation and washing steps, thus allowing small volumes to be used. The tubes were gently rotated end-over-end on a rotary mixer. The strips were first incubated at room temperature for 1 h in 1 ml of 10 mM Tris/HCl pH 7.5, 0.35 M NaCl containing 3% (w/v) BSA to saturate remaining protein binding sites and then for 12 – 16 h at room temperature in 400 μl of RIA buffer containing 100 μl of either anti-RdRp serum or preimmune serum. After washing with RIA buffer (three 5-ml portions, 30 min each) to remove unbound antibodies, the strips were placed on filter paper and air dried at room temperature. If desired, strips containing antibody bound by the immobilized antigen can be stored at 4°C until further use at this stage.

The strips were equilibrated with polymerase buffer (50 mM Tris-acetate pH 8.2, 25% (v/v) glycerol, 50 mM KOAc and 1 mM EDTA) by washing batchwise with two changes of 150 ml for 30 min each at room temperature. Next, they were placed back into polypropylene tubes and incubated for 12 – 16 h at 4°C in 50 μl of a partially purified RdRp preparation (0.16 units) diluted to 400 μl with polymerase buffer containing 5 mM dithioerythritol (DTE) and 0.1% (v/v) NP40. The latter was included by way of precaution to prevent aspecific binding of enzyme to proteins on the nitrocellulose strip. Following extensive washing with polymerase buffer containing 5 mM DTE and 0.1% (v/v) NP40 (five times with 5 ml, 30 – 40 min each) to remove unbound enzyme, the strips were assayed for antibody-linked RdRp by incubating in 200 μl of a reaction mixture containing 50 mM Tris-acetate pH 8.2, 12.5% (v/v) glycerol, 50 mM $(NH_4)_2SO_4$, 8 mM $Mg(OAc)_2$, 1 mM EDTA, 2 mM DTE, 4 μg actinomycin D, 1 mM each ATP, GTP and CTP, 2 μM unlabeled UTP, 0.12 – 0.15 μM [α-^{32}P]UTP and 10 μg CPMV RNA. Viral RNA was used as a convenient template since it is routinely obtained in purified form in our laboratory. The tubes containing the reaction mixtures were placed in a waterbath for 2 min at 30°C to warm up quickly and then placed in a rotary mixer in a stove at 30°C for 30 min.

At the end of the incubation period, the strips were removed from the tubes and transferred to ice cold 10% (w/v) TCA-1% (w/v) $Na_4P_2O_7$ to precipitate *in situ* newly synthesized RNA chains. The strips were washed twice with 150 ml of this solution. This was followed by washing in ice cold 5% (w/v) TCA-1% $Na_4P_2O_7$ with five changes of 200 ml for 30 – 45 min each. After drying for 1 h at 70°C, the strips were exposed to Sakura Medical X-ray film.

To determine the amount of [^{32}P]UMP incorporated into RNA chains which had been released from the antibody-linked polymerase during incubation, samples were removed from the reaction mixture, spotted onto Whatman DE-81 filter discs and processed as previously described (Dorssers *et al.*, 1982).

Acknowledgements

We thank Albert van Kammen and Rob Goldbach for stimulating discussions and critical reading of the manuscript, J.Haas for help in preparing the antiserum, Piet Madern for art work and photography and Marie-José van Neerven for typing the manuscript. This work was supported by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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VIROLOGY 125, 155–174 (1983)

V. The Cowpea Mosaic Virus RNA Replication Complex and the Host-Encoded RNA-Dependent RNA Polymerase–Template Complex Are Functionally Different

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Received July 20, 1982; accepted November 3, 1982

Purification of the putative cowpea mosaic virus (CPMV) RNA replicase previously started with an RNA-dependent RNA polymerase activity which had been solubilized from a crude membrane fraction of CPMV-infected cowpea leaves by extraction with a Mg^{2+}-deficient buffer. This led to the identification of a host-encoded, 130,000-dalton monomeric enzyme, the activity of which was highly enhanced upon infection. As the role of this enzyme in viral replication was questionable, we reverted to the template-associated RNA-dependent RNA polymerase in the crude membrane fraction in order to characterize in detail its *in vitro* products. We now demonstrate that the crude membrane fraction of CPMV-infected cowpea leaves harbors two functionally different, RNA-dependent RNA polymerase activities that are both associated with endogenous template RNA and can be separated from each other without affecting their distinct properties. One of the RNA polymerase activities was specific for CPMV-infected leaves and constituted a CPMV RNA replication complex; enzyme activity *in vitro* allowed for the completion of nascent chains initiated *in vivo*. Full-length viral RNAs (B- and M-RNA) were produced which were recovered mainly in double-stranded form. Solution- and Northern blot hybridization demonstrated that the *in vitro*-labeled RNA chains were viral RNAs of positive polarity. The other template-associated RNA-dependent RNA polymerase activity occurred in both uninfected and infected leaves and transcribed *in vitro* endogenous plant and viral RNAs only into small RNAs (4-5 S) of negative polarity. Northern blot analysis revealed the RNA products of plant origin to be transcribed from two major RNA templates of approximately 0.26 and 0.14×10^6 daltons, respectively. Washing of the crude membrane fraction in a Mg^{2+}-deficient buffer did accomplish the complete release of the low-molecular-weight RNA-synthesizing activity but did not solubilize the CPMV RNA replication complex. We tentatively conclude that the RNA-dependent RNA polymerase which has previously been purified from the buffer-soluble fraction and has been identified as a host-encoded enzyme is not involved in viral RNA replication. Solubilization of the viral replication complex was achieved with Triton X-100. By taking advantage of the characteristic conformation of the replication complex, we applied Sepharose 2B chromatography as a highly efficient and simple means for purifying the detergent-solubilized complex. We anticipate that this purification step should also be applicable to replication complexes of other RNA viruses.

INTRODUCTION

Replication of cowpea mosaic virus (CPMV) RNA is associated with vesicular membranes of a characteristic cytopathic structure appearing in the cytoplasm of cowpea cells early in infection with CPMV (Assink *et al.*, 1973; De Zoeten *et al.*, 1974; Rezelman *et al.*, 1982). This structure contains virus-specific double-stranded RNAs (Assink *et al.*, 1973; De Zoeten *et al.*, 1974) and RNA-dependent RNA polymerase molecules bound to endogenous template RNA and capable of synthesizing *in vitro*

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double-stranded and, to a lesser extent, single-stranded RNAs (Zabel *et al.*, 1974; Stuik, 1979).

In an attempt to purify and characterize the polymerase components involved in viral RNA replication, we have previously demonstrated that the bulk of the membrane-associated RNA-dependent RNA polymerase activity can be solubilized by washing the membranes in a Mg^{2+}-deficient buffer (Zabel *et al.*, 1976, 1979) and subsequently can be freed of endogenous template RNA and purified to apparent homogeneity (Dorssers *et al.*, 1982). The purified enzyme appeared to consist of a single, host-encoded 130,000-dalton (130K) polypeptide, the activity of which was greatly stimulated upon infection. However, as none of the known virus-encoded polypeptides (Rezelman *et al.*, 1980; Fransen *et al.*, 1982; Goldbach *et al.*, 1982; Zabel *et al.*, 1982) was found to be associated with the purified RNA polymerase, we presently have no indication as to which role, if any, this host-encoded RNA polymerase might play in viral replication. This uncertainty is dictated by observations indicating that replication and expression of the middle-component RNA (M-RNA) of CPMV does not occur in the absence of bottom-component RNA (B-RNA) expression whereas, on the other hand, B-RNA is capable of self-replication (Goldbach *et al.*, 1980; Stanley *et al.*, 1980; Rezelman *et al.*, 1982). Thus, replication does not seem to be mediated by the host-encoded RNA polymerase per se but apparently requires the involvement of B-RNA-encoded polypeptide(s).

To account for these findings we have recently discussed (Dorssers *et al.*, 1982) two possible explanations, namely, (i) that during isolation of the RNA polymerase we have lost essential virus-encoded polypeptides which should render the host-encoded core polymerase a bona fide replicase and (ii) that the host-encoded RNA polymerase is not involved in viral replication but has been masking the real replicase—consisting of a viral-encoded core—because of improper assay conditions applied during isolation (see also Romaine and Zaitlin, 1978; Duda, 1979).

Since no conclusive evidence is available as to the origin of the core polymerase of the replicase, we set out to reinvestigate more thoroughly the membrane-bound, RNA-dependent RNA polymerase associated with endogenous template and known to be capable of synthesizing viral RNA *in vitro* (Zabel *et al.*, 1974). Characterization and purification of this enzyme-template complex, the viral RNA replication complex, would permit the identification of core polymerase molecules engaged in viral RNA chain elongation. Such an approach has proved useful in identifying proteins that are involved in animal virus replication (Lundquist *et al.*, 1974; Clegg *et al.*, 1976; Clewly and Kennedy, 1976; Newman *et al.*, 1979; Ranki and Kääriäinen, 1979; Etchison and Ehrenfeld, 1980) but has not been pursued with plant viruses mainly because attempts to prepare template-free enzymes from solubilized complexes were successful (Hadidi and Fraenkel-Conrat, 1973; Zaitlin *et al.*, 1973; Clark *et al.*, 1974; Mouches *et al.*, 1974; Bol *et al.*, 1976; Zabel *et al.*, 1976; Le Roy *et al.*, 1977; White and Murakishi, 1977; Clerx and Bol, 1978; Hardy *et al.*, 1979; Kumarasamy and Symons, 1979; Gill *et al.*, 1981; Sommer *et al.*, 1981).

We now provide evidence showing that the crude membrane fraction of CPMV-infected cowpea leaves actually harbors two functionally different RNA-dependent RNA polymerase activities, only one of which is associated with replicating viral RNA to constitute the virus-specific replication complex. The other RNA polymerase activity which accounts for the bulk of the membrane-associated RNA polymerase activity differs from the viral replication complex in that it is transcribing plant as well as viral RNA into small RNA fragments of negative polarity. In addition, we present results indicating that the host-encoded RNA-dependent RNA polymerase activity, which has previously been purified (Dorssers *et al.*, 1982), is not associated with the viral RNA replication complex and has no function in synthesizing viral RNAs of positive polarity.

MATERIALS AND METHODS

Materials. The following chemicals were used in addition to those mentioned pre-

viously (Dorssers *et al.*, 1982): cetyltrimethylammonium bromide (CTAB) and dimethylsulfoxide (E. Merck); CF-11 cellulose (Whatman Ltd.); Sepharose 2B (Pharmacia Fine Chemicals); [α-^{32}P]UTP, code PB.163, spec act > 400 Ci/mmol (Radiochemical Centre, Amersham, England); [α-^{32}P]UTP, code NEG-007X, [α-^{32}P]ATP, code NEG-003X, spec act > 400 Ci/mmol, and Na^{125}I, code NEZ-033A (New England Nuclear); aminobenzyloxymethyl(ABM) paper, 2-aminophenylthioether(APT) paper, and mixed bed resin, AG 501-X8 (Bio-Rad); yeast RNA type III, Triton X-100, RNase T_1 grade IV, and RNase A, Type I-A (Sigma Chemical Co.); glyoxal (Fluka A. G.); Sarkosyl NL 97 (Ciba-Geigy); disodium triisopropylnaphthalene sulfonate, Control G (Serva Feinbiochemica); 4-aminosalicylic acid, sodium salt (BDH Chemicals Ltd.); agarose, Sea-Kem (FMC Corp.); DNase I (Worthington Biochemical Corp.).

Virus and plants. Cowpea plants (*Vigna unguiculata* L., Walp. var. California Blackeye) were grown and inoculated with an SB isolate of cowpea mosaic virus as described previously (Dorssers *et al.*, 1982). Primary leaves were usually harvested between 88 and 93 hr after inoculation and used directly for the preparation of enzyme extracts.

Purification and iodination of CPMV-RNA. Total virion RNA was prepared from purified virus as described by Zabel *et al.* (1982). Fifty micrograms of viral RNA was iodinated according to Commerford (1980), purified on a Whatman CF-11 cellulose column (Prensky, 1976), and stored at −20° in TNE buffer (0.05 *M* Tris/HCl, pH 7.5, 0.1 *M* NaCl, 1 m*M* Na_3EDTA) containing 25% (v/v) ethanol at −20°.

Purification of leaf RNA. A crude membrane fraction, prepared as described below, from CPMV-infected or mock-inoculated cowpea leaves was diluted with an equal volume of twice concentrated detergent mixture (final concentrations: 0.088 *M* glycine, 0.012 *M* NaOH, 0.088 *M* NaCl, 0.01 *M* Na_3EDTA, pH 9.0, containing 2%, w/v, Sarkosyl, 2%, w/v, 4-aminosalicylic acid and 1%, w/v, disodiumtriisopropylnaphthalene sulfonate) and extracted three to four times with 1 vol of phenol–cresol (500 ml of phenol, 70 ml of cresol, 0.5 g of hydroxyquinoline, and 55 ml of water). The aqueous layer was adjusted to 0.25 *M* ammonium acetate and RNA was precipitated with 2.5 vol of ethanol at −20° for 16 hr. RNA precipitate was collected by centrifugation (20,000 *g*, 15 min), dissolved in sterile water, and reprecipitated twice with ethanol.

Purification of CPMV double-stranded RNAs (RF_B and RF_M). Total RNA was isolated from 400 g of CPMV-infected cowpea leaves using the cetyltrimethylammonium bromide (CTAB) procedure of Murray and Thompson (1980) as originally developed for the rapid isolation of high-molecular-weight plant DNA. The CTAB–nucleic acid precipitate was washed three times with 70% ethanol containing 1 *M* Na-acetate (Bellamy and Ralph, 1968) and dissolved in water and then the nucleic acids were precipitated with ethanol. The nucleic acids were dissolved in 1–2 ml of 10 m*M* Tris/HCl, pH 7.5, 10 m*M* Mg-acetate containing 25 μg/ml DNase I and incubated at 37° for 1 hr. The solution was diluted 10-fold with TNE buffer containing 17.5% (v/v) ethanol and passed twice through a Whatman CF-11 cellulose column (1.5 × 12 cm) equilibrated with the same buffer. Under these conditions, most DNA and single-stranded RNA molecules are not bound to the column. After extensive washing of the column with TNE-buffer containing 17.5% ethanol, double-stranded RNAs were eluted with water, lyophilized, dissolved in 0.5 ml of TNES (TNE buffer containing 0.5%, w/v, SDS) and layered onto 15–30% (w/v) sucrose gradients in TNES. Centrifugation was for 20 hr at 27,000 rpm in a Beckman SW-28 rotor at 20°. Peak fractions were pooled, dialyzed against water, lyophilized, and dissolved in 0.5 ml of TNES. Full-size double-stranded RNAs were then obtained by gel filtration on a Sepharose 2B column (1.5 × 50 cm) in TNES buffer. Peak fractions containing double-stranded CPMV B-RNA (RF_B) and CPMV M-RNA (RF_M) were pooled, lyophilized, washed three times with 80% ethanol, dissolved in water, and stored in aliquots at −20°. Approximately 200 μg of purified double-stranded CPMV RNA was obtained from 400 g of leaves.

Isolation and solubilization of mem-

brane-bound RNA-dependent RNA polymerases. (i)The crude membrane fraction of the cellular extract was prepared from primary leaves as described (Dorssers *et al.*, 1982; Zabel *et al.*, 1979) and suspended in TGKED buffer (50 m*M* Tris/acetic acid, pH 8.2, 25% v/v, glycerol, 50 m*M* K-acetate, 1 m*M* Na_3EDTA, and 5 m*M* dithioerytritol) using a Thomas homogenizer and 0.5 ml of buffer per gram of fresh leaf tissue. The suspension was stored in aliquots at −80° and is referred to as the crude membrane fraction.

(ii) Solubilization of host-encoded RNA-dependent RNA polymerase activity was carried out by washing the crude membrane fraction with a Mg^{2+}-deficient buffer (0.625 ml/g leaf) as described in detail elsewhere (Zabel *et al.*, 1976; Dorssers *et al.*, 1982). This procedure results in the extraction of approximately 80% of the total RNA polymerase activity from the crude membrane fraction (Zabel *et al.*, 1976) and yields a preparation which is referred to as the buffer-soluble fraction. The remaining membrane fraction containing the residual RNA polymerase activity was resuspended in TGKED buffer (0.5 ml/g of leaf) as described above and is referred to as the washed membrane fraction.

(iii) To solubilize the CPMV RNA replication complex, the washed membrane fraction was collected by centrifugation (31,000 *g*, 30 min, 4°), resuspended in TGKED buffer containing 0.2% (v/v) Triton X-100 (0.25 ml/g of fresh leaf tissue), stirred for 1 hr at 4°, and centrifuged at 31,000 *g* for 1 hr. The clear supernatant was removed, and the pellet was reextracted once more using 0.25 ml of the same buffer per gram of leaf tissue. The combined supernatants were stored at −80° and are referred to as the detergent soluble fraction.

(iv) The detergent-solubilized replication complex was further purified by gel filtration on a Sepharose 2B column (1 × 50 cm) in TKED buffer containing 15% (v/v) glycerol and 0.1% (v/v) Triton X-100; 0.2-1.0 ml of the detergent soluble fraction was applied and eluted at a flow rate of about 2.5 ml/hr using a peristaltic pump. Fractions of 0.4-0.8 ml were collected and 45-μl aliquots were assayed for polymerase activity using [^{3}H]UTP.

RNA-dependent RNA polymerase assay. The standard assay mixture (60 μl) contained the following: 40-60 m*M* Tris/acetic acid, pH 8.2, 8 m*M* Mg-acetate, 0.8-1.2 m*M* Na_3EDTA, 25 m*M* ammonium sulphate, 1.25 μg of actinomycin D, 2.5-10 μ*M* of either [α-^{32}P]UTP (8-130 Ci/mmol) or [α-^{32}P]ATP (20-50 Ci/mmol), 1 m*M* each of the other three unlabeled ribonucleotides and 20 to 47 μl of the enzyme fraction. Reaction mixtures were incubated at 30° for times as indicated in the legends of the figures and Table 1, and incorporation was measured by spotting aliquots on DE-81 filter disks as described by Dorssers *et al.* (1982).

Purification and fractionation of in vitro synthesized RNA. (i) The reaction mixture was diluted with 1 vol of twice concentrated detergent mixture (see Purification of leaf RNA) and extracted two or three times with 1/2 vol phenol-cresol. The aqueous layer was adjusted to 0.25 *M* ammonium acetate and RNA was precipitated with ethanol, using 10 μg/ml phenol-treated yeast RNA as carrier. The RNA pellet was dissolved in sterile water and reprecipitated twice with ethanol to remove unincorporated nucleoside triphosphates.

(ii) To obtain partially double-stranded RNA (RI) and double-stranded RNA (RF), the precipitate of RNA was dissolved in 0.2-0.5 ml of TNES buffer and fractionated by Sepharose 2B column (0.9 × 15 or 1.0 × 27 cm) chromatography. CPMV RI and RF, which are both excluded from Sepharose 2B, were recovered by ethanol precipitation in the presence of carrier RNA.

(iii) Fractionation of RNA by Whatman CF-11 cellulose column chromatography was performed according to Mouches *et al.* (1974). RNA in TNE buffer containing 35% (v/v) ethanol was passed twice through a 0.8 × 2-cm column of CF-11 cellulose and eluted successively with TNE buffer containing 15% (v/v) ethanol and water to provide single-stranded RNAs (15% ethanol fraction) and double-stranded RNAs (0% ethanol fraction), respectively

(Franklin, 1966). RNA was recovered by ethanol precipitation.

(iv) Poly(U)-Sepharose 4B chromatography was used for isolation of poly(A)-containing RNAs. Purified RNA from a CF-11 cellulose column was denatured in 0.1 ml of water (3 min at 100°), diluted with 1 ml of ice-cold high-salt buffer (0.05 *M* Tris/HCl, pH 7.5, 0.7 *M* NaCl, 0.01 *M* Na_3EDTA, and 25% (v/v) formamide), applied to a 0.8 × 2-cm poly(U)-Sepharose 4B column and processed as described by Phillips *et al.* (1980). Radioactivity was determined by Cerenkov counting.

RNA gel electrophoresis. RNA samples to be analyzed under nondenaturing conditions were dissolved in sterile water, mixed with 1/3 to 1/5 vol of layer mix (7 *M* urea, 20%, w/v, sucrose and 0.1% bromophenol blue) and electrophoresed in horizontal, "submarine," 1.25% agarose gels (12.5 × 12.5 × 0.6 cm) in Tris–borate buffer, pH 8.3 (Peacock and Dingman, 1968). Electrophoresis was for 3–4 hr at 60–70 V.

RNA samples to be analyzed under denaturing conditions were fully denatured in 1 *M* glyoxal, 50% (v/v) dimethyl sulfoxide, 10 m*M* Na-phosphate buffer, pH 7.0, for 1 hr at 50° (McMaster and Carmichael, 1977), mixed with 1/3 vol of 25% (w/v) Ficoll–0.1% bromophenol blue and subjected to electrophoresis in horizontal, "submarine," 1.5% agarose gels in 10 m*M* Na-phosphate buffer, pH 7.0, for 3 hr at 70 V. Control experiments using unlabeled RF_B and RF_M revealed that under these conditions both double-stranded RNAs were completely denatured as inferred from their migration to the position of single-stranded viral RNAs.

If required, nonglyoxalated RNAs were visualized by staining with 1–2 μg/ml ethidium bromide in water. Glyoxalated RNAs were stained as described below. For autoradiography gels were dried onto Whatman 3MM paper and exposed to Sakura X-ray films.

Transfer of RNA to DBM and DPT paper. CPMV double-stranded RNAs (0.5–1 μg), CPMV virion RNAs (1–2 μg), and total RNA (5–10 μg) prepared from crude membrane fractions of both CPMV-infected and mock-inoculated cowpea leaves were denatured by glyoxalation and fractionated by agarose gel electrophoresis as described above. After electrophoresis the gel was gently shaken in 200 ml of 50 m*M* NaOH containing 5 μg/ml ethidium bromide for 30 to 45 min at room temperature, neutralized with two washes (15 min each) of 200 ml of 0.2 *M* Na-acetate buffer (pH 4.0), and photographed. RNA was then transferred electrophoretically (10 V, overnight at 4°) onto either diazobenzyloxymethyl (DBM) paper or diazophenylthioether (DPT) paper in 0.2 *M* Na-acetate buffer (pH 4.0) using the Bio-Rad Trans-Blot electrophoretic blotting apparatus. Activation of aminobenzyloxy (ABM) paper to DBM paper and of 2-aminophenylthioether (APT) paper to DPT paper was carried out as described by Alwine *et al.* (1979) and Rezaian and Jackson (1981), respectively. Unreacted diazonium groups were blocked by incubation with a solution of 1% (w/v) glycine at 37° for 16 hr.

Blot hybridization. Blots (4.5 × 10 cm) were prehybridized at 50° for 16 hr with 3 ml of 1.1 times concentrated hybridization buffer (50% formamide (deionized by treatment with mixed bed resin), 5× SSC (1× SSC = 0.15 *M* NaCl, 0.015 *M* Na-citrate, pH 7.2), 50 m*M* Na-phosphate, pH 7.0, 0.3% SDS, and 100–200 μg/ml of phenol-treated yeast RNA) in a sealed bag. The labeled probe, dissolved in 0.3 ml of water, was denatured at 100° for 10 min, cooled in an ice-water bath and added to the prehybridization mixture. After resealing, incubation was continued at 50° for 64 hr. After hybridization blots were washed four times with 100-ml portions of 2× SSC containing 0.1% SDS at 50–55° for 1 hr, dried, and exposed to Sakura X-ray film for autoradiography.

Solution hybridization. In vitro synthesized RNAs were lyophilized, washed two or three times with 80% ethanol to remove residual salts, dissolved in sterile water and transferred to 1.5 ml conical Eppendorf tubes containing unlabeled RNA as indicated in Table 1. The total sample volume was adjusted to 36 μl with water and the reaction mixture was overlaid with 30 μl of paraffin oil. After heating at 100° for

TABLE 1

SOLUTION HYBRIDIZATION OF RNA SYNTHESIZED *IN VITRO* BY RNA-DEPENDENT RNA POLYMERASE PRESENT IN CRUDE MEMBRANE FRACTION, BUFFER-SOLUBLE FRACTION AND WASHED MEMBRANE FRACTION

Conditions	RNase-resistent RNA (%) synthesized by RNA polymerase in			
	Crude membrane fraction[a]			
	0% ETOH CF-11 fraction	15% ETOH CF-11 fraction	Buffer-soluble fraction[b]	Washed membrane fraction[c]
No re-annealing[d]				
Before melting, no RNase (2× SSC)	100 (3192)[e]	100 (5074)	100 (2970)	100 (1377)
Before melting + RNase (2× SSC)	101.8	75.0	81.0	101.9
After melting + RNase (2× SSC)	2.6	3.9	2.5	<1
After melting + RNase (0.1× SSC)	1.6	1.9	1.0	ND
Re-annealing[f]				
Self-annealing	30.7	33.7	23.9	12.8
+CPMV RNA (10 μg)	14.7-16.7[g]	56.7	42.9	7.8
+BMV RNA (10 μg)	ND	36.7	ND	ND
+Yeast RNA (10 μg)	31.4	ND	21.6	ND
+RF (0.5 μg)	73.2	43.3	34.3	91.0
+RF + CPMV RNA (10 μg)	16.3	56.0	42.5	6.0
+RF + BMV RNA (10 μg)	77.8	47.0	ND	ND
+RF + yeast RNA (10 μg)	77.2	ND	34.5	96.7
Hybridization temperature (°)	80	70	70	80

[a] RNA products as obtained from the experiment described in Fig. 4 were used.

[b] RNA products prepared from the particular reaction mixture as described in Fig. 5A (lane 1), after elution from a CF-11 cellulose column by a single step with water. RNA was recovered by ethanol precipitation, dissolved in 2× SSC and digested with a mixture of RNase A and T_1 as described in Fig. 4.

[c] RNA products prepared from the particular reaction mixture described in Fig. 5A (lane 3), purified by Sepharose 2B column chromatography. Double-stranded viral RNA (the major reaction product, see Fig. 5A) was recovered from the void volume and used without additional RNase treatment.

[d] Reaction mixtures were heated as indicated, adjusted to the appropriate SSC concentration, treated with RNase A plus T_1 as specified, and then spotted onto DE-81 filter disks.

[e] cpm in parentheses.

[f] After addition of competitor RNAs, the reaction mixtures were heated, quickly cooled, and incubated as described under Materials and Methods.

[g] RNase resistance figures correspond to annealing in the presence of 5 and 20 μg of CPMV virion RNA, respectively.

10 min and quick cooling in dry ice–ethanol, the reaction mixtures were adjusted to 2× SSC by adding 4 μl of 20× SSC and incubated for 210 min at either 70° or 80° as indicated in Table 1. Unhybridized RNA was completely digested by adding 1/10 vol of a RNase mixture containing 200 μg/ml RNase A and 60 U/ml RNase T_1 and incubation at 37° for 1 hr. The complete contents of the reaction vial was then transferred onto DE-81 filter disk and processed as described previously (Dorssers *et al.*, 1982). All hybridizations were assayed in duplicate.

RESULTS

Time Course of Appearance of Membrane-Bound RNA Polymerase Activity

In search for replicase activity in CPMV-infected cowpea leaves we initially detected, by measuring incorporation of

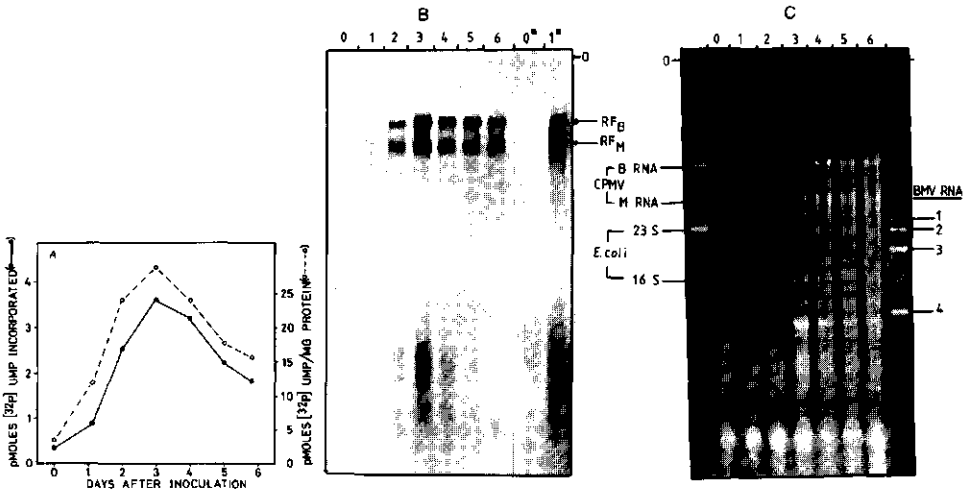

FIG. 1. Time course of appearance of RNA-dependent RNA polymerase in crude membrane fraction of cowpea leaves after inoculation with CPMV. Nine-day-old primary leaves were inoculated with CPMV and portions of four primary leaves were harvested 0, 26, 48, 72, 96, 120, and 140 hr after inoculation. After removal of the midribs, the leaves were washed, wiped dry, and stored at −80°. The crude membrane fraction was prepared as described under Materials and Methods and suspended in 2 ml of TGKED buffer, and a 30-μl aliquot was assayed for 30 min in a standard assay mixture containing 10 μM of [α-^{32}P]UTP (spec act, 8.7 Ci/mmol). A 6-μl sample was removed and spotted onto a DE-81 filter disk to determine the amount of incorporation. (A) Results are expressed as number of picomoles of [^{32}P]UMP incorporated per assay (● —— ●) or per milligram of protein (○ - - - ○), respectively. Protein contents were determined according to Bramhall *et al.* (1969) using ovalbumin as a standard. (B, C) The remainder of each reaction mixture was mixed with an equal volume of a detergent mixture (see Materials and Methods), RNA was prepared by phenol–cresol extraction and half of the preparation was analyzed in a 1.25% agarose gel. The following RNAs were used as marker: brome mosaic virus (BMV) RNA 1–4 (MW 1.1, 1.0, 0.7, and 0.3×10^6, respectively; Ahlquist *et al.*, 1981), CPMV B- and M-RNA (MW 2.39 and 1.39×10^6, respectively; Murant *et al.*, 1981) and *Escherichia coli* 23 S and 16 S ribosomal RNA (MW 1.009 and 0.534×10^6, respectively; Murant *et al.*, 1981). After electrophoresis, the gel was stained, photographed (C), dried onto Whatman 3 MM paper, and autoradiographed (B). Numbers on top of the gel indicate days after inoculation. Lanes 0* and 1* refer to lane 0 and 1, respectively, but after a fourfold longer exposure to reveal weak zones. The 0 indicated at the side of the gel refers to the origin and RF_B and RF_M indicate the position of the double-stranded RNAs of CPMV B- and M-RNA, respectively.

[^{3}H]UMP, a membrane-associated RNA-dependent RNA polymerase activity that appeared on Day 1 after inoculation and increased rapidly to reach a maximum on Days 3 and 4 (Zabel *et al.*, 1974). We have now reexamined the appearance of this RNA polymerase activity with special emphasis on the nature of its *in vitro* RNA products.

Nine-day-old primary cowpea leaves were inoculated with CPMV and portions of primary leaves were harvested at daily intervals. The crude membrane fraction was isolated, assayed *in vitro* using [α-^{32}P]UTP, and the labeled RNA products were resolved in a nondenaturing agarose gel. From Day 1 on, enzyme preparations from infected leaves were found to synthesize two major size classes of RNA, one of which comprised two distinct high-molecular-weight RNA species which comigrated with double-stranded CPMV B-RNA (RF_B) and M-RNA (RF_M) (Fig. 1B). Incorporation of label into RF_B and RF_M was maximal 3 days after inoculation, coincided with total RNA polymerase activ-

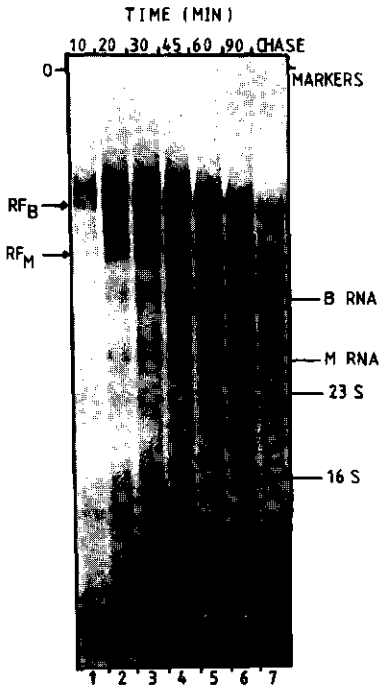

FIG. 2. Time course of RNA synthesis by RNA-dependent RNA polymerase in crude membrane fraction. Seven reaction mixtures (120 μl) each containing 40 μl of crude membrane fraction and 10 μM of [α-^{32}P]UTP (spec act, 7.8 Ci/mmol) were incubated under standard conditions for the times indicated. After 20 min, one reaction mixture was adjusted to 0.8 mM UTP and incubation was continued for 40 min (chase). RNA products were prepared as described and resolved by electrophoresis in a 1.25% agarose gel. RF_B, RF_M, B-RNA, M-RNA, 23 S RNA, and 16 S RNA refer to the position of marker RNAs as described in Fig. 1.

ity as measured by [^{32}P]UMP incorporation (Fig. 1A), and preceded the appearance of single-stranded viral RNAs as revealed by staining (Fig. 1C). In addition to these known viral RNA products, considerable incorporation of [^{32}P]UMP was observed into RNA species rather small ($< 0.3 \times 10^6$ dalton) and heterogeneous in size (Fig. 1B).

Membrane-fractions prepared from uninfected leaves displayed virtually no RNA polymerase activity (Figs. 1A and B, lane 0). RNA products were only detectable after exposing the autoradiogram for a long period and appeared to be similar in size to the class of small RNAs synthesized by the membrane fraction from CPMV-infected leaves (Fig. 1B, lane 0* and 1*). Apparently, infection by CPMV leads both to the appearance of an RF-synthesizing activity (hereafter also referred to as viral replication complex) and to a dramatic increase of a host-encoded RNA-dependent RNA polymerase activity that is synthesizing only small RNAs. It should be noted that incorporation of [^{32}P]UMP required the presence of all four ribonucleoside triphosphates (data not shown) and thus was not due to terminal uridylyl transferase activity (Zabel *et al.*, 1981). Moreover, similar results were obtained using [α-^{32}P]ATP as the labeled substrate (see Fig. 5B).

Characterization of RNA Products of RNA-Dependent RNA Polymerase in Crude Membrane Fraction

Analysis by agarose gel electrophoresis. Since the major *in vitro* product of the viral replication complex as described above consisted of RF_B and RF_M, it was of interest to study the kinetics of appearance of other viral RNAs including replicative intermediate (RI) and single-stranded RNAs. Hence, a time course experiment was performed in which reaction mixtures were incubated for various times and RNA products were analyzed in nondenaturing agarose gels (Fig. 2). After short periods of incubation a smear of radioactivity upward from the RF bands (presumably RI) was observed, which disappeared with time and apparently faded into RF_B and RF_M and possibly some single-stranded B- and M-RNA. A pulse–chase experiment demonstrated that the pulse-labeled smear (Fig. 2, lane 2) indeed could be chased into discretely sized RF_B and RF_M (Fig. 2, lane 7) and, therefore, should represent the replicative intermediate.

The results presented so far show that the crude membrane fraction of CPMV-infected cowpea leaves harbors the viral replication complex and is capable of synthesizing viral RNAs *in vitro*. To establish the actual size of the *in vitro*-labeled RNA

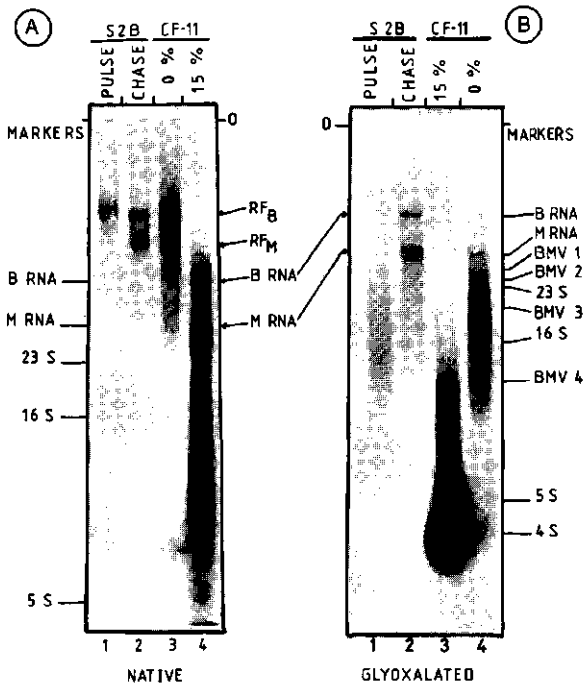

FIG. 3. Characterization of RNA products of RNA-dependent RNA polymerase in crude membrane fraction by nondenaturing and denaturing gel electrophoresis following fractionation by Sepharose 2B or cellulose CF-11 chromatography. A reaction mixture (120 μl) containing 50 μl of crude membrane fraction, 10 μM of [α-^{32}P]UTP (spec act, 13 Ci/mmol) and 8 μg/ml human placental RNase inhibitor was incubated under standard conditions. Half of the reaction mixture was removed after 11 min (pulse, lane 1 in A and B), whereas the other half was incubated for an additional 19 min in the presence of excess (1.6 mM) unlabeled UTP (chase, lane 2 in A and B). RNA products were extracted and fractionated on a Sepharose 2B (S2B) column (0.9 × 15 cm). Double-stranded RNA (RI and RF) was recovered from the void volume of the column by ethanol precipitation and analyzed both under nondenaturing (A) and denaturing conditions (B) using a 1.25 and 1.5% agarose gel, respectively. Another reaction mixture (300 μl) containing 125 μl of crude membrane fraction and 10 μM of [α-^{32}P]UTP (spec act, 13 Ci/mmol) was incubated for 30 min. RNA products were extracted, fractionated by CF-11 cellulose chromatography to obtain single-stranded RNA (15% ethanol fraction; lane 4 in A, lane 3 in B) and double-stranded RNA (0% ethanol fraction; lane 3 in A, lane 4 in B), and subsequently analyzed by electrophoresis as described above.

strands, purified double-stranded pulse-labeled and pulse–chase products (RI and RF) were analyzed by agarose gel electrophoresis under denaturing conditions as described by McMaster and Carmichael (1977). The pulse-labeled RI (Fig. 3A, lane 1) appeared to consist of growing nascent chains (Fig. 3B, lane 1) which were converted into full-length B- and M-RNA upon the chase (Fig. 3B, lane 2). Obviously, polypeptide(s) required for proper elongation of nascent chains are associated with the RI so as to generate *in vitro* full-length viral RNAs.

To determine the size of the RNA products not associated with RI and RF, RNA was extracted from a reaction mixture which had been incubated for 30 min and subsequently fractionated by CF-11 cellulose column chromatography. Nucleic acids were eluted successively with buffer containing 15% ethanol and buffer without ethanol to obtain single-stranded and double-stranded RNAs, respectively. Gel

electrophoresis under nondenaturing conditions showed that the 15% ethanol fraction was free of RF_B and RF_M and comprised a wide variety of differently sized RNA products, some of which migrated to the position of single-stranded viral RNAs (Fig. 3A, lane 4). After glyoxalation, however, the newly synthesized RNA actually appeared to consist of rather small molecules migrating mainly to the position of 4-5 S RNA with some species up to the position of the BMV RNA 4 marker (0.3×10^6 dalton) (Fig. 3B, lane 3). Presumably, the 15% ethanol fraction contained unlabeled endogenous RNA template strands complexed to short complementary RNA transcripts synthesized *in vitro*. This result also implies that CF-11 cellulose chromatography does not discriminate between true single-stranded RNAs and those bearing short complementary pieces of RNA as was also suggested by Mouches *et al.* (1974).

The 0% ethanol fraction predominantly consisted of RF plus RI (Fig. 3A, lane 3), which upon glyoxal treatment appeared to contain growing strands and some completed B- and M-RNA chains (Fig. 3B, lane 4).

Analysis by poly(U)-Sepharose chromatography. CPMV virion RNAs have been shown to contain a 3'-terminal poly(A) sequence (El Manna and Bruening, 1973; Ahlquist and Kaesberg, 1979). To investigate whether RNA synthesis *in vitro* included generation of a poly(A) tail, [^{32}P]AMP-labeled RNA products as obtained from a CF-11 cellulose column were denatured and applied to a poly(U)-Sepharose column. Less than 1% of the RNA products present in the 15% ethanol fraction was bound by the column. In contrast, 26% of the labeled RNA in the 0% ethanol fraction was bound and subsequently recovered upon elution with buffer containing 90% (v/v) formamide (Phillips *et al.*, 1980). This result suggests that only the labeled RNA products derived from the double-stranded RNA fraction contained poly(A).

Analysis by solution and blot hybridization. We have shown so far that the *in vitro* products of the RNA-dependent RNA polymerase(s) in the crude membrane fraction resolve into two distinct classes comprising growing viral RNA strands and small RNA molecules complexed to a short region of single-stranded RNAs. However, the results do not bear upon the polarity of the newly synthesized viral strands nor on the nature of the small RNA products. In an attempt to solve this question, solution and blot hybridization experiments were performed. Total *in vitro* synthesized RNA was extracted from a large-scale reaction mixture and fractionated by CF-11 cellulose chromatography into a single-stranded RNA (15% ethanol fraction) and a double-stranded RNA (0% ethanol fraction). Both RNA fractions were then treated with a mixture of RNase A and RNase T1 to destroy single-stranded RNA which would interfere in the hybridization reaction. To compensate for the smaller average length of the class of small RNA products as compared to the labeled RNA in the double-stranded RNA fraction, the 15% ethanol RNA fraction was hybridized for 3.5 hr at 70° and the 0% ethanol RNA fraction for 3.5 hr at 80°, respectively (Davis *et al.*, 1980). The results of the solution hybridization experiments are presented in Table 1 and expressed as the percentage of RNA products in an RNase-resistant hybrid. With the 0% ethanol fraction, self-annealing amounted to 30.7% and was found to diminish to 16.7% in the presence of excess unlabeled CPMV RNA but not yeast RNA. By adding purified RF, annealing was strongly enhanced to 73.2%, a stimulation which in turn was abolished by CPMV RNA but not by BMV RNA nor yeast RNA. From these data we conclude that the majority of the *in vitro*-labeled RNA that is present in the double-stranded RNA fraction represents plus-type CPMV RNA. A minor portion, however, seems to be either nonviral or minus strand CPMV RNA as judged from the fraction (16%) that remained RNase resistant after annealing with either CPMV RNA alone or CPMV RNA plus RF. In contrast to the 0% ethanol fraction, annealing of the RNA in the 15% ethanol fraction was enhanced both by the addition of single-stranded virion RNA and RF (Table 1) indicating the

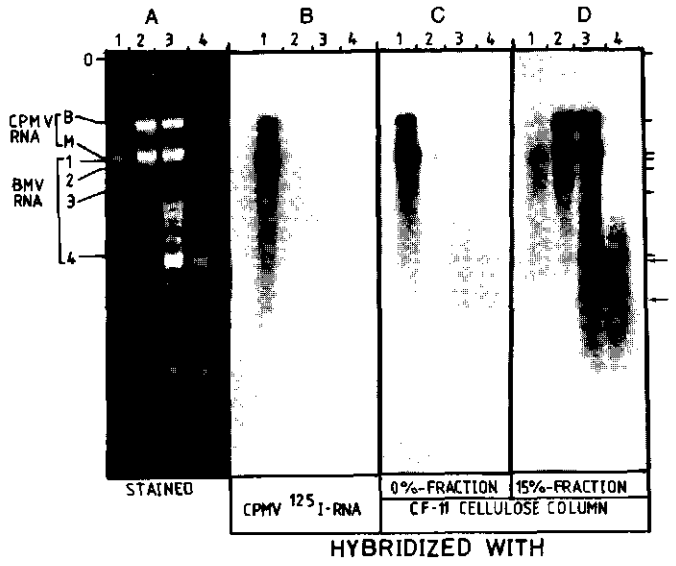

FIG. 4. Hybridization of RNA products of RNA-dependent RNA polymerase in crude membrane fraction to Northern blots of plant and viral RNA species. A reaction mixture (240 μl) containing 150 μl of crude membrane fraction, 10 μM of [α-^{32}P]UTP (spec act, 38 Ci/mmol) and 2.5 μg/ml human placental RNase inhibitor was incubated for 2 hr. RNA products were prepared as described and fractionated by CF-11 cellulose column chromatography to provide single-stranded RNA (15% ethanol fraction) and double-stranded RNA (0% ethanol fraction). RNA was recovered by ethanol precipitation, dissolved in 2× SSC buffer, and digested with 0.5 μg/ml RNase A and 50 U/ml RNase T1 at 37° for 30 min. After two cycles of phenol extraction, RNA was recovered by ethanol precipitation and applied in the hybridization reaction as described. Purified CPMV RF_B and RF_M (lane 1), CPMV virion RNA (lane 2), and total RNA extracted from the membrane fraction of CPMV-infected (lane 3) and uninfected leaves (lane 4) were treated with glyoxal and resolved by agarose gel electrophoresis. After staining (A), the RNAs were transferred to DBM paper and incubated as described with 1×10^5 cpm of ^{125}I-labeled CPMV virion RNA (B), 3×10^4 cpm of the 0% ethanol fraction RNA (C), and 4×10^4 cpm of the 15% ethanol fraction RNA (D). Autoradiography was for 12 (B) and 22 days (C and D), respectively, at room temperature.

presence of minus-type viral RNAs among the *in vitro* products. However, more than 40% of the RNA was not made ribonuclease resistant by annealing with excess RF or viral RNA and, hence, may contain nonviral RNA sequences (Table 1).

We next characterized the nature of the *in vitro* synthesized RNA by blot hybridization. In control experiments, iodinated virion RNA hybridized strongly, as might be expected, with purified RF (Fig. 4B, lane 1) but not with virion RNA (Fig. 4B, lane 2) nor with RNA from mock-inoculated leaves (Fig. 4B, lane 4). The *in vitro* synthesized RNA from the 0%-ethanol fraction hybridized strongly with purified RF (Fig. 4C, lane 1) and only very weakly with virion RNA (Fig. 4C, lane 2). These results corroborate our previous conclusion that the *in vitro* labeled RNA chains of the RF are predominantly viral RNAs of positive polarity. In contrast, *in vitro* synthesized RNA from the 15% ethanol fraction hybridized strongly both with purified virion RNAs (Fig. 4D, lane 2) and with viral RNAs present in the extract from infected leaves (Fig. 4D, lane 3). Remarkably, the 15% ethanol fraction also hybridized strongly with various plant RNA species of heterogeneous size, among which two bands (marked with an arrow) at the position of approximately 0.26 and 0.14×10^6 daltons

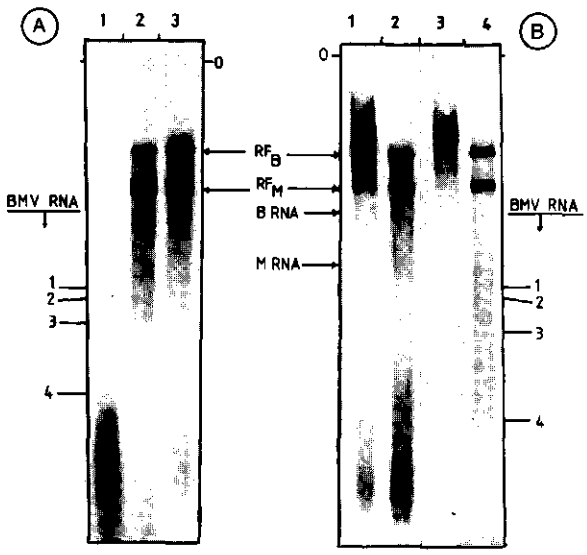

FIG. 5. Agarose gel electrophoresis of RNA synthesized *in vitro* by RNA-dependent RNA polymerase present in crude membrane fraction, washed membrane fraction, and buffer-soluble fraction. (A) Reaction mixtures (60 μl 5 μM [α-^{32}P]UTP, spec act, 50 Ci/mmol; 2.5 μg/ml human placental RNase inhibitor) containing 43 μl of enzyme (buffer-soluble fraction, lane 1; crude membrane fraction, lane 2; washed membrane fraction, lane 3) were incubated for 2 hr. RNA products were prepared and separated by nondenaturing agarose gel electrophoresis as described under Materials and Methods. The buffer-soluble and washed membrane fraction were derived from a crude membrane preparation that was different but comparable to the one shown in lane 2. (B) A washed membrane fraction was prepared by washing a crude membrane fraction (same as in A, lane 2) for three times with a total volume of 6.5 ml of TGKED buffer per gram of original leaf tissue. Sixty microliters of the original crude membrane fraction and 40 μl of the washed membrane fraction were assayed in 120-μl reaction mixtures containing 10 μM [α-^{32}P]ATP (spec act, 8 Ci/mmol). After 15 min, half of the reaction mixture was removed (lane 1, crude membrane fraction; lane 3, washed membrane fraction) and the other half was incubated for an additional 20 min in the presence of 0.5 mM unlabeled ATP (lane 2, crude membrane fraction; lane 4, washed membrane fraction). RNA products were prepared and separated by nondenaturing agarose gel electrophoresis as described under Materials and Methods.

were most prominent (Fig. 4D, lanes 3 and 4). These results suggest that besides the RNA polymerase which is associated with the viral replication complex, there is a class of membrane-associated RNA polymerase molecules transcribing both plant and viral RNA species into short complementary RNA pieces of negative polarity.

Solubilization of RNA-Dependent RNA Polymerase Activity and Characterization of RNA Products

We have previously shown that washing of the crude membrane fraction with a Mg^{2+}-deficient buffer renders more than 80% of the RNA polymerase activity soluble (Zabel *et al.*, 1976, 1979). Upon further purification, the buffer-solubilized RNA polymerase activity turned out to be a host-encoded enzyme, the activity of which is greatly stimulated in infected cells (Dorssers *et al.*, 1982). As our current strategy of identifying the RNA polymerase responsible for replicating viral RNA requires solubilization of the replication complex and, hence, of the RF-synthesizing activity, we next compared the products of the buffer-solubilized RNA polymerase activity with those of the mem-

brane-associated activity. As in the earlier experiments, RNA polymerase in the crude membrane fraction synthesized viral double-stranded RNAs (RF_B and RF_M) (Fig. 5A, lane 2). However, the *in vitro* products of the buffer soluble fraction did not include RF_B and RF_M but exclusively consisted of small RNAs (Fig. 5A, lane 1). The *in vitro* synthesis of RF_B and RF_M was completely confined to the washed membrane fraction (Fig. 5A, lane 3); even extensive washing of the membrane fraction (three times with a total of 6.5 ml of buffer per gram of original leaf tissue instead of the usual 0.625–1.25 ml/g) did not alter this distribution (Fig. 5B). Furthermore, the replication complex in the washed membrane fraction was capable of synthesizing RI and RF in a manner indistinguishable from that in the crude membrane fraction as shown in Fig. 5B. Thus, the viral replication complex with the major functions required for proper elongation of nascent viral RNA chains remains firmly membrane bound under conditions which allow the complete release of the host-encoded RNA polymerase activity.

To further characterize the RNA products, hybridization experiments were carried out. Evaluation of the data shown in Table 1 reveals that the *in vitro* products of the buffer soluble fraction are similar to the 15% ethanol fraction RNA products of the crude membrane fraction in containing both CPMV-specific and nonviral RNA sequences. A strikingly different picture was obtained after analysis of the purified double-stranded products of the RNA polymerase in the washed membrane fraction. Annealing was diminished from 12.8% (self-annealing) to 7.8% by virion RNA, whereas up to 91% of the label was driven into hybrid by adding RF (Table 1). Since hybridization to RF could almost completely be abolished by adding excess of CPMV virion RNA we conclude that virtually all of the RNA synthesized *in vitro* by the washed membrane fraction consists of viral RNA of positive polarity.

As the nature of the small RNA products of the buffer-solubilized RNA polymerase was only partially resolved by solution hybridization, we next applied blot hybridization. To bypass treatment of the labeled reaction products with RNase prior to hybridization—a procedure that is required to degrade unlabeled single-stranded RNAs present in the reaction mixture but on the other hand also degrades approximately 40–60% of the small reaction products (data not shown)—the RNA products were glyoxalated and purified by agarose gel electrophoresis. The labeled RNA species were located on the wet gel by autoradiography and the RNA products at the position of 4–5 S (see Fig. 3B) were eluted, and subsequently applied in the hybridization reaction. Just as was the case with the small RNA molecules synthesized by the crude membrane fraction (Fig. 4D and 6B), the RNA products of the buffer-soluble fraction hybridized strongly with RNA of both plant and viral origin (Fig. 6D). From the hybridization patterns we conclude that the products in both cases are similar. Taken together, the separate data on the products made by the buffer solubilized RNA polymerase and the replication complex present in the washed membrane fraction apparently complement each other so as to provide together the composite picture of the RNA products made in the crude membrane fraction.

In discussing the time course of appearance of the membrane-bound RNA polymerase activities in virus-infected cells, we have shown that the membrane fraction from uninfected cells displayed a very low level of RNA polymerase activity (Fig. 1B, lane 0). It was nevertheless of importance to compare the products of this "silent" polymerase with those of the membrane-associated RNA polymerases from infected cells. Figure 6C shows that the small RNA products of the "silent" polymerase from uninfected cells exhibit a hybridization pattern that is virtually similar (of course, except for the CPMV-specific sequences) to the pattern obtained with the small RNA products of the RNA polymerase of infected leaves. We presently have no indication as to whether the plant RNA templates, the most prominent being approximately 0.26 and 0.14×10^6 daltons, represent messenger- or nonmessenger-type RNA molecules.

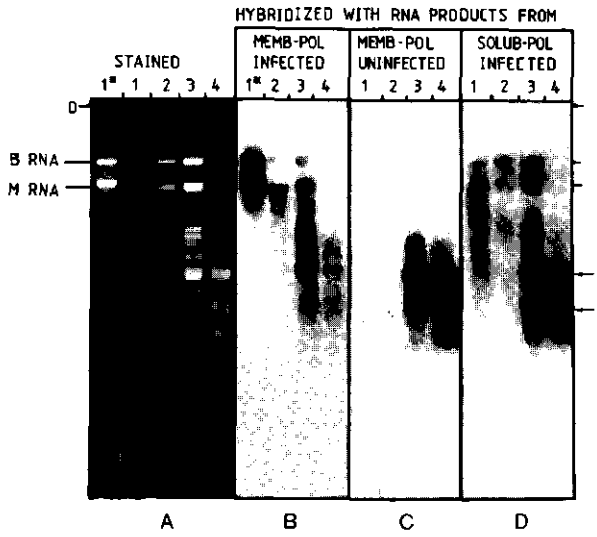

FIG. 6. Hybridization of small RNAs synthesized *in vitro* by RNA-dependent RNA polymerase in crude membrane fraction and buffer-soluble fraction to Northern blots of plant- and virus-specific RNAs. Three reaction mixtures (120 μl 10 μM [α-^{32}P]UTP, spec act, 70 Ci/mmol; 2.5 μg/ml human placental RNase inhibitor) containing 84 μl of enzyme (crude membrane fraction, MEMB-POL, from mock-inoculated and infected leaves, or buffer-soluble fraction, SOLUB-POL, from infected leaves) were incubated for 2 hr. RNA products were extracted, denatured by glyoxalation, and resolved by agarose gel electrophoresis as described. After autoradiography of the wet gel for 2 hr at 4° the radioactive band corresponding to the 4-5 S region was excised and homogenized by pressing through a syringe and the RNA eluted with three 5-ml portions of 0.5 *M* ammonium acetate containing 0.1% SDS for 48 hr at room temperature. The RNA was then lyophilized, dissolved in 1 ml of 0.2 *M* ammonium bicarbonate (pH 10), and incubated for 2 hr at 30° to remove the glyoxal adduct. After lyophilization, the RNA was dissolved in water and applied in the hybridization reaction. Virus- and plant-specific RNA species (as described in Fig. 4, lanes 1-4) were fractionated by gel electrophoresis, stained (A), and transferred to DPT paper. Lane 1* contains purified CPMV RF without prior glyoxalation. Blots were hybridized with 4.5 and 1×10^4 cpm of RNA products of the RNA polymerase in the crude membrane fraction prepared from infected (B) and mock-inoculated (C) leaves, respectively, and with 6×10^4 cpm of RNA products of the buffer-solubilized RNA polymerase (D). Autoradiography was for 3 weeks at room temperature (B and D) and for 9 days at −80° using a Cronex lightning Plus intensifying screen (C).

Solubilization and Isolation of CPMV RNA Replication Complex

We have shown so far that washing of the membranes with a Mg^{2+}-deficient buffer effectively accomplished the release of a loosely bound host-encoded RNA-dependent RNA polymerase activity but did not affect the membrane association of the viral RNA replication complex. Solubilization of the replication complex has been achieved with Triton X-100. The detergent-solubilized complex retained its capacity to synthesize viral double-stranded RNA, implying that protein(s) responsible for chain elongation were not detached from the template during solubilization (Fig. 7, inset, lane 2). Glycerol gradient centrifugation revealed the detergent-solubilized replication complex to sediment between 20 and 30 S and thus to be free of small membrane fragments (data not shown).

It is well known that Sepharose 2B chro-

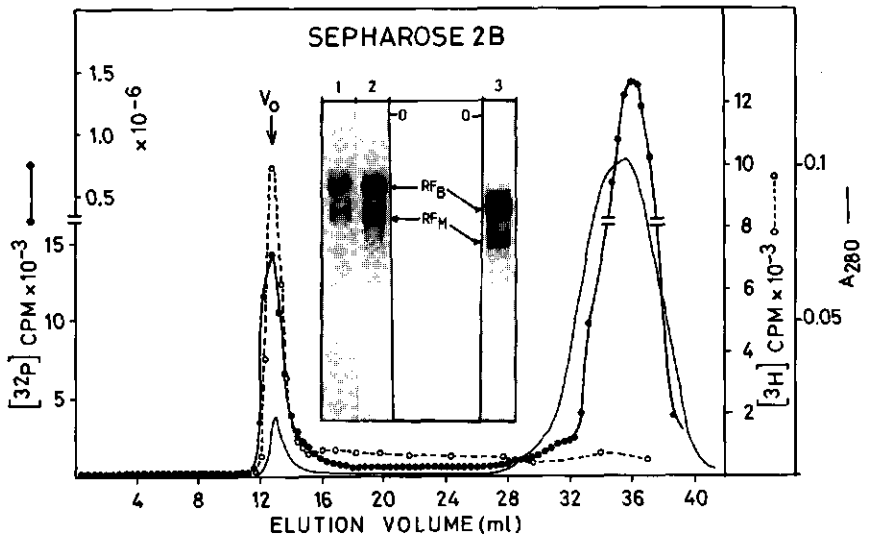

FIG. 7. Purification of CPMV RNA replication complex by Sepharose 2B column chromatography. Detergent-soluble fraction (200 μl) was pulse-labeled for 10 min (assay mixture of 240 μl 5 μM [α-^{32}P]UTP, spec act, 8 Ci/mmol) and, after cooling on ice, chromatographed on a Sepharose 2B column as described. Radioactivity in the column fractions (0.4 ml) was determined by Cerenkov counting (● —— ●). In a separate experiment, detergent-soluble fraction (500 μl) was applied to the same column without prior pulse-labeling. Fractions (0.8 ml) were collected and 45-μl aliquots were assayed for RNA polymerase activity in a standard assay mixture containing 8.8 μM [^{3}H]UTP (spec act, 4.7 Ci/mmol) (○ - - - ○). Inset shows an autoradiograph of a nondenaturing agarose gel of products synthesized by the original washed membrane fraction (lane 1) and the detergent-solubilized replication complex before and after purification through Sepharose 2B column (lanes 2 and 3, respectively). The assay mixtures (60 μl) contained 47 μl of the respective enzyme preparations and 5 μM [α-^{32}P]UTP (spec act, 15 Ci/mmol) and were incubated for 30 min.

matography provides a simple and highly efficient means for the purification of double-stranded RNA (RF and RI) (Erikson and Gordon, 1966; Baltimore, 1968; Girard, 1969; Oberg and Philipson, 1969). Both RNA species are by virtue of their configuration excluded from the column while single-stranded RNAs, proteins, and globular particles such as ribosomes and virions are retained. Accordingly, one would expect that a CPMV RNA replication complex containing RNA polymerase molecules associated with RI also elutes in the void volume of a Sepharose 2B column and is separable from the majority of the solubilized proteins. Preliminary experiments, using a detergent solubilized complex pulse-labeled *in vitro* with [^{32}P]UTP prior to gel filtration, indeed demonstrated that most of the incorporated radioactivity was recovered in the void volume of the column (Fig. 7). When an unlabeled detergent-solubilized replication complex was subjected to Sepharose 2B chromatography, RNA polymerase activity eluted in the void volume, clearly separated from the bulk of protein (Fig. 7). This highly purified replication complex was still capable of synthesizing viral double-stranded RNAs (Fig. 7 inset, lane 3).

DISCUSSION

The results presented in this paper underline the importance of studying the viral replication complex. We have shown that the crude membrane fraction from CPMV-infected cowpea leaves contains

two functionally different, RNA-dependent RNA polymerase activities which are both associated with endogeneous template and can be separated from each other without affecting their distinct properties. We tentatively conclude that the RNA polymerase activity which has previously been purified from the buffer-soluble fraction and has been identified as a host-encoded, 130K-dalton protein (Dorssers *et al.*, 1982) is not involved in viral RNA replication. This conclusion is based on the observation that the RNA polymerase activity displaying the essential features of a viral replication complex (Girard *et al.*, 1967; Girard, 1969; McDonnell and Levintow, 1970; Bishop and Levintow, 1971; Michel and Gomatos, 1973; Etchison and Ehrenfeld, 1981) remains tightly associated with the membrane fraction under conditions which allow release of the loosely bound host-encoded RNA polymerase. Recently immunological studies using antibodies against the host-encoded RNA polymerase have shown that indeed this enzyme does not form part of the viral replication complex purified through a Sepharose 2B column (J. van der Meer, R. Hanemaaijer, L. Dorssers and P. Zabel, unpublished results).

Since more than 80% of the crude membrane-associated RNA polymerase activity became solubilized by washing the membranes with a Mg^{2+}-deficient buffer (Zabel *et al.*, 1976), purification of the presumed replicase previously started with the buffer-soluble fraction, thereby not taking into account—wrongly as it appears now—that the 20% of the polymerase activity still associated with the membrane fraction, might represent specifically the CPMV replication complex.

The function of the RNA-dependent RNA polymerase in uninfected leaves remains as yet an enigma. Enzymes with similar activity have been detected in a variety of plants but have not been related to any physiological role (for references see Zaitlin, 1979; Dorssers *et al.*, 1982; Linthorst, 1982). Remarkably, their activity is greatly enhanced upon virus infection but, as mentioned by Romaine and Zaitlin (1978) the same occurs, albeit less, in response to injury from inoculation. The results presented in this paper lead us to conclude that the host-encoded RNA-dependent RNA polymerase in cowpea is not associated with viral replication. The enzyme appears to be engaged in transcribing plant RNA species (and after infection also viral ones) into short complementary pieces of negative polarity. At this stage, however, we can not exclude that the small size of the *in vitro* synthesized RNAs is due to nicks in the template strand generated during isolation of the enzyme-template complex. Further characterization of the endogenous template RNAs transcribed by this host enzyme and the products made may provide the first clue to understand its role.

The following features of the viral replication complex are worth mentioning.

(i) The complex consists of polymerase molecules which are bound to endogeneous template strands and can resume *in vitro* the elongation of nascent strands initiated *in vivo*. The *in vitro* synthesized RNA is virus specific and mainly of plus-strand polarity. A similar conclusion has been reached for a variety of plant viral replication complexes (Gilliland and Symons, 1968; Bradley and Zaitlin, 1971; Semal and Kummert, 1971; Jacquemin, 1972; Lafleche *et al.*, 1972; Zaitlin *et al.*, 1973; Mouches *et al.*, 1974; Weening and Bol, 1975; Stussi-Garaud *et al.*, 1977; Clerx and Bol, 1978). The low level of minus strand RNA synthesized by the replication complex may indicate that *in vivo* mainly virion RNAs are being produced at the time the cellular extract is made. Synthesis of a greater proportion of minus strands may be expected to occur at earlier stages of infection (Zaitlin *et al.*, 1973). Surprisingly, the replication complex of black beetle virus produces *in vitro* exclusively minus strand RNA (Guarino and Kaesberg, 1981).

(ii) The pulse-chase experiment shown in Fig. 3 clearly demonstrates that the polymerase molecules are capable of elongating the nascent chains completely; full-length viral RNAs were produced, many of them containing a poly(A) tract as ob-

served with *in vitro* products of the replication complex of poliovirus (Spector and Baltimore, 1975; Dorsch-Häsler *et al.*, 1975).

(iii) From the three known types of virus-specific RNA, namely the replicative intermediate (RI), the replicative form (RF) and single-stranded RNA, the former two were found to be the major *in vitro* products. In general, most of the RNA synthesized by membrane-bound plant viral RNA replication complexes is present in RNase-resistant double-stranded form (RF) (for review see references in Zaitlin, 1979, and Linthorst, 1982), suggesting that most of the nascent strands have been lost and/or degraded during isolation of the replication complex. Alternatively recent studies by Etchison and Ehrenfeld (1981) show that disruption of the membrane structure which normally provides the matrix for the replicase to function properly can affect the ability of the replicase to synthesize full-size single-stranded RNAs (see also Caliguiri and Tamm, 1970; Caliguiri, 1974; Clewley and Kennedy, 1976).

(iv) Following release of the loosely bound host RNA polymerase-template complex by extraction with a Mg^{2+}-deficient buffer, solubilization of the virus-specific replication complex required treatment of the membrane fraction with a nonionic detergent. Triton X-100 appeared to meet our requirements of rendering the replication complex soluble into an enzymatically active form without loss of the endogenous template.

By taking advantage of the characteristic conformation of the replication complex which causes it to be excluded from Sepharose 2B despite its relatively low sedimentation rate (20–30 S), we have been able to purify the detergent-solubilized replication complex extensively in a single step. Further studies are undertaken now to reveal its polypeptide composition.

ACKNOWLEDGMENTS

We are grateful to Dr. P. Blackburn for the generous gift of human placental RNase inhibitor, Ton van Berkel and Roeland Hanemaaijer for their participation in the characterization of the replication complex, Ab Hoogeveen for photography, Dr. Rob Goldbach for critical reading of the manuscript, and Marie-José van Neerven for typing the manuscript. This research was supported by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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VI. RNA-DEPENDENT RNA POLYMERASE FROM COWPEA IS NOT INVOLVED IN COWPEA MOSAIC VIRUS RNA REPLICATION

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Albert van Kammen and Pim Zabel

SUMMARY

An antiserum raised against the purified host-encoded 130K RNA-dependent RNA polymerase (RdRp) from cowpea leaves, was applied to examine a possible role of this enzyme in cowpea mosaic virus (CPMV) RNA replication.

Using ^{125}I-labeled protein A to detect immunocomplexes on protein blots it is shown that the amount of host-encoded RdRp increases more than twenty-fold in cowpea leaves upon infection with CPMV. This increase of RdRp appeared to be restricted to tissue cells infected with CPMV and actively engaged in virus multiplication. In striking contrast, no increase of RdRp was found to accompany CPMV RNA replication in cowpea mesophyll protoplasts after infection with CPMV, indicating that increased production of RdRp is not a prerequisite for CPMV RNA replication. Furthermore, by using the anti-RdRp serum, it was demonstrated that the host-encoded RdRp is not present in the partially purified CPMV RNA replication complex, which is capable of synthesizing full-length viral RNAs. We conclude that the host-encoded RdRp is not responsible for RNA polymerase activity in the viral replication complex and thus is not involved in viral RNA replication. The strong increase of RdRp seems to represent a tissue-specific host response to virus infection, the physiological significance of which is yet not clear.

INTRODUCTION

Since the discovery of an RNA-dependent RNA polymerase (RdRp) activity in chinese cabbage plants by Astier-Manifacier and Cornuet in 1971, a wide variety of plant species has been found to contain a similar enzyme, including tobacco (Duda *et al.*, 1973; Le Roy *et al.*, 1977; Clerx and Bol, 1978; Ikegami and Fraenkel-Conrat, 1978a; Romaine and Zaitlin, 1978), cowpea (Ikegami and Fraenkel-Conrat, 1978b; White and Dawson, 1978; Dorssers *et al.*, 1982), tomato (Boege and Sänger, 1980), cauliflower (Astier-Manifacier and Cornuet, 1978), *Phaseolus* (Lazar *et al.*, 1979), broad bean (Weening and Bol, 1975), barley (Hardy *et al.*, 1979) and cucumber (Linthorst, 1982; Takanami and Fraenkel-Conrat, 1982). The physiological role of RdRp in plants is unknown, but a striking feature is that the RdRp activity strongly increases upon virus infection. As different researchers aiming at the elucidation of the mechanism of plant viral RNA replication were faced with this remarkable increase of host-encoded RdRp activity, whereas, on the other hand, efforts to identifying a virus-specific RNA replicase in virus-infected plants were unsuccesfull, involvement of the host-encoded RdRp in plant viral RNA replication was proposed (Duda *et al.*, 1973; Fraenkel-Conrat 1976; Romaine and Zaitlin, 1978; Clerx and Bol, 1978; Ikegami and Fraenkel-Conrat, 1978a, 1978b, 1979; Symons *et al.*, 1983; Hall *et al.*, 1982; Fraenkel-Conrat, 1983).

We have previously described the purification of a membrane-associated host-encoded RdRp from cowpea leaves, the activity of which was strongly stimulated upon infection with cowpea mosaic virus (CPMV) (Dorssers *et al.*, 1982). The enzyme appeared to consist of a single 130K polypeptide. Recently, an immunochemical assay (antibody-linked polymerase assay, ALPA) has been developed to identify RNA polymerase activity following SDS-polyacrylamide gel electrophoresis (Van der Meer *et al.*, 1983). By using an antiserum directed against a preparation of purified RdRp, it could thus be firmly established that the 130K polypeptide is indeed responsible for host-encoded RdRp activity in cowpea leaves (Van der Meer *et al.*, 1983).

Previously, we have shown that the membrane fraction of CPMV-infected cowpea leaves contains two different RNA-dependent RNA polymerase activities, which are associated with endogenous template RNA and can be distinguished on basis of the products synthesized *in vitro* (Dorssers *et al.*, 1983a). The host-encoded RdRp appeared to be engaged in transcribing plant RNA species, and after infection also viral ones, into short complementary pieces of negative polarity. Besides this polymerase activity, the membrane fraction of CPMV-infected leaves was found to contain an RNA polymerase-template complex, referred to as CPMV RNA replication complex, which is characterized

by its capability of elongating nascent strands to full-length viral RNAs of positive polarity. The host-encoded RdRp can be released from the membranes by washing in a Mg^{2+}-deficient buffer whereas, under these conditions the CPMV RNA replication complex remains membrane-bound (Dorssers *et al.*, 1983a).

Using an antiserum directed against the host-encoded RdRp as a probe to detect the 130K polypeptide on protein blots, we present evidence indicating that the amount of host-encoded RdRp increases more than twenty-fold in cowpea leaves upon infection with CPMV, whereas, in contrast, no such increase is oberved when cowpea mesophyll protoplasts are inoculated with CPMV. Furthermore, we show that the 130K RdRp is not found in the CPMV RNA replication complex and thus is not involved in viral RNA replication.

MATERIALS AND METHODS

Virus, plants and protoplasts.

Cowpea plants (*Vigna unguiculata* L., Walp. var. "California Blackeye") were grown and inoculated with CPMV, Sb isolate, as previously described (Dorssers *et al.*, 1982). Cowpea mesophyll protoplasts from uninfected cowpea leaves were prepared and inoculated with a mixture of bottom (B) and middle (M) component of CPMV or with purified B component alone, according to Rezelman *et al.* (1980). Protoplasts from CPMV-infected leaves were prepared from primary cowpea leaves 72 hr after inoculation and incubated for 45 hr under standard conditions (Rezelman *et al.*, 1980). The protoplasts were collected by centrifugation and frozen at -80^{o}.

Purification of RdRp from cowpea leaves.

The purification procedure is summerized in Fig. 1. At each step RdRp activity was assayed as described by Dorssers *et al.* (1982). The standard buffer used throughout the purification was TGKEDP containing 50 mM Tris/acetic acid, pH 8.2, 1 mM EDTA, 5 mM DTE (dithioerythritol), 0.5 mM PMSF (phenylmethylsulfonyl fluoride) and variant concentrations of K-acetate (mM) and percentages (v/v) of glycerol as indicated by subscripts.

Up to the poly(U)-Sepharose 4B column chromatography-step (Fig. 1), purification was performed as described by Dorssers *et al.* (1982). Then either of the two following steps were used for further purification. (i) The fractions eluted from the poly(U)-Sepharose 4B column containing RdRp activity were pooled and dialyzed against $TG_{35}K_{250}ED$. The RdRp preparation was then concentratred by adsorption on a 300 µl poly(U)-Sepharose 4B column followed by elution in a single step with $TG_{25}K_{1250}ED$. The concentrated enzyme pre-

paration was dialyzed against $TG_{12}K_{250}ED$ for 2-4 hr and then subjected to glycerol gradient centrifugation as described previously (Dorssers *et al.*, 1982). (ii) The fractions from the poly(U)-Sepharose 4B column containing RdRp activity were dialyzed against $TG_{35}K_{125}ED$ and applied to a 400 µl phosphocellulose (Bethesda Research Laboratories, Inc.) column equilibrated with $TG_{35}K_{125}ED$. As not all RdRp activity was bound to the phosphocellulose in a single run, the flow-through material was collected and applied once more to the column. The phosphocellulose column was washed with $TG_{35}K_{125}ED$

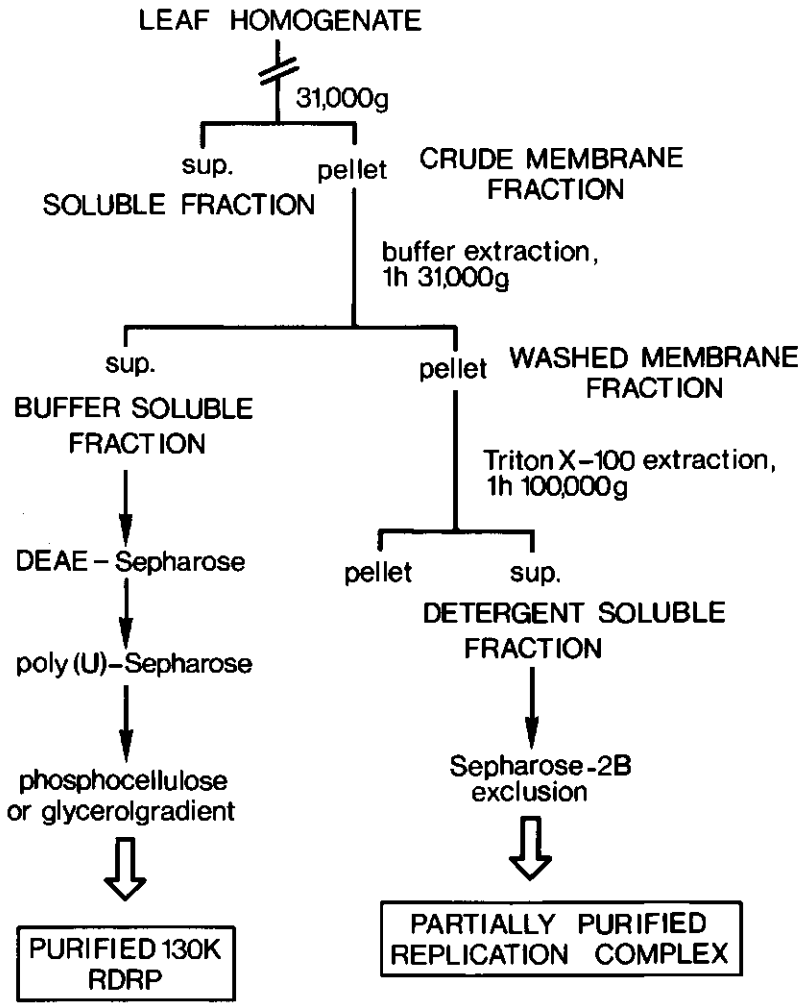

Fig. 1. Scheme for purification of host-encoded RNA-dependent RNA polymerase (RdRp) from cowpea and the cowpea mosaic virus RNA replication complex. The latter was purified according to Dorssers *et al.* (1983a, b).

and the RdRp activity was eluted with $TG_{30}K_{300}ED$. The enzyme preparation thus obtained was analyzed by SDS-polyacrylamide gel electrophoresis. If silver staining of the gel (Oakley *et al.*, 1980) revealed that RdRp preparations contained significant amounts of polypeptides besides the 130K RdRp polypeptide, the phosphocellulose chromatography-step was repeated once more using $TG_{35}K_{150}ED$ as loading buffer and $TG_{35}K_{300}ED$ for eluting the enzyme.

Antisera.

Immunization of New Zealand white rabbits with RdRp involved two injections with 7.5 µg and 12 µg of glycerol gradient-purified RdRp, respectively, followed by three additional injections with 5-15 µg of phosphocellulose column-purified RdRp. Injections were given at two month intervals. Appropriate peak fractions from the glycerol gradient or the phosphocellulose column were pooled, adjusted to 1 ml with $TG_{25}K_{250}ED$, emulsified with an equal volume of Freunds incomplete adjuvant and injected subcutaneously. Protein blots to be incubated with ^{125}I-labeled protein A were reacted with antiserum obtained after the fourth injection, whereas ALPA-experiments involved antiserum obtained after the fifth injection. For detection of CPMV B-RNA-encoded proteins (Goldbach and Rezelman, 1983), an antiserum was used which was raised against the virus-specific 170K polypeptide purified by SDS-polyacrylamide gel electrophoresis (F. van Straaten, M. Moerman and P. Zabel, unpublished results).

SDS-polyacrylamide gel electrophoresis and protein blotting.

SDS-polyacrylamide gel electrophoresis on 7.5%, 10% or 12.5% acrylamide gels containing 0.17%, 0.13% and 0.1% methylene bisacrylamide, respectively, was performed as described by Dorssers *et al.* (1982). The polypeptides were transferred electrophoretically (55V, 6-16 hr at 4^{o}) to a sheet of nitrocellulose using 20 mM Tris, 150 mM glycine, pH 8.2, and 20% (v/v) methanol as transfer buffer (Van der Meer *et al.*, 1983). In later experiments, transfer was carried out in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol and 0.1% (w/v) SDS, as this buffer was found to improve transfer of high molecular weight (> 100K) polypeptides (Erickson, 1982).

Immunological detection of polypeptides on protein blots using ^{125}I-labeled protein A.

Protein blots (12 x 15 cm) were preincubated with gelatin, incubated using 0.2 - 0.3 ml of antiserum for 6-16 hr and ^{125}I-labeled protein A for 2-16 hr, respectively, as described previously (Zabel *et al.*, 1982; Van der Meer *et al.*, 1983). Blots could be regenerated and probed with another antiserum by washing twice in 100 ml 8 M urea-0.5% bovine serum albumin (BSA)

(Sigma, fraction V)-100 mM β-mercaptoethanol at 60° for 30 min (Erickson, 1982).

Antibody-linked polymerase assay (ALPA).

The procedure described in detail elsewhere (Van der Meer *et al.*, 1983) was modified to allow the use of protein blots (3 x 12 cm) corresponding to 5 or 6 lanes from an SDS-polyacrylamide gel. The nitrocellulose sheets were incubated in polypropylene trays (4 x 16 cm) holding 2-4 ml solution on a Labline oscillating shaker. After successive incubations with buffers containing BSA, excess antiserum and DEAE-Sepharose CL-6B-purified RdRp, respectively, as decribed previously (Van der Meer *et al.*, 1983), the blots were subsequently incubated in 1.8 ml reaction mixture containing 50 mM Tris/acetate, pH 8.2, 12.5% (v/v) glycerol, 50 mM $(NH_4)_2SO_4$, 8 mM Mg-acetate, 1 mM EDTA, 2 mM DTE, 36 µg of actinomycin D, 1 mM each of ATP, GTP and CTP, 2 µM unlabeled UTP, 0.07 µM [α-^{32}P]UTP (550 Ci/mmol) and 90 µg of CPMV RNA. After incubation for 25 min at 30°, the blots were transferred to icecold 10% (w/v) trichloroacetic acid (TCA) - 1% (w/v) $Na_4P_2O_7$, washed in 5% (w/v) TCA - 1% (w/v) $Na_4P_2O_7$ dried and autoradiographed as described (Van der Meer *et al.*, 1983). The use of large excess of DEAE-Sepharose CL-6B-purified enzyme during incubation of antiserum-treated protein blots was avoided as it was found to reduce the formation of a specific enzyme-sandwich. The native enzyme may possess a greater affinity for the antibody binding sites and thus may compete for these sites with the denatured polypeptide on the nitrocellulose filter.

Protein blots used for ALPA could be regenerated by washing in 50 mM Tris/acetate, pH 8.2 - 1 mM EDTA for 20 min. Such washing did remove both ^{32}P-labeled RNA products and antibody molecules as was revealed by the lack of subsequent binding of ^{125}I-labeled protein A to the washed blots. Apparently, the TCA-treatment had disrupted the binding of antibodies, whereupon denatured antibodies were readily washed from the blots. It should be noted, however, that a similar TCA-treatment was not effective in regenerating protein blots which had been dried after successive treatment with antiserum and ^{125}I-labeled protein A.

RESULTS

RdRp purification and characterization of anti-RdRp serum.

RdRp purification as described previously (Dorssers *et al.*, 1982) was considerably improved by replacing glycerol gradient centrifugation by phos-

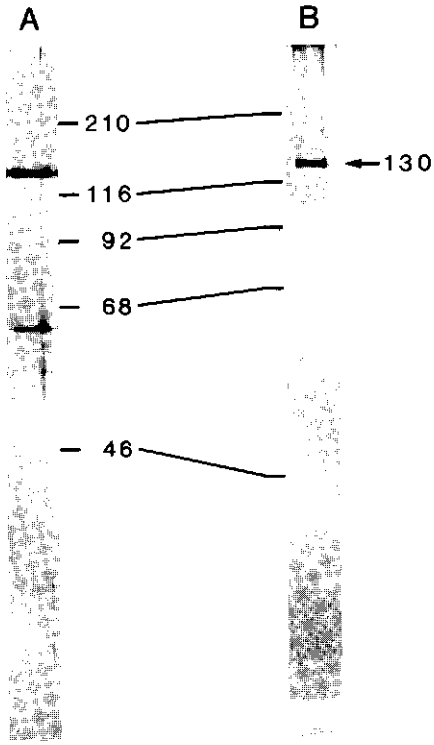

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified RNA-dependent RNA polymerase (RdRp).
Glycerol gradient-purified RdRp according to Dorssers *et al.* (1982) (0.03 units lane A) and phosphocellulose column-purified RdRp (0.04 units, lane B) were electrophoresed on 12.5% SDS-polyacrylamide gels and visualized by silver staining. Numbers between lanes A and B refer to molecular weights (x 10^{-3}) of marker proteins.

phocellulose column chromatography (Fig. 1). Glycerol gradient centrifugation required concentration of the poly(U)-Sepharose-purified RdRp, whereas this step could be omitted in case of phosphocellulose chromatography. In addition, this purification step resulted in an improved recovery of enzyme activity and a higher degree of purification (Fig. 2). Recovery of enzyme activity was 50-60%, which is twice as much as after glycerol gradient centrifugation (Dorssers *et al.*, 1982). SDS-polyacrylamide gel electrophoresis of the phosphocellulose-purified enzyme revealed the RdRp preparations to consist almost exclusively of the 130K polypeptide (Fig. 2B), which has recently been identified as the RdRp from cowpea leaves (Van der Meer *et al.*, 1983). The specific activity of the most purified enzyme preparations was 800 units/mg protein and the maximal yield using the modified purification procedure was approximately 25 µg of RdRp protein/Kg of CPMV-infected cowpea leaves.

Figure 3 presents the results of blotting experiments with RdRp preparations at various stages of purification. Using anti-RdRp serum and ^{125}I-labeled protein A to detect immunocomplexes, the phosphocellulose-purified enzyme did show only the 130K polypeptide (Fig. 3A, lane 1). This 130K polypeptide was similarly found in poly(U)-Sepharose- and DEAE-Sepharose-purified enzyme preparations from CPMV-infected cowpea leaves (Fig. 3A, lanes 2 and 3) and could also be detected, though with some difficulty, in

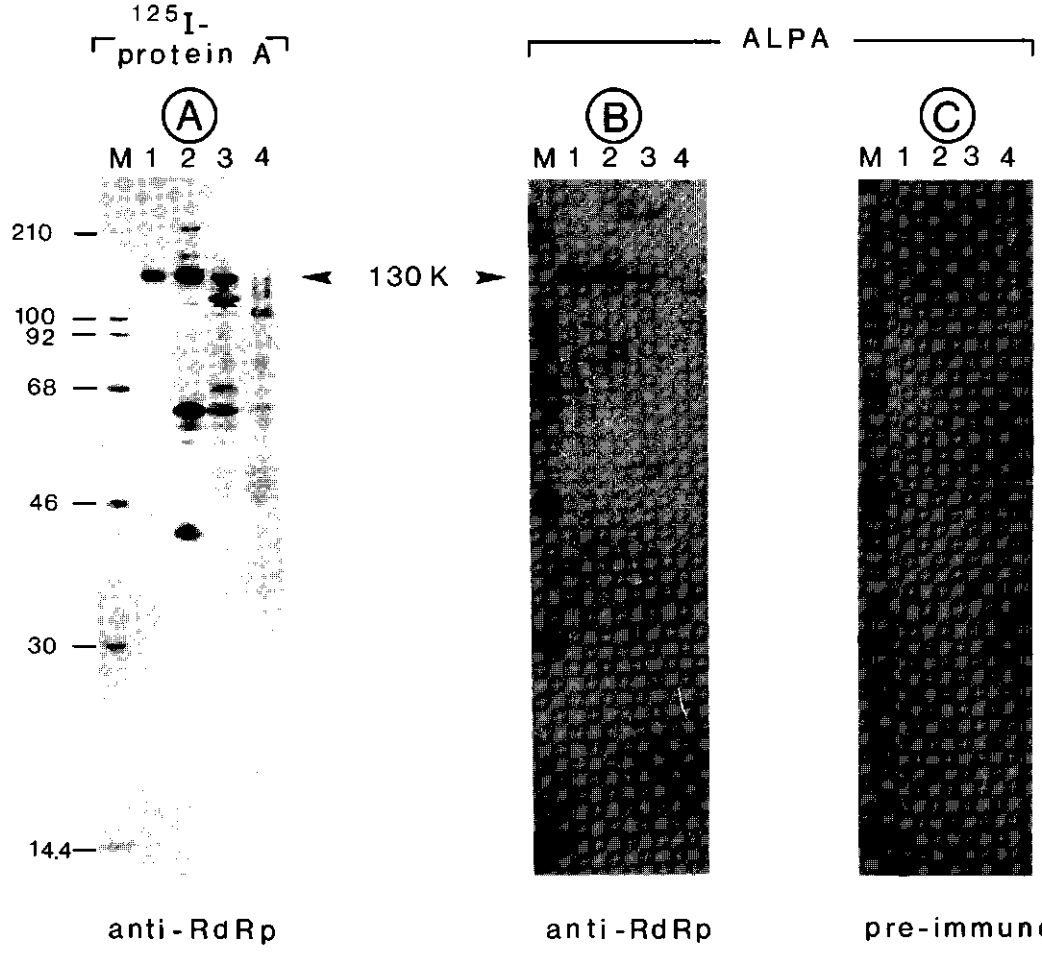

Fig. 3. Immunological detection of host-encoded RNA-dependent RNA polymerase on protein blots using ^{125}I-labeled protein A and ALPA.
Enzyme preparations from CPMV-infected cowpea leaves (unless specified otherwise) were electrophoresed on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose. Lane M, ^{14}C-methylated marker proteins (Amersham). Lane 1, 0,2 µg of phosphocellulose column-purified RdRp. Lane 2, 1 µg of poly(U)-Sepharose-purified RdRp. Lane 3, 10 µg of DEAE-Sepharose-purified RdRp. Lane 4, 10 µg of DEAE-Sepharose-purified RdRp from mock-inoculated leaves. Protein determinations were performed according to McKnight (1977). (A). Autoradiogram of protein blot incubated with anti-RdRp serum followed by treatment with ^{125}I-labeled protein A to reveal immunocomplexes as described in Materials and Methods. (B, C). Autoradiograms of protein blots subjected to antibody-linked polymerase assay (ALPA). Blots were incubated, successively, with anti-RdRp serum (B) or preimmune serum (C) and DEAE-Sepharose-purified RdRp, and then assayed for antibody-linked polymerase activity using [^{32}P]UTP. The molecular weights (x10^{-3}) of ^{14}C-methylated marker proteins are indicated at the left. Note that the protein blot used in (B) was regenerated (see Materials and Methods) and then used in (A) by incubating successively with anti-RdRp serum and ^{125}I-labeled protein A.

DEAE-Sepharose-purified enzyme from mock-inoculated cowpea leaves (Fig. 3A, lane 4). The anti-RdRp serum appeared to be not specific for the 130K polypeptide, as was apparent from its reaction with several other polypeptides present in the DEAE-Sepharose- and poly(U)-Sepharose-purified RdRp preparations (Fig. 3A). Some of these additional polypeptides became visible on protein blots of phosphocellulose-purified enzyme upon very long exposures. Apparently, trace amounts of these polypeptides had contaminated the RdRp preparations used for raising the antiserum.

Anti-RdRp serum obtained after the fourth injection with purified RdRp, turned out to be incapable of neutralizing RdRp activity in DEAE-Sepharose-purified enzyme preparations (data not shown). When DEAE-Sepharose-purified enzyme was incubated with saturating amounts of anti-RdRp serum followed by removal of immunocomplexes using *Staphylococcus aureus* cells, RdRp activity decreased with 30-40%. Nevertheless, this particular antiserum preparation proved very useful as a probe for detecting RdRp on protein blots.

Recently, we have developed a new immunological method, referred to as ALPA (antibody-linked polymerase assay, Van der Meer *et al.*, 1983), which allows detection of RdRp activity on protein blots following polyacrylamide gel electrophoresis in the presence of SDS. Figure 3B shows the results when the same protein blot used in Fig. 3A was subjected to ALPA. It is apparent that the 130K polypeptide is the only polypeptide band in the various RdRp preparations correlating with RNA polymerase activity as detected by ALPA. These results confirm the earlier identification of the 130K polypeptide as the RdRp from cowpea leaves (Dorssers *et al.*, 1982; Van der Meer *et al.*, 1983).

Whereas ALPA allows the 130K polypeptide to be identified as RdRp, the Western blotting procedure using ^{125}I-labeled protein A to visualize immunocomplexes provides a more sensitive detection method. Thus, the tiny bit of 130K polypeptide in the DEAE-Sepharose purified RdRp from mock-inoculated cowpea leaves could still be detected (Fig. 3A, lane 4), which was not feasible using ALPA (Fig. 3B, lane 4). The identity of the 130K polypeptide as RdRp being established, we applied the more sensitive method with antiserum and ^{125}I-labeled protein A in further experiments.

Host-encoded 130K RdRp in CPMV-infected leaf tissue.

The membrane-associated RdRp activity is very low in uninfected primary cowpea leaves but increases rapidly after inoculation of the leaves with CPMV to reach a maximum on day 3 and 4 (Zabel *et al.*, 1974; Dorssers *et al.*, 1982). The amount of 130K polypeptide in the crude membrane fraction from inoculated leaves (Fig. 1) as detected by Western blotting, increased concurrently with the RdRp activity, also reaching a maximum at day 3 and 4 (not shown). By comparing the amounts of protein from crude membrane fractions

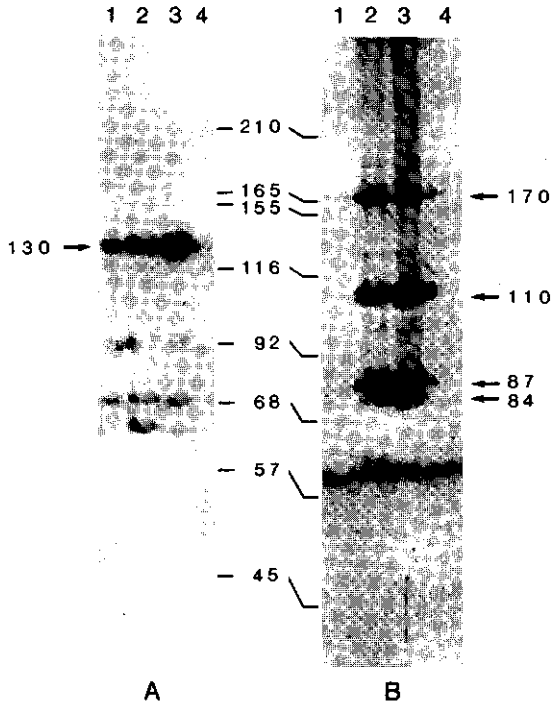

Fig. 4. Appearance of host-encoded 130K RNA-dependent RNA polymerase and CPMV B-RNA encoded polypeptides in the membrane fraction of cowpea leaves. Dark green and light green areas of young, systemically-infected, cowpea leaves were excised, 30 days after inoculation of the primary leaves, and the crude membrane fraction was prepared as described. Samples of the crude membrane fraction of dark green tissue (lane 1), light green tissue (lane 2), CPMV-inoculated primary leaves (lane 3) and mock-inoculated primary leaves (lane 4), containing 30 µg of protein were electrophoresed on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose. Identical protein blots were incubated with anti-RdRp serum (A) and anti-170K serum (B), treated with ^{125}I-labeled protein A and autoradiographed, Numbers between panels A and B refer to molecular weights ($x10^{-3}$) of marker proteins. Note that the band above the 57K marker polypeptide in panel B represents a background band. The appearance of this band is due to the use of antiserum prepared against polypeptides which have been purified by preparative SDS-polyacrylamide gel electrophoresis (unpublished results). Protein determinations were performed according to Bradford (1976).

of uninfected leaves and CPMV-infected leaves 3-4 days after inoculation, the increase of 130K polypeptide was estimated to be at least 20-fold (Fig. 4A, lanes 3 and 4), in good agreement with earlier estimates (Dorssers *et al.*, 1982).

Mock-inoculation of primary cowpea leaves using buffer and carborundum as an abrasive, resulted in a 1.5 to 2-fold increase in the amount of 130K polypeptide detectable on protein blots. Previously, a similar increase in RdRp activity has been observed following mock-inoculation of tobacco and cowpea leaves (Romaine and Zaitlin, 1978; White and Dawson, 1978).

To examine a possible correlation between the appearance of host-encoded 130K RdRp and viral replication more closely, various leaf tissues were also tested for the appearance of virus-specific polypeptides using an antiserum raised against the B-RNA encoded 170K polypeptide. The virus-specific 170K

polypeptide is the precursor of 110K, 87K, 84K, 60K, 58K and VPg polypeptides (Rezelman *et al.*, 1980; Zabel *et al.*, 1982; Goldbach and Rezelman, 1983), among which the 170K, 110K, 87K and 84 K polypeptides are immunoreactive against the anti-170K serum. Since these polypeptides are known to be synthesized in cells that are engaged in viral RNA replication (Goldbach *et al.*, 1980), the anti-170K serum thus provides a powerful probe to screen tissue or isolated cells for the occurrence of viral replication.

The anti-170K serum was particularly useful in analyzing the different tissue types of systemically-infected cowpea leaves. Cowpea plants of which the primary leaves have been inoculated at an early stage (nine days after sowing), develop systemically-infected leaves which often show a bright mosaic pattern consisting of dark green and light green tissue. The crude membrane fraction from light green tissue appeared to contain considerable amounts of 170K, 110K, 87K and 84K B-RNA encoded polypeptides (Fig. 4B, lane 2), thus indicating virus replication to occur in this particular tissue. In contrast, virtually none of the virus-specific 170K, 110K, 87K and 84K polypeptides could be detected in dark green areas of the mosaic of systemically-infected leaves (Fig. 4B, lane 1). Similarly, the amount of host-encoded 130K polypeptide was found to increase strongly in light green tissue (Fig. 4A, lane 2) and only slightly in darkgreen tissue (Fig. 4A, lane 1). These results indicate that the marked increase of 130K polypeptide is typical for leaf cells actively engaged in virus multiplication, suggesting a direct correlation with CPMV RNA replication.

No increase of host-encoded 130K RdRp in CPMV-infected cowpea protoplasts.

In cowpea mesophyll protoplasts inoculated with either purified B components of CPMV or with a mixture of B + M components, the appearance of virus-specific polypeptides (170K, 110K, 87K and 84K) could be easily demonstrated using anti-170K serum and ^{125}I-labeled protein A as described above (Fig. 5B, lanes 2 and 3). Remarkably, virtually no 130K polypeptide was detectable in such protoplasts (Fig. 5A, lanes 2 and 3) and there was no indication of an increase of the amount of 130K polypeptide in the protoplasts inoculated with either B or B + M components as compared with mock-inoculated protoplasts (Fig. 5A, lane 1). These findings seemed in contradiction with the results obtained with infected leaves, which suggested a direct correlation between an increase of 130K RdRp and CPMV RNA replication. To gain more insight in the behaviour of 130K polypeptides in isolated leaf cells, mesophyll protoplasts were isolated from primary cowpea leaves which had been inoculated with CPMV 72 hr earlier. Such preparations showed an increased amount of 130K polypeptide as compared with protoplasts prepared from healthy leaves (Fig. 5A, lane 4). However, upon further incubation of the protoplasts *in vitro* for 25hr (Fig. 5A, lane 5) and 45 hr (Fig. 5A, lane 6), respectively,

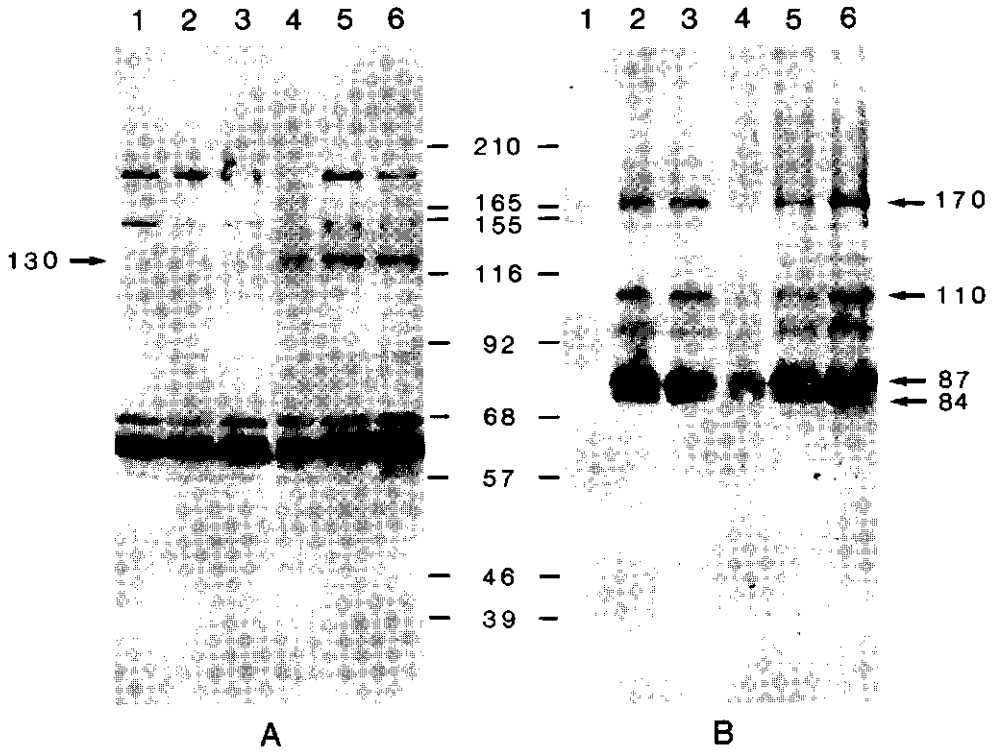

Fig. 5. Appearance of host-encoded 130K RNA-dependent RNA polymerase and CPMV B-RNA-encoded polypeptides in protoplasts.
Cowpea mesophyll protoplasts were prepared from primary leaves and were either mock-inoculated (lane 1), inoculated with purified CPMV B components (lane 2) or CPMV B + M components (lane 3) as described by Rezelman *et al.* (1980). The protoplasts were incubated for 45 hr to allow replication of viral RNAs. Another batch of protoplasts was prepared from CPMV-infected primary cowpea leaves, 72 hr after inoculation, and further incubated for 0 hr (lane 4), 25 hr (lane 5) and 45 hr (lane 6). Samples of the protoplasts containing 60 µg of protein were electrophoresed on a 7.5% SDS-polyacrylamide gel and subsequently transferred to nitrocellulose. (A) Protein blot incubated with anti-RdRp serum and ^{125}I-labeled protein A. (B) The protein blot used in (A) was regenerated and probed with anti-170K serum.

the amount of 130K polypeptide did neither measurably increase or decrease whereas the production of B-RNA encoded polypeptides continued and increased to the level detected in protoplasts inoculated with CPMV *in vitro* (Fig. 5B, lane 6 versus lanes 2 and 3). The conclusion must be that the correlation between the strong increase of RdRp and CPMV RNA replication is confined to leaf-tissue, whereas in isolated protoplasts CPMV RNA replication is not accompanied by an increase of host-encoded RdRp.

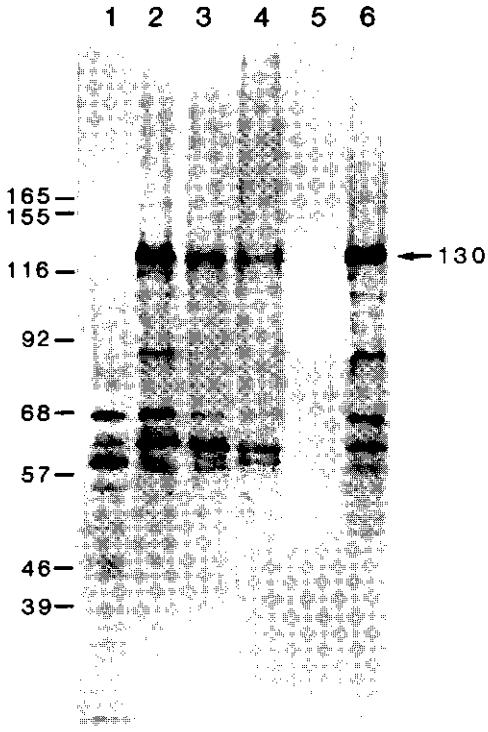

Fig. 6. Appearance of host-encoded 130K RNA-dependent RNA polymerase in preparations of the CPMV RNA replication complex at various stages of purification.
Samples of the soluble fraction (lane 1), the crude membrane fraction (lane 2), the washed membrane fraction (lane 3), the detergent-soluble fraction (lane 4) and the buffer-soluble fraction (lane 6) containing 30 µg of protein (determined according to Bradford, 1976) were electrophoresed on a 7.5% SDS-polyacrylamide gel, along with 10 µl of the concentrated, Sepharose 2B-purified CPMV RNA replication complex (lane 5). Polypeptides were then transferred to nitrocellulose and the protein blot was incubated with anti-RdRp serum and ^{125}I-labeled protein A as described. Samples of the purified replication complex (lane 5) and the buffer-soluble fraction (lane 6) contained equal amounts (0.003 units) of RNA polymerase activity as determined by measuring incorporation of ^{3}H-CMP for 30 min in a standard RdRp assay containing 20 µM of ^{3}H-CTP and added CPMV RNA. Note that 30 µg of protein in the buffer-soluble fraction and 10 µl of the concentrated replication complex were derived from 17 mg and 550 mg of CPMV-infected cowpea leaves, respectively.

Host-encoded RdRp is not present in the functional CPMV RNA replicaton complex.

When the crude membrane fraction (Fig. 1) of CPMV-infected cowpea leaves is extracted with a Mg^{2+}-deficient buffer, host-encoded RdRp is released from the membranes, whereas the CPMV RNA replication complex remains membrane bound (Dorssers *et al.*, 1983a). The CPMV RNA replication complex can subsequently be solubilized using Triton X-100 and partially purified by Sepharose 2B chromatography without loss of its capability of elongating *in vitro* nascent RNA strands to full-sized viral RNAs (Fig. 1; Dorssers *et al.*, 1983a). Various fractions containing the viral replication complex were examined for the occurrence of the 130K polypeptide, using Western blot analysis (Fig. 6). The 130K polypeptide was hardly detectable in the soluble fraction of a leaf homogenate of CPMV-infected leaves (Fig. 6, lane 1), but occurred exclusively in the crude membrane fraction (lane 2). As expected, following extraction of the crude membrane fraction with Mg^{2+}-deficient buffer, the bulk of 130K polypeptide was found in the buffer-soluble fraction (lane 6) containing the RdRp activity. However, some of the 130K polypeptide remained associated with the washed membranes (lane 3) as did the CPMV RNA replication complex. When

the RNA replication complex was subsequently solubilized from the washed membrane fraction by treatment with Triton X-100, the residual 130K polypeptide was similarly released in the detergent-soluble fraction (lane 4). Sepharose 2B chromatography, however, resolved completely the remainder of the host-encoded RdRp activity from the viral replication complex. The 130K polypeptide was retarded on the column, along with the bulk of proteins, whereas the CPMV-RNA replication complex eluted in the void volume (Dorssers *et al.*, 1983 a/b). In comparing samples of the Sepharose 2B void fraction (Fig. 6, lane 5) and the buffer soluble fraction (lane 6) containing equal amounts of RNA polymerase activity, no 130K polypeptide was detected in the Sepharose void fraction (lane 5). Thus, the results presented in Figure 6, warrant the conclusion that host-encoded RdRp activity associated with the 130K polypeptide is not responsible for the polymerase activity of the CPMV RNA replication complex. The composition of the CPMV RNA replication complex will further be discussed in a separate paper (Dorssers *et al.*, 1983b).

DISCUSSION

In this paper we have shown that the host-encoded RNA-dependent RNA polymerase of cowpea does not copurify with the CPMV RNA replication complex that is capable of elongating nascent chains to full-sized viral RNA strands, even though the amount of RdRp is elevated at least twenty-fold compared to uninfected leaves. Thus, the conclusion should be that the host-encoded RdRp, which has often been assigned a role as core RNA polymerase in plant viral RNA replication, is not responsible for the RNA polymerase activity of the viral RNA replication complex. Sensitive immunochemical detection methods, using antiserum raised against purified RdRp, enabled us to draw this conclusion.

Previously, we have shown that the host-encoded RdRp from cowpea leaves is a monomeric 130,000-dalton protein associated with the membrane fraction (Dorssers *et al.*, 1982; Van der Meer *et al.*, 1983). The results presented in this paper confirm and strengthen this conclusion. Whereas the recently developed antibody-linked polymerase assay (ALPA) has been invaluable in correlating the 130K polypeptide with RdRp activity, the use of ^{125}I-labeled protein A to detect immunocomplexes on protein blots proved to be the more sensitive detection method. The latter method was, therefore, applied to detect the host-encoded 130K RdRp in preparations of the CPMV RNA replication complex. We have, thus, been able to demonstrate that the 130K RdRp can be resolved completely from the viral replication complex by Sepharose 2B

chromatography without affecting the viral RNA-synthesizing capability of the CPMV RNA replication complex (Dorssers *et al.*, 1983a). It, therefore, seems justified to conclude that the host-encoded RdRp is not engaged in viral RNA replication.

Various findings have suggested that plant RNA viruses encode information essential to viral RNA replication: (i) Temperature-sensitive mutants of tobacco mosaic virus (Dawson and White, 1978, 1979) and alfalfa mosaic virus (Sarachu *et al.*, 1983; Roosien and Van Vloten-Doting, 1982) have been described with defects in viral RNA synthesis (ii) For plant viruses with a bipartite or tripartite RNA genome, including cowpea mosaic virus (Goldbach *et al.*, 1980), tobacco rattle virus (Lister, 1968), tomato black ring virus (Robinson *et al.*, 1980), alfalfa mosaic virus (Nassuth *et al.*, 1981) and brome mosaic virus (Kiberstis *et al.*, 1981), it has been demonstrated that replication of at least one of the genomic RNAs is completely dependent on the expression and replication of other genomic RNAs. (iii) Replication of the RNA of the satellite virus of tobacco necrosis virus (Kassanis, 1962) and of satellite RNA of tomato black ring virus (Murant and Mayo, 1982) is entirely dependent on the expression and replication of the helper virus.

In spite of these findings, several authors have proposed the involvement of host-encoded RdRp in plant viral RNA replication, mainly because they were unsuccessfull in identifying a virus-specific RNA polymerase and were confronted with a strong enhancement of host-encoded RdRp activity upon viral infection (for references, see introduction). Usually it is then assumed, that the virus contributes information to allow the host-encoded RdRp to recognize viral RNA as template and to initiate properly. This concept is challenged by our findings that the functional CPMV RNA replication complex does not contain the host-encoded RdRp. It seems, therefore, plausible that also plant RNA viruses, by analogy to RNA bacteriophages (Blumenthal and Carmichael, 1979) and animal picornaviruses (Van Dijke and Flanegan, 1980; Lowe and Brown, 1981; Lowe *et al.*, 1981) encode an RNA-dependent RNA polymerase to act as core of the viral RNA replicase. Host proteins and other viral proteins may be required for specific recognition of the template and initiation of RNA synthesis.

Immunological detection of the host-encoded 130K RdRp was also useful for examining the appearance and behaviour of RdRp in cowpea leaves and mesophyll protoplasts. Surprisingly, the amount of 130K RdRp was not increased in cowpea protoplasts upon infection with CPMV. This finding further supports the conclusion that host-encoded RdRp is not involved in viral replication. Previously, it was noted that the RNA-dependent RNA polymerase activity of CPMV-infected cowpea protoplasts was only three-fold higher than in uninfected protoplasts (Goldbach *et al.*, 1980). From the results presented in this paper, it follows that the increase in RNA polymerase activity in CPMV-in-

fected protoplasts can not be due to stimulation of host-encoded RdRp, but should be attributed to induced RNA polymerase activity involved in viral replication. In contrast, the twenty-fold increase of RNA-dependent RNA polymerase activity in cowpea leaves following infection with CPMV, is mainly due to the increase of host-encoded RdRp. Indeed, the RNA polymerase activity associated with the CPMV RNA replication complex has been estimated to represent less than 5% of total RNA-dependent RNA polymerase activity in the crude membrane fraction of CPMV-infected cowpea leaves (Dorssers *et al.*, 1983b).

About the physiological role of the host-encoded RdRp and the significance of the dramatic increase of this enzyme upon infection with CPMV, we are completely in the dark. Previously, we have shown (Dorssers *et al.*, 1983a) that the host-encoded RdRp transcribed *in vitro* endogenous plant and viral RNAs only into small (4-5 S) RNAs of negative polarity. In addition, RNAs synthesized *in vitro* by poly(U)-Sepharose-purified RdRp (see Fig. 1) on exogenous CPMV RNA as template, also appeared to be small and to have arisen from initiation at multiple sites (L. Dorssers, unpublished results). Since the added template RNA remained intact during RNA synthesis, the small size of the RNA products is apparently not due to an endonuclease activity either contaminating or inherent to the RdRp as suggested by Takanami and Fraenkel-Conrat (1982). Similar small and heterogenous RNAs were shown to be produced by extensively purified RNA-dependent RNA polymerase preparations from CMV-infected cucumbers (Kumarasamy and Symons, 1979). Perhaps, the production of small sized complementary RNAs is an intrinsic property of the host-encoded RdRp.

The increase of host-encoded 130K RdRp appears to be restricted to CPMV-infected cells in leaf tissue, whereas no such increase is evoked in isolated cowpea mesophyll protoplasts. Such differences in response to virus inoculation have also been observed with respect to resistance and hypersensitivity, which are not expressed in the usual manner in isolated leaf cell protoplasts (Otsuki *et al.*, 1972; Motoyoshi and Oshima, 1975, 1977; Beier *et al.*, 1977, 1979; for review see Matthews, 1981). If it would be possible to determine the nature of the endogenous template of the 130K RdRp in healthy plants, this might help to propose a hypthesis for a function of the 130K RdRp in the RNA metabolism of plants cells. Since the host-encoded RdRp transcribes after infection also small parts of viral RNAs, one may wonder whether the RdRp interferes somehow with virus multiplication. Sofar, the physiological function of the host-encoded RdRp remains an enigma.

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VII. THE COWPEA MOSAIC VIRUS B-RNA-ENCODED 110K POLYPEPTIDE IS INVOLVED IN VIRAL RNA REPLICATION

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SUMMARY

The cowpea mosaic virus (CPMV) RNA replication complex has been solubilized from the washed membrane fraction of CPMV-infected cowpea leaves using Triton X-100 and purified extensively by Sepharose 2B column chromatography and glycerol gradient centrifugation. The RNA replication complex retained the capability to elongate nascent RNA strands to full-length viral RNA throughout the purification procedure. Analysis of the polypeptide composition of the purified replication complex by SDS-polyacrylamide gel electrophoresis and silver staining revealed three major polypeptides with molecular weights of approximately 110K, 68K and 57K. Using antisera directed against CPMV B-RNA-encoded proteins, the 110K polypeptide was found to be the only viral polypeptide functionally associated with the RNA replication complex. As the host-encoded 130K RNA-dependent RNA polymerase is not engaged in viral RNA replication, we tentatively conclude that the 110K polypeptide constitutes the core RNA polymerase of the CPMV RNA replication complex.

INTRODUCTION

In extracts from cowpea leaves infected with cowpea mosaic virus (CPMV) the viral RNA replication complex is contained in the crude membrane fraction. Previous studies (Zabel *et al.*, 1974; Dorssers *et al.*, 1983) have shown that the replication complex consists of RNA polymerase molecules which are bound to endogenous template strands and are capable of resuming *in vitro* the elongation of nascent viral RNA strands already initiated *in vivo*. The *in vitro* synthesized RNA is virus-specific and has mainly plus-strand polarity (Dorssers *et al.*, 1983).

Identification of the RNA polymerase involved in the replication of CPMV RNA has been hampered by the occurrence of a host-encoded RNA-dependent RNA polymerase (RdRp), the activity of which is greatly enhanced upon virus infection (Dorssers *et al.*, 1982). This enzyme is also membrane-bound and associated with endogenous template RNAs. Our previous studies, however, have shown that the host-encoded RdRp is functionally different from the polymerase activity in the CPMV RNA replication complex (Dorssers *et al.*, 1983). The host-encoded enzyme appeared to be engaged in transcribing plant RNA species (and after infection also viral ones) into short complementary pieces of negative polarity. In addition, the host-encoded RdRp could be released from the membranes by washing with a Mg^{2+}-deficient buffer without affecting the membrane-association of the CPMV RNA replication complex (Dorssers *et al.*, 1983). The RdRp has been purified and identified as a host-encoded 130K-dalton protein (Dorssers *et al.*, 1982). Using an antiserum raised against the purified enzyme and a newly developed antibody-linked polymerase assay on protein blots, the 130K polypeptide was firmly established to constitute the host-encoded RdRp (Van der Meer *et al.*, 1983a).

The CPMV RNA replication complex has been solubilized from the washed membranes by treatment with Triton X-100. The soluble complex retained its endogenous template and was still capable of elongating *in vitro* nascent CPMV RNA strands to full-length viral RNAs (Dorssers *et al.*, 1983). By means of antibodies raised against the 130K RdRp it was demonstrated, that the host-encoded RNA polymerase was absent in the CPMV RNA replication complex (Van der Meer *et al.*, 1983b). In this paper we describe the further purification of the CPMV RNA replication complex and we present evidence for a CPMV B-RNA-encoded 110K polypeptide associated with the CPMV RNA replication complex.

MATERIALS AND METHODS

Purification of CPMV RNA replication complex.

(i) The crude membrane fraction from CPMV-infected cowpea leaves was prepared and washed twice with a Mg^{2+}-deficient buffer to extract the bulk of host-encoded RNA-dependent RNA polymerase activity as described by Dorssers *et al.* (1982, 1983). The washed membrane fraction was resuspended in TKED buffer (50 mM Tris/acetic acid, pH 8.2, 50 mM K-acetate, 1 mM Na_3EDTA and 5 mM dithioerytritol) containing 25% (v/v) glycerol, using approximately 0.2 ml/g of fresh leaf tissue, and stored at -80°. To solubilize the replication complex, portions of the washed membrane fraction were adjusted to 0.5% (v/v) Triton X-100 (Sigma Chemical Co.), stirred for 1 hr at 4° and clarified at 31,000 g for 1 hr. The supernatant was centrifuged at 123,000 g (35,000 rpm in a Beckman 60 Ti rotor) for 70 min at 4°. The resulting dark green supernatant (referred to as the detergent-soluble fraction) was stored at -80°, either directly or after concentrating two to three-fold in an Amicon ultrafiltration system (model 8 MC) using an YM 30 filter.

(ii) The replication complex in the detergent-soluble fraction was further purified by chromatography on a Sepharose 2B column (1.6 x 60 cm) in TKED buffer containing 15% (v/v) glycerol and 0.5% Triton X-100; 2.5 - 5 ml of the concentrated detergent-soluble fraction was applied to the column and eluted at a flow rate of about 10 ml/hr using a peristaltic pump. Fractions of about 1 ml were collected and 30 µl aliquots were assayed for RNA-dependent RNA polymerase activity using [^{3}H]UTP. The void fractions containing the viral replication complex were pooled (in the text referred to as the Sepharose void fraction) and stored at -80°.

(iii) The Sepharose void fraction was concentrated five to fifteenfold by ultrafiltration, and 0.3 - 0.5 ml was then loaded onto an 11 ml linear 17.5-40% (v/v) glycerol gradient in TKED buffer containing 0.5% Triton X-100 and centrifuged in a Beckman SW-41 rotor at 36,000 rpm (220,000 g) for 17 hr at 4°. The gradient was pumped out from the bottom and collected into 0.5 ml fractions; 30 µl aliquots of the fractions were assayed for polymerase activity. Sedimentation markers (CPMV double-stranded RNAs, RF_B and RF_M, Dorssers *et al.*, 1983) were run on a parallel gradient and their position in the gradient was determined by electrophoresing aliquots of the fractions on an agarose gel (Dorssers *et al.*, 1983).

RNA-dependent RNA polymerase assay and characterization of in vitro products.

The standard assay for RNA polymerase activity on endogenous template RNA has been previously described (Dorssers *et al.*, 1983). Whenever product ana-

lysis was not required, polymerase activity was measured using 6 μM of [5-^{3}H]UTP or 6 μM of [5,6-^{3}H]UTP (Amersham, 4.8 or 6.9 Ci/mmol, respectively) instead of [α-^{32}P]UTP. One unit of RNA-dependent RNA polymerase activity is defined as that amount of enzyme which incorporated 1 nmol of labeled UMP in 30 min at 30°.

The *in vitro* synthesized RNA was purified and analyzed on nondenaturing 1% agarose gels as decribed (Dorssers *et al.*, 1983). Analysis under denaturing conditions was performed on horizontal 1.5% agarose gels containing methylmercury hydroxide (Alfa products, Ventron GmbH, Karlsruhe, West Germany) (Van Dijke *et al.*, 1982).

SDS-polyacrylamide gel electrophoresis.

Proteins were analyzed on 10 or 12.5% polyacrylamide gels as described previously (Dorssers *et al.*, 1982). Polypeptide bands were visualized by silver staining (Morrissey, 1981). In some experiments, gels were fixed and stained with Coomassie brilliant blue prior to silver staining (Irie *et al.*, 1982).

Immunological techniques.

Preparation of antiserum directed against the 130K, host-encoded RNA-dependent RNA polymerase (anti-130K serum) and against the genome-linked protein (VPg) of CPMV (anti-VPg serum) have been described previously (Van der Meer *et al.*, 1983a; Zabel *et al.*, 1982). Antisera directed against the CPMV B-RNA-encoded 170K, 110K and 87K polypeptides (anti-170K, anti-110K and anti-87K serum, respectively) were raised by immunizing rabbits with each of the viral polypeptides purified by phosphocellulose chromatography and SDS-polyacrylamide gel electrophoresis (to be published elswhere; see also Franssen *et al.*, 1982).

Protein blots on nitrocellulose were prepared as described (Van der Meer *et al.*, 1983b) and probed with antiserum and ^{125}I-labeled protein A (Zabel *et al.*, 1982; Van der Meer *et al.*, 1983a and b).

RESULTS

Purification of the CPMV RNA replication complex.

Solubilization of the membrane-bound CPMV RNA replication complex by the nonionic detergent Triton X-100 turned out to be highly effective in that no loss of RNA polymerase activity occurred and virtually no polymerase activity remained associated with the detergent-insoluble membrane residue (Fig. 1A).

Like the membrane-bound replication complex, the solubilized replication complex was capable of elongating *in vitro* nascent RNA chains to full-length CPMV RNAs (Fig. 1B/C), the only difference being that less replicative intermediate (RI) was recovered after short labeling periods. This might be due to nuclease(s) which are active in the solubilized enzyme preparation, but not in the membrane fraction, and convert the RI's into RNase-resistant double-stranded CPMV B-RNA (RF_B) and M-RNA (RF_M). Although Triton X-100 concentrations of 0.1-1% (v/v) were found to be equally effective in solubilizing the replication complex, the use of concentrations

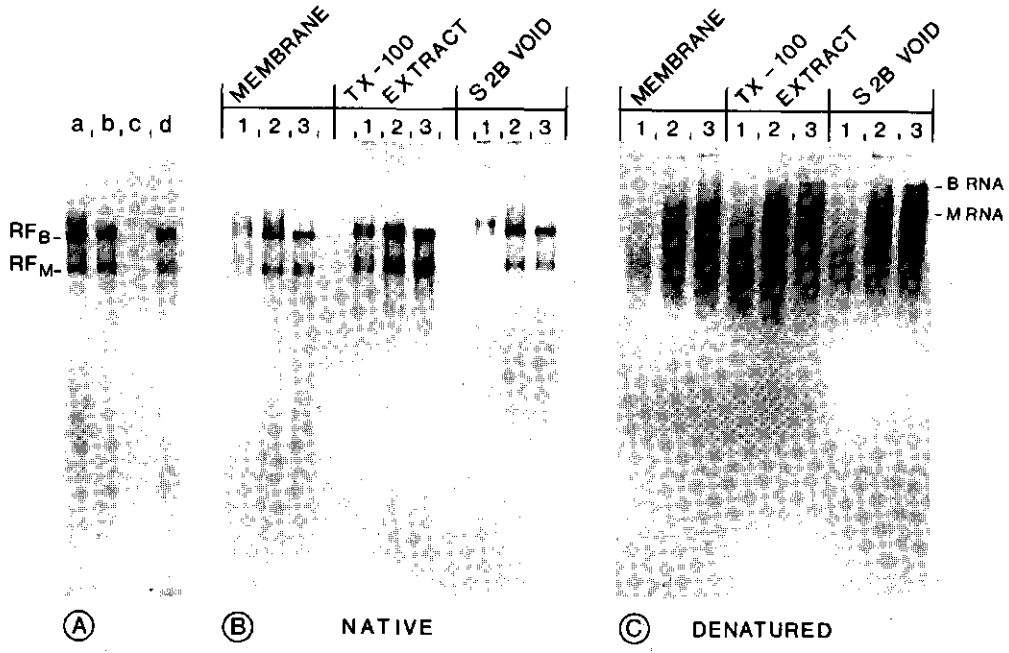

Fig. 1. Characterization of RNA synthesized *in vitro* by CPMV RNA replication complex at various stages of purification.
(A) Four reaction mixtures (60 µl, 5 µM [α-^{32}P]UTP, spec.act. 8.2 Ci/mmol) containing 33 µl of enzyme (washed membrane fraction, lane a; washed membrane fraction adjusted to 0.5% (v/v) Triton X-100, lane b; detergent-insoluble membrane residue, lane c; detergent-soluble fraction, lane d) were incubated for 30 min. RNA products were prepared and resolved by nondenaturing agarose gel electrophoresis as described.
(B, C) Reaction mixtures (180 µl, 5 µM [α-^{32}P]UTP, spec.act. 15.9 Ci/mmol, 2.5 µg/ml human placental RNase inhibitor) containing either 60 µl of the washed membrane fraction (MEMBRANE), 60 µl of the detergent-soluble fraction (TX-100 EXTRACT) or 90 µl of the Sepharose 2B void fraction (S2B VOID) were incubated under standard conditions. The amount of enzyme of the respective fractions was chosen so as to represent equivalent amounts (0.3 g) of leaf tissue. Samples (60 µl) were taken at 10 min (lanes 1) and 30 min (lanes 2) and the remainder of the reaction mixtures was incubated for an additional 10 min in the presence of 0.5 mM unlabeled UTP (lanes 3). RNA was prepared and analyzed by agarose gel electrophoresis under nondenaturing (B) and denaturing (C) conditions as described.

in the range of 0.5-1% (v/v) was preferable, as it resulted in enzyme preparations which were less contaminated by small membrane fragments upon subsequent Sepharose 2B column chromatography.

The CPMV RNA replication complex eluted in the void volume of the Sepharose 2B column as a clear and colourless enzyme preparation, clearly separated from the bulk of solubilized proteins (Fig. 2). At least 50% of the viral RNA polymerase activity present in the crude membrane fraction was recovered after Sepharose 2B column chromatography as estimated from the amount of RF_B and RF_M produced *in vitro*. Sepharose 2B column-purified enzyme was not stimulated by adding template RNA and appeared to be still associated with endogenous viral RNA template. Analysis of the *in vitro* products by pulse-labeling and pulse-chase experiments revealed full-length viral RNA strands to be made by elongation of nascent chains present in the replication complex (Fig. 1B/C). Following gel filtration through Sepharose 2B, nuclease activity apparently no longer contaminated the replication complex preparation, as the presence of RI's could again be demonstrated after short labeling periods (Fig. 1B).

To purify the replication complex further, glycerol gradient centrifugation was chosen to resolve the replication complex - expected to display a rather low sedimentation rate - from the fast sedimenting residual small membrane fragments coeluting with the replication complex in the void volume of the Sepharose 2B column. Active fractions from the gel filtration column were pooled, concentrated by ultrafiltration and loaded onto a linear glycerol gradient. RNA polymerase activity sedimented as a peak at 20-25S just ahead of purified (protein-free) RF_B and RF_M with a recovery of at least 65% (Fig. 3A). Analysis of RNA synthesized *in vitro* by the glycerol gradient fractions revealed the 20-25S peak to represent the viral replication complex consisting of RNA polymerase molecules which are completing viral RNA chain elongation (Fig. 3B). As expected, RNA polymerase molecules associated with replicating B-RNA did sediment slightly faster than those involved in transcribing M-RNA.

Protein composition of CPMV RNA replication complex.

Although Sepharose 2B chromatography did accomplish the separation of replication complex from residual host-encoded RdRp activity (Van der Meer *et al.*, 1983b) and, in addition, afforded a high degree of purification as revealed by SDS-polyacrylamide gel electrophoresis and silver staining (Fig. 2), the presence of contaminating small membrane fragments in the void volume did not allow us yet to identify the RNA polymerase polypeptide(s). This was particularly evident on analyzing enzyme preparations from different isolations, which showed a variable polypeptide pattern depending on the

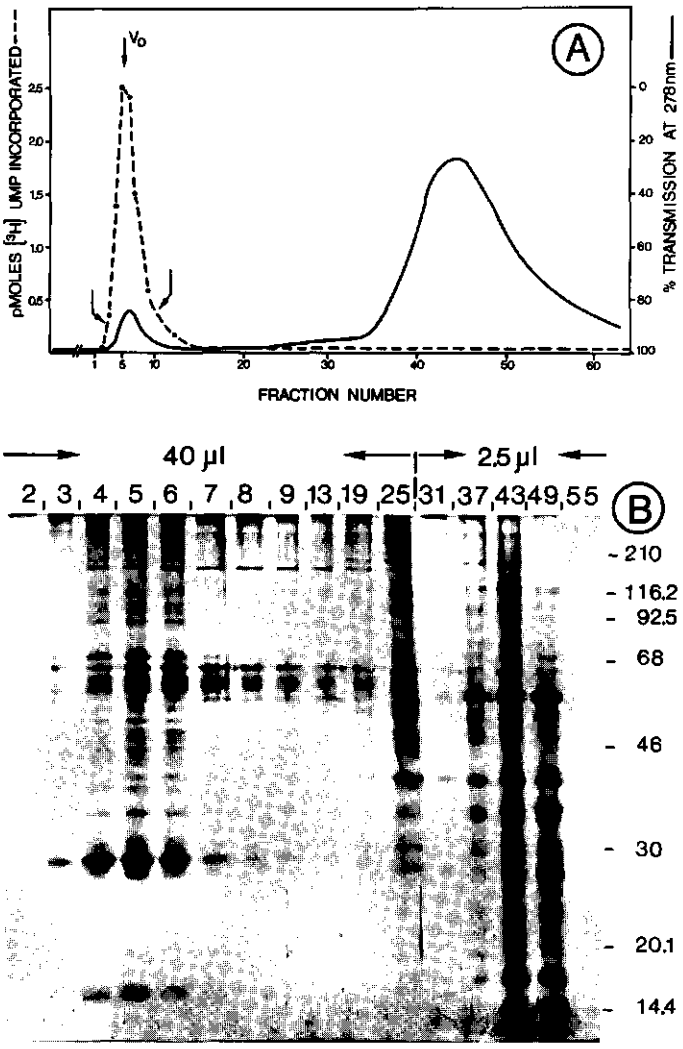

Fig. 2. Purification of CPMV RNA replication complex by Sepharose 2B chromatography.
(A) Two ml of a three-fold concentrated detergent-soluble fraction, derived from 29 gram of CPMV-infected cowpea leaves, was loaded onto a Sepharose 2B column (1.5 cm x 49 cm) and eluted at a flow rate of 8.5 ml/hr. After 21.5 ml had emerged, fractions (number 1-10, 0.85 ml; number 11-60, 1.3 ml, respectively) were collected and assayed for RNA polymerase activity under standard conditions using 30 µl aliquots and [^{3}H]UTP.
(B) Aliquots of the column fractions as indicated were adjusted to 1 x sample buffer (Dorssers *et al.*, 1982) and analyzed on a 12.5% SDS-polyacrylamide gel. Polypeptides were visualized by silver staining. The position of marker polypeptides (Dorssers *et al.*, 1982) is indicated at the right side (molecular weight x 10^{-3}). Some of the bands in the 53K-65K region represent background staining due to 2-mercaptoethanol used in the sample buffer (Tasheva and Dessev, 1983).

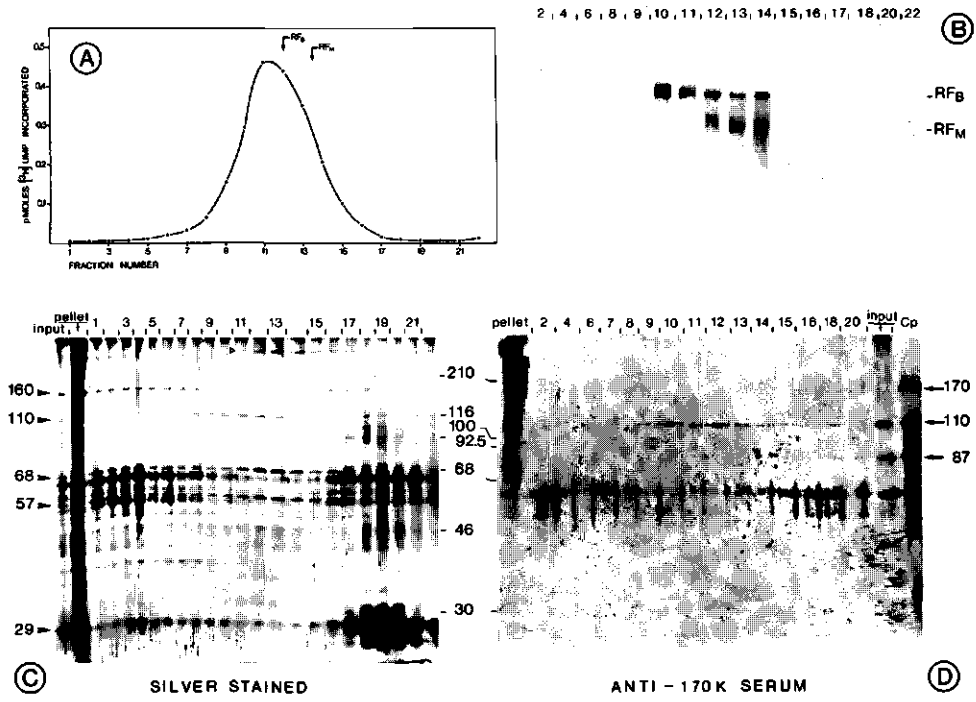

Fig. 3. Characterization of CPMV RNA replication compex by glycerol gradient centrifugation.
(A) Sepharose 2B-purified replication complex, prepared from 29 g of CPMV-infected cowpea leaves, was concentrated to a volume of 0.3 ml, layered onto an 11 ml linear 17.5-40% (v/v) glycerol gradient in TKED buffer containing 0.5% Triton X-100 and centrifuged at 36,000 rpm for 17 hr at 4° in a SW-41 rotor. Fractions (0,5 ml) were collected and 30 µl aliquots were assayed for RNA polymerase activity in a reaction mixture containing only 1 µM [^{3}H]-UTP (spec. act. 41 Ci/mmol). These assay conditions result in a three-fold reduction of the number of pmoles [^{3}H]UMP incorporated as compared to our standard conditions. Recovery of polymerase activity after glycerol gradient centrifugation was approximately 68%.
(B) Reaction mixtures (40 µl, 2.2 µM [α-^{32}P]UTP, spec.act 155 Ci/mmol) containing 20 µl of the glycerol gradient fractions as indicated, were incubated for 30 min. RNA products were prepared and analyzed by agarose gel electrophoresis under nondenaturing conditions, as described.
(C) Fifty µl aliquots of the glycerol gradient fractions, 2 µl of the concentrated Sepharose void fraction (input) and 5 µl of the pellet fraction (dissolved in 100 µl 0.5% SDS) of the glycerol gradient were analyzed on a 10% SDS-polyacrylamide gel. Polypeptide bands were visualized by silver staining following prestaining with Coomassie brilliant blue. Some of the bands present throughout the gradient in the 53K-68K region are due to background staining. ▷ Indicates the position of CPMV dsRNA (RF$_B$ and RF$_M$).
(D) One hundred µl aliquots of the glycerol gradient fractions as indicated, 10 µl of the pellet fraction of the glycerol gradient (see above), 20 µl of the concentrated Sepharose void fraction (input) and 30 µl of a 30,000 g supernatant from CPMV-infected cowpea protoplasts (Cp; Franssen *et al.*, 1982) were fractionated on a 10% SDS-polyacrylamide gel. After electrophoresis, polypeptides were blotted onto nitrocellulose and the blot was incubated, successively, with 100 µl crude anti-170K serum and ^{125}I-protein A as described. The position of marker polypeptides is indicated between panels C and D (molecular weight x 10^{-3}).

extent to which the membranes were disintegrated by treatment with Triton X-100. The vast majority of these contaminating proteins, however, were readily removed by subsequent glycerol gradient centrifugation (Fig. 3C).

Only three polypeptide bands with molecular weights of approximately 110K, 68K and 57K were detected by silver staining in fractions containing RNA polymerase activity. Among these polypeptides, only the 110K polypeptide was found to copurify consistently with the CPMV RNA replication complex in all glycerol gradients analyzed so far, the other two polypeptides being present throughout the gradient. In some experiments, the 68K and 57K bands were not distinguishable, owing to strong background staining in the 53K-65K region of the gel which particularly appeared to interfere at low protein concentrations. Similar background bands produced by silver staining have been observed by others (Morrissey *et al.*, 1981) and have been attributed to 2-mercaptoethanol used in the sample buffer (Tasheva and Dessev, 1983). Background bands in the same size region have also been observed when polypeptide bands were visualized after blotting onto nitrocellulose filters, using antisera which had been raised by immunizing rabbits with antigens purified by polyacrylamide gel electrophoresis (see below). On using silver staining to detect polypeptides, we have also been able to detect the RNA moiety of the viral replication complex in fractions containing RNA polymerase activity, as shown by the two bands at the top of the gel (Fig. 3C). These bands comigrated with purified RF_B and RF_M and were only observed if the samples were not heated at $100^{o}C$ prior to gel electrophoresis. Indeed, silver staining has recently been reported to be applicable to nucleic acids (Somerville and Wang, 1981).

CPMV B-RNA-encoded 110K polypeptide is associated with the CPMV RNA replication complex.

Previously, it has been found that CPMV B-RNA encodes information essential to viral RNA replication, suggesting that one or more of the B-RNA-encoded polypeptides with molecular weights of 170K, 110K, 87K, 84K, 60K, 58K, 32K and 4K (VPg), respectively, might be component(s) of the viral replication complex (Goldbach *et al.*, 1980). In Fig. 5 it is shown how these polypeptides arise from a common 200K polyprotein precursor by specific proteolytic cleavages and how these polypeptides are related to each other (Goldbach and Rezelman, 1983).

Antisera raised against the separate 170K, 110K, 87K and VPg polypeptides were used to study the occurrence of virus-specific proteins in preparations of the CPMV RNA replication complex at various stages of purification. Following SDS-polyacrylamide gel electrophoresis, polypeptides of different fractions were transferred to nitrocellulose and blots were treated with antisera directed against B-RNA-encoded polypeptides, using ^{125}I-labeled protein A to detect immunocomplexes.

Incubation with anti-170K serum revealed the presence of 170K, 112K 110K, 87K and 84K B-RNA-encoded polypeptides in the crude membrane fraction, among which the 112K polypeptide represents a novel viral protein not detected before (Fig. 4A, lane 2). The bulk of these viral proteins was released into the buffer-soluble fraction by washing the crude membrane fraction with Mg^{2+}-deficient buffer, whereas the remainder was solubilized, along with the viral replication complex, by treating the washed membrane fraction with Triton X-100. Only very small amounts of the virus-specific proteins were found to copurify with the CPMV RNA replication complex upon Sepharose 2B chromatography (Fig. 4A, lane 6). The 170K, 112K and 84K polypeptides were virtually absent in the void fraction of the column which contained all the replication complex activity, whereas the 110K polypeptide and, to a lesser extent also the 87K polypeptide were clearly detectable.

In addition to these viral polypeptides, considerable reaction was obser-

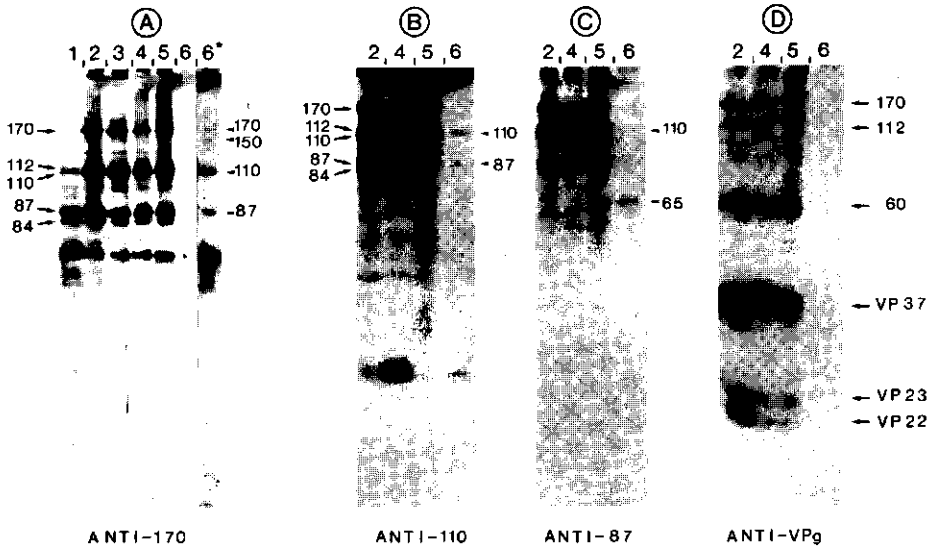

Fig. 4. Immunological analysis of partially purified CPMV RNA replication complex.
Samples of CPMV RNA replication complex at various stages of purification were analyzed on a 12.5% SDS-polyacrylamide gel, blotted onto nitrocellulose and probed with crude antisera as indicated. Lane 1, cytoplasmic fraction; lane 2, crude membrane fraction; lane 3, buffer-soluble fraction; lane 4, washed membrane fraction; lane 5, detergent-soluble fraction; lane 6, concentrated Sepharose 2B void fraction.
(A) Lane 1-5 each contained 30 µg of protein derived from 4 mg, 12 mg, 16 mg, 28 mg and 59 mg of CPMV-infected cowpea leaves, respectively. The amount of protein (derived from 1100 mg of CPMV-infected leaves) applied to lane 6 was unknown.
(B, C, D) Lane 2 contained 17.5 µg of protein (derived from 7 mg of CPMV-infected leaves); lane 4, 30 µg of protein (28 mg); lane 5, 29 µg of protein (57 mg); lane 6, unknown amount of protein (derived from 1540 mg of CPMV-infected leaves). *: autoradiograph obtained after four-fold longer exposure; → indicates position of CPMV-specific polypeptides.

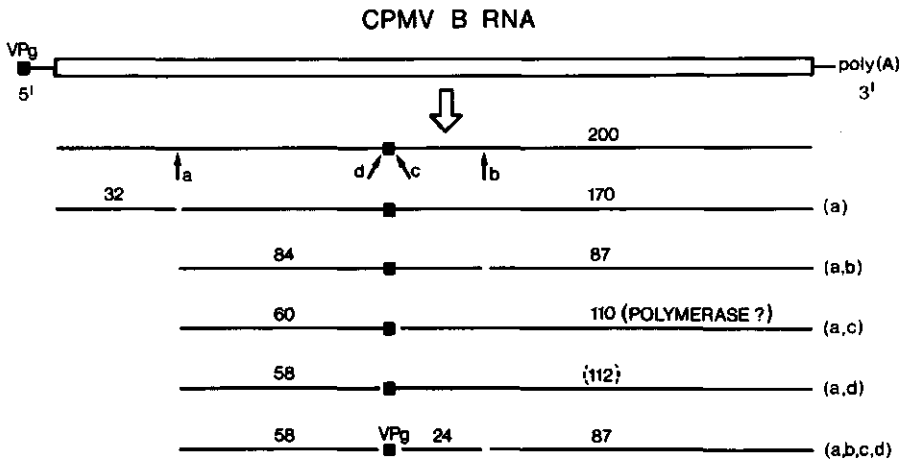

Fig. 5. Translation and processing map of CPMV B-RNA.
This map is taken from Goldbach and Rezelman (1983) and slightly modified to include some recent unpublished results. Proteolytic cleavage sites in the 200K polyprotein are indicated by arrows (a-d). Cleavage at position 'a' occurs first, the order of the other three cleavages remains to be established. The map position of the 112K polypeptide is tentative.

ved in the region of apparent molecular weight of 53K - 65K. However, this blackening turned out to be due to a strong background reaction - probably similar to the one obtained with silverstaining - and thus precluded establishing the presence of the B-RNA-encoded 60K VPg-precursor polypeptide in the different fractions. Since anti-VPg serum did not display the background reaction observed with the anti-170K serum, this particular antiserum was used to detect the 60K polypeptide (Zabel *et al.*, 1982).

Anti-VPg serum reacted rather weakly with 170K and 112K polypeptides and very strongly with the 60K VPg-precursor present in the crude membrane fraction, the washed membrane fraction and the solubilized replication complex (Fig. 4D). The 112K polypeptide which reacted both with anti-VPg serum and anti-170K serum (Figs. 4A and 4D) and was more abundant in leaf tissue than in isolated protoplasts, might represent a B-RNA-encoded polypeptide not detected previously, and arising by alternative cleavage of the 170K polypeptide as indicated in Fig. 5. By using crude anti-VPg serum, which had not been preadsorbed with virion particles to remove antibodies directed against CPMV capsid proteins (Zabel *et al.*, 1982), the presence of the VP37 and VP23/22 capsid proteins could be concomitantly detected in the different fractions. Following Sepharose 2B chromatography, both the 60K VPg-precursor and the capsid proteins were no longer detectable in the replication complex (Fig. 4D).

The results obtained with both anti-110K and anti-87K serum confirm the conclusions derived from the blot treated with anti-170K serum, that low amounts of 110K and 87K polypeptides were detected in the Sepharose void

fraction (Figs. 4B and 4C). A 150K polypeptide detected by the anti-170K serum in the void fraction of the Sepharose 2B-column (Fig. 4A, lane 6), also reacted weakly with the anti-110K and anti-87K sera. In addition to the immunoreactive B-RNA-encoded polypeptides mentioned above, the anti-87K serum also reacted with a 65K polypeptide of unknown origin (Fig. 4C). Whether the 150K and 65K polypeptides represent as yet unidentified viral polypeptides remains to investigated.

After glycerol gradient centrifugation, the B-RNA-encoded 110K polypeptide was the only viral polypeptide detectable in gradient fractions containing the functional replication complex (Fig. 3D). The 170K and 87K viral polypeptides present in the concentrated enzyme preparation applied to the glycerol gradient (Fig. 3D, input) were completely resolved from the replication complex and recovered in the pellet fraction. Upon treatment of a protein blot of the glycerol gradient fractions with anti-VPg serum, no polypeptide bands could be detected (data not shown). This indicates that the 110K polypeptide is not - nor is it contaminated with - the B-RNA-encoded 112K polypeptide containing VPg sequences. Similarly, no host-encoded 130K RdRp could be detected in the glycerol gradient fractions using either silver staining or anti-RdRp serum. Since the sedimentation profile of the 110K polypeptide as detected by both silver staining and reaction with anti-170K serum completely coincides with the sedimentation profile of the RNA polymerase activity of the viral RNA replication complex (Fig. 3), we tentatively conclude that the CPMV B-RNA-encoded 110K polypeptide represents the core RNA polymerase of the replication complex.

DISCUSSION

The specific purpose of the purification procedure described here was to maintain a functional CPMV RNA replication complex capable of elongating *in vitro* nascent viral RNA strands throughout all purification steps. The rationale for this approach was that other approaches which aimed at the purification of template-free RNA-dependent RNA polymerases (RdRp), have indeed led to the identification of a host-encoded enzyme, the activity of which is greatly stimulated upon viral infection, but have failed so far in demonstating this particular enzyme to be actually involved in viral replication (Gordon *et al.*, 1982; Takanami and Fraenkel-Conrat, 1982; Dorssers *et al.*, 1982; Van der Meer *et al.*, 1983a). On the other hand, one may be sure to be dealing with the real core RNA polymerase of the viral RNA replicase, if one purifies a functional enzyme-template complex that is completing

in vitro the synthesis of viral RNA chains which were initiated *in vivo*. As no conclusive evidence is available regarding the origin of the core polymerase of any of the plant viral RNA replicases, such an approach seemed both obvious and promising to pursue. Once the core of the replicase has been identified, one may aim at the purification and characterization of polypeptides involved in template recognition and initiation of RNA synthesis.

As a prelude to purifying the CPMV RNA replication complex, we have previously characterized in detail the crude membrane fraction from CPMV-infected cowpea leaves and shown the occurrence of two, functionally different, RNA polymerase-template complexes, only one of which appeared to display the essential features of a typical viral RNA replication complex (Dorssers *et al.*, 1982). The other RNA polymerase activity, which could be separated from the viral replication complex by washing the membrane fraction in a Mg^{2+}-deficient buffer, has recently been purified and identified as a host-encoded 130K polypeptide (Dorssers *et al.*, 1982; Van der Meer *et al.*, 1983a, 1983b).

We have now succeeded in purifying the CPMV RNA replication complex following its solubilization from the washed membrane fraction with Triton X-100. The solubilized viral replication complex was found to be contaminated with some host-encoded 130K RdRp, but subsequent gel filtration on Sepharose 2B appeared to be highly effective in resolving the remainder of this activity from the replication complex. As discussed elsewhere (Van der Meer *et al.*, 1983b) these results clearly demonstrate that the host-encoded RdRp, which has often been imputed a role in viral RNA replication (for references see Dorssers *et al.*, 1982; Fraenkel-Conrat, 1983) does not constitute the core polymerase of the viral replicase.

Virus-specific RNA polymerase activity represents less than 5% of total RNA-dependent RNA polymerase activity present in the crude membrane fraction from CPMV-infected cowpea leaves four days after inoculation. The large excess of host-encoded RdRp makes it therefore imperative to isolate a functional viral RNA replication complex free of host RdRp before identification of the RNA polymerase involved in viral replication can be undertaken.

The purification procedure presented in this paper has been designed so as to take advantage of both the characteristic conformation of the solubilized CPMV RNA replication complex, consisting of RNA polymerase molecules associated with RI's, and its low sedimentation rate. The former has been achieved by Sepharose 2B chromatography, a purification step well known for isolating protein-free double-stranded RNA (RF and RI), the latter by glycerol gradient centrifugation. In conjunction, the two steps were sufficient to allow analysis of the polypeptide composition of the CPMV RNA replication complex.

Among the three major polypeptides (110K, 68K and 57K) detected in the glycerol gradient fractions containing the replication complex, the 110K

polypeptide was identified as a virus-specific polypeptide encoded by CPMV B-RNA. This polypeptide was the only viral polypeptide found to cosediment consistently with the RNA polymerase activity of the replication complex, even though the other two viral polypeptides (170K and 87K) present in the Sepharose void fraction are closely related to the 110K polypeptide (Fig. 5). Apparently, association of the 110K polypeptide with replicating RNA is functional rather than based on adventitious binding. Furthermore, by comparing the sedimentation profiles of replication complex preparations from different experiments, it became apparent that the amount of polymerase activity was correlated with the amount of 110K polypeptide detectable in the replication complex (data not shown). From these observations, we tentatively conclude that the CPMV B-RNA-encoded 110K polypeptide constitutes the core RNA polymerase of the replication complex. Whether the 68K and 57K polypeptides represent host proteins still contaminating the replication complex or are functional subunits required for polymerase activity remains to be determined.

To further define the role of the 110K polypeptide in viral RNA replication, we have attempted to neutralize the *in vitro* RNA polymerase activity of the replication complex by using antibodies raised against the B-RNA-encoded 170K, 110K and 87K polypeptides. However, none of the antisera preparations was capable of inhibiting the polymerase activity (data not shown). Since on the other hand, all these antisera preparations did react readily with viral proteins following blotting onto nitrocellulose filters, these results indicate that the antigenic sites on the 110K polypeptide, that is present in the replication complex, either do not comprise the active site of the enzyme molecule or are not accessible to antibodies. The latter may be due to association of the 110K polypeptide with endogenous RNA templates and/or with other polypeptides in the replication complex. It should also be noted, however, that the antisera were raised against denatured polypeptides present within polyacrylamide and, therefore, the antibodies may not have been as reactive against native polypeptides in the replication complex.

It is obvious from Fig. 4, that a considerable part of the virus-specific 110K polypeptide present in the crude membrane fraction from CPMV-infected cowpea leaves does not copurify with the replication complex. Apparently, the bulk of 110K polypeptide is not associated, or does not remain associated, with replicating viral RNA molecules. Both the 110K polypeptide, and any of the other virus-specific proteins occurring outside the functional replication complex, were found to be incapable of transcribing *in vitro* added RNA (data not shown). Such observations indicate that the 110K polypeptide alone is not sufficient to act as a polymerase, but requires other proteins in order to carry out its function. Another plausible explanation might be, that the 110K polypeptide is unable to start RNA synthesis on an added

template and needs additional proteins for initiation. If initiation of CPMV RNA synthesis is mediated by the generation of VPg from its precursor, by analogy to what has been suggested for poliovirus RNA (for review see Wimmer, 1982), then it is conceivable that this step does not occur on added template RNA.

We have not been able to demonstrate *de novo* initiation of viral RNA synthesis to occur with the CPMV RNA replication complex either (data not shown). Similarly, the core polymerase involved in the replication of bacteriophage Qβ RNA and poliovirus RNA are incapable of transcribing their homologous templates in the absence of specific subunits and/or primers required for initiation (Blumenthal and Carmichael, 1979; Wimmer, 1982). As the only activity of the CPMV RNA replication complex involves elongation of nascent viral RNA chains, the synthesis of which has been started *in vivo*, the isolated replication complex apparently represents only a part of the viral RNA replicating machinary. Future studies may reveal the role of other host- and viral-encoded subunits in viral replication.

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VIII. CONCLUDING REMARKS

The aim of the research described in this thesis was the purification and identification of the RNA-dependent RNA polymerase engaged in replicating viral RNA in cowpea mosaic virus (CPMV)-infected cowpea leaves.

Previously, an RNA-dependent RNA polymerase produced upon infection of *Vigna unguiculata* plants with CPMV, was partially purified (Zabel, 1978). This enzyme was thought to be responsible for the replication of viral RNA and believed to be encoded, at least in part, by the virus genome.

In chapter III, it is shown that this enzyme is actually a host-encoded enzyme that occurs in small amounts in uninfected cowpea leaves and is greatly enhanced upon infection with CPMV. The host-encoded RNA-dependent RNA polymerase was extensively purified by DEAE-Sepharose, poly(U)-Sepharose and phosphocellulose column chromatography. Using antiserum directed against the purified host-encoded enzyme and a novel antibody-linked polymerase assay (ALPA), the host-enzyme was found to consist of a monomeric polypeptide with a molecular weight of 130,000 dalton (chapter IV).

Since it was uncertain whether the purified host-encoded RNA polymerase plays any role in viral RNA replication, we reinvestigated the membrane-bound RNA-dependent RNA polymerase activity associated with endogenous template and known to be capable of synthesizing viral RNAs *in vitro*. Detailed analysis of the RNAs synthesized *in vitro* by RNA-dependent RNA polymerase activity associated with the crude membrane fraction of CPMV-infected leaves revealed the existence of two functionally different enzyme-template complexes (chapter V). One of the activities constitutes the CPMV RNA replication complex engaged in the production of full-length (+)-type viral RNA. The other activity occurs both in uninfected and infected leaves and transcribes endogenous plant RNAs into small RNAs of negative polarity. The occurrence of two distinct RNA-synthesizing activities in infected leaves is supported by their respective behaviour upon extracting the crude membrane fraction with a Mg^{2+}-deficient buffer. Whereas the host-enzyme-template complex is solubilized, the viral RNA replication complex remains membrane-bound.

A further analysis as to the role of the host-enzyme in viral RNA replication is presented in chapter VI. Using an antiserum directed against the purified host-encoded RNA polymerase to identify the 130K polypeptide on protein blots, it was confirmed that the amount of host-enzyme is greatly enhanced in leaves upon invasion of the virus. In contrast, no increase of 130K polypeptide occurs in isolated cowpea protoplasts infected with CPMV,

indicating that the increase of host-enzyme represents a tissue-specific response to virus inoculation. Furthermore, the host-encoded 130K RNA polymerase is not found associated with the CPMV RNA replication complex. Thus, it is concluded that the RNA-dependent RNA polymerase of cowpea does not participate in CPMV RNA replication.

The purification procedure and the analysis of the protein composition of the viral replication complex is described in chapter VII. After solubilization from the membranes with a nonionic detergent and extensive purification by chromatography on Sepharose 2B and glycerol gradient centrifugation, an RNA replication complex preparation is obtained that is still capable of elongating nascent RNA chains *in vitro*. The purified replication complex appeared to contain three polypeptides, as revealed by SDS-polyacrylamide gel electrophoresis and silver staining. One of these polypeptides was found to be identical to the CPMV B-RNA encoded 110K polypeptide. Since the host-encoded RNA-dependent RNA polymerase of cowpea is not involved in viral RNA replication, it is tempting to speculate that the 110K viral protein, by analogy to bacterial- and animal RNA viruses, is the core polymerase engaged in elongating CPMV RNAs in the viral RNA replication complex. Further experiments are required to verify this conclusion.

The data presented in this thesis dispute the hypothesis that plant virus RNA replication might be mediated by a host-encoded RNA-dependent RNA polymerase. Contradictory to this hypothesis, CPMV RNA replication does not require the host-encoded RNA polymerase of cowpea but uses a different polymerase, probably of viral origin. This virus-specific RNA polymerase is present in very low amounts in infected tissue in comparison with the host-enzyme, and has previously been overlooked during purification of the viral replicase (Dorssers *et al.*, 1982). Only by employing an assay which keeps track of the synthesis of viral RNA, we have been able to distinguish between these RNA-dependent RNA polymerase activities.

In other host plants inoculated with different viruses, a virus-specific RNA polymerase may similarly be present in low amounts in the particulate fraction of the leaves in addition to an enhanced amount of host-encoded RNA-dependent RNA polymerase. It seems conceivable, that in some cases where the purification of a plant viral replicase has been attempted without success, as for example with TMV, AlMV and CMV, the virus-specific RNA polymerase has been overlooked because of improper assay conditions applied during isolation and the very small amount of viral replicase relative to the host-encoded RNA-dependent RNA polymerase. It seems almost imperative in purifying plant viral RNA replicases to use test methods which keep control on the ability of the enzyme to produce full-length viral RNAs. In particular, this should apply when the purification of a viral RNA replication complex turns out to be impossible because the endogenous template is rapidly lost (Zaitlin

et al., 1973). By using a proper assay, a BMV replicase preparation from BMV-infected barley displaying a high degree of template-specificity has been obtained (Bujarski *et al.*, 1982; Miller and Hall, 1983).

In those cases where it is not possible to isolate intact viral RNA replication complexes, an appropriate primer complexed to the template may be useful to test for the presence of a different (viral ?) core polymerase. Thus, the first step in viral RNA replication - i.e. specific template recognition and initiation of RNA synthesis for which specific factors are required that are often readily lost - is bypassed and the core polymerase is allowed to start transcription by elongating the primer. This approach has proven to be successful in identifying the core polymerase of several picornaviruses (Flanegan and Baltimore, 1977; Van Dijke and Flanegan, 1980; van Dijke *et al.*, 1982; Baron and Baltimore, 1982; Lowe and Brown, 1981) and it is rather surprising that it has not yet been used in the study of plant viral RNA replication.

Concerning CPMV RNA replication, the mechanism of initiation of CPMV RNA synthesis is not yet understood. The occurrence of a protein covalently linked to the 5'-end of the virion RNAs suggest that VPg might be of vital importance for the initiation of CPMV RNA synthesis. Evidence to support this hypothesis may be obtained by determining the nature of the 5'-termini of RNA replication intermediates. *De novo* initiation of CPMV RNA synthesis *in vitro* has not been detected sofar, not even in the viral RNA replication complex still being an intergral part of the cellular membranes. The lacking of *de novo* initiation of CPMV RNA synthesis, suggests that some factor(s) required for initiation are already lost or inactivated upon the first fractionation of CPMV-infected leaves.

A CPMV RNA-specific binding activity has previously been detected by using a nitrocellulose filter-binding assay (Zabel *et al.*, 1979). This specific binding activity was found in preparations devoid of the CPMV RNA replication complex and can therefore not be ascribed to functions of the viral replication complex. However, this binding activity, which should be identified further, may well be involved in initiation of CPMV RNA synthesis.

In conclusion, the replication of CPMV RNA may be described by the following model, taking into account the well established features of the process. (i) Infecting CPMV B-RNA directs the synthesis of a large B-RNA-specific polyprotein which is subsequently processed. Some mature protein then induces the expansion of cellular membranes. (ii) Proteolytic processing of the B-RNA-encoded precursors seems to occur in association with membranes, as virtually all 170K polypeptides are found in the particulate fraction (chapter VII, fig. 4A). (iii) The CPMV RNA replication complex is assembled using the viral RNA, the putative 110K core polymerase and probably other virus-encoded and host proteins, and is integrated in the cellular membranes

to start RNA synthesis. Since the B-RNA-encoded proteins do not seem to be typical membrane proteins - in majority, they are solubilized by extraction with a Mg^{2+}-deficient buffer - host proteins should play an important role in the compartimentalization of viral RNA synthesis. (iv) Initiation of viral RNA synthesis may involve VPg to be donated by either the 60K VPg-precursor or the 112K VPg-containing polypeptide. The latter seems to be largely overlapping with the putative 110K core polymerase and may be converted into the active core polymerase upon donating VPg.

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IX. SAMENVATTING

Het in dit proefschrift beschreven onderzoek had tot doel meer inzicht te verkrijgen in het mechanisme van de RNA replicatie van cowpea mozaïek virus (CPMV). CPMV is een virus bestaande uit kleine isometrische deeltjes en een enkelstrengig RNA genoom van het (+)-type. CPMV leent zich goed voor onderzoek naar de replicatie van dit type virussen, daar het snel wordt vermenigvuldigd in zijn waardplant *Vigna unguiculata* (cowpea), en bovendien in grote hoeveelheden wordt gemaakt.

In hoofdstuk II wordt een overzicht gegeven van wat er bekend is over de replicatie van bacterievirussen, diervirussen en plantevirussen met een enkelstrengig RNA genoom van het (+)-type. Bij bacterievirussen is het replicatie mechanisme tot in detail opgehelderd, bij diervirussen is deze kennis nog fragmentarisch terwijl bij plantevirussen hierover nog erg weinig bekend is. Voor de synthese van virus RNA is een RNA-afhankelijk RNA polymerase nodig. Bij bacteriële RNA virussen codeert het infecterende RNA voor een RNA-afhankelijk RNA polymerase, dat samen met enige gastheer-eiwitten een virus RNA replicase vormt. Dit replicase is verantwoordelijk voor de vermeerdering van het virusgenoom RNA. Recentelijk is gevonden dat ook een aantal diervirussen (o.a. poliovirus en mond- en klauwzeer virus) in geïnfecteerde cellen zorgen voor de synthese van een virus-specifiek RNA polymerase met behulp waarvan het virus RNA vermenigvuldigd wordt.

Een van de vragen die ten grondslag lagen aan het onderzoek voor dit proefschrift was of het replicatie mechanisme van CPMV volgens een overeenkomstig patroon verloopt of daarvan afwijkt. In planten komt, in tegenstelling tot bacteriële en dierlijke cellen, namelijk in kleine hoeveelheden een RNA-afhankelijk RNA polymerase voor, dat sterk toeneemt na virus-infectie. Daarom wordt door een aantal onderzoekers wel verondersteld, dat plantevirussen met een RNA genoom voor hun replicatie gebruik zouden maken van het RNA-afhankelijk RNA polymerase van de waardplant en niet zelf voor zo'n enzym zorgen, zoals bacterie en dier RNA virussen doen.

Het onderzoek vormt de voortzetting van onderzoek zoals dat beschreven is in het proefschrift van Dr. P. Zabel. In zijn proefschrift wordt o.a. beschreven hoe uit met CPMV-geïnfecteerde cowpea bladeren een membraan-gebonden RNA-afhankelijk RNA polymerase geïsoleerd kan worden, dat in staat is om *in vitro* CPMV RNA te maken. Deze RNA-afhankelijk RNA polymerase activiteit kon, voor het grootste gedeelte, losgemaakt worden uit de membranen door deze te wassen met een Mg^{2+}-deficiënte buffer en daarna gedeeltelijk gezuiverd worden.

In hoofdstuk III van dit proefschrift wordt beschreven hoe dit RNA-afhanke-

lijk RNA polymerase uit de membraanfraktie van met CPMV-geïnfecteerde bladeren gezuiverd kon worden, met behulp van achtereenvolgens DEAE-Sepharose CL-6B chromatografie, poly(U)-Sepharose affiniteitschromatografie en glycerol-gradiënt centrifugatie. Het gezuiverde enzym preparaat bleek slechts één eiwit met een molecuul gewicht van 130,000 (130K) dalton te bevatten en identiek te zijn aan het RNA-afhankelijk RNA polymerase, dat, zij het in veel lagere concentratie, aanwezig is in gezonde bladeren. De conclusie moest dus wel zijn dat het RNA-afhankelijk RNA polymerase dat wij gezuiverd hadden, een gastheer- en niet een virus-gecodeerd enzym is.

Uit proeven van andere medewerkers van ons laboratorium over de vermenigvuldiging van CPMV in geïsoleerde bladcelprotoplasten was echter gebleken dat wel degelijk een of meer virus-specifieke eiwitten betrokken moeten zijn bij de replicatie van CPMV RNA. Omdat in het gezuiverde enzym preparaat geen virus-specifieke eiwitten waren te onderkennen, leken er twee mogelijkheden te zijn: of de virus-specifieke eiwitten waren verloren gegaan bij de zuivering of met het gezuiverde enzym hadden we niet het RNA-afhankelijk RNA polymerase dat deel uitmaakt van het virus RNA replicase te pakken.

Om na te gaan of het door ons gezuiverde RNA-afhankelijk RNA polymerase al dan niet een rol speelt bij de replicatie van CPMV RNA, hebben we een antiserum bereid tegen het gezuiverde gastheer-enzym. In hoofdstuk IV wordt beschreven hoe we er in geslaagd zijn om met behulp van dit antiserum en een nieuw ontwikkelde immunologische detectie methode aan te tonen dat het 130K eiwit het enige bestanddeel is van het gastheer-specifieke RNA-afhankelijk RNA polymerase. Dit resultaat stelde ons in de gelegenheid het antiserum te gebruiken als een heel gevoelige toets om het gastheer enzym aan te tonen in verschillende enzym preparaten.

In hoofdstuk V wordt beschreven hoe toen eerst de membraan-gebonden RNA-afhankelijk RNA polymerase aktiviteit uit CPMV-geïnfecteerde bladeren nader is onderzocht. Die RNA polymerase aktiviteit was immers in staat om *in vitro* virus RNA te maken. Op basis van een zeer grondige analyse van de produkten die *in vitro* gemaakt worden door de membraan-gebonden RNA-afhankelijk RNA polymerase aktiviteit, konden we konkluderen dat er niet één maar twee verschillende RNA synthetiserende aktiviteiten in de membranen van met CPMV-geïnfecteerde bladeren voorkomen. De ene aktiviteit bleek ook voor te komen in gezonde cowpea bladeren en was dus het gastheer-enzym. Dit enzym kopieërt plante RNA's, en na virus-infectie ook virus RNA's, maar de produkten zijn korte, heterogene stukjes RNA. De andere aktiviteit maakt, zoals al eerder gevonden was, *in vitro* virus RNA's met de lengte zoals die ook in virus deeltjes gevonden wordt. Deze aktiviteit had bovendien de kenmerken van een zgn. viraal replicatie complex. Daarmee wordt bedoeld een complex van een RNA-afhankelijk RNA polymerase met een endogene virus RNA matrijs, dat bezig is door overschrijving van die matrijs nieuwe RNA ketens te maken. Zo'n com-

plex kan *in vitro* de synthese van virus RNA, waar het *in vivo* mee begonnen is, afmaken. Het membraan-gebonden RNA-afhankelijk RNA polymerase van met CPMV geïnfecteerde bladeren bleek de nascente virus RNA ketens te verlengen tot volledige lengte. Er vond geen initiatie van de synthese van nieuwe RNA ketens plaats.

Dit virale replicatie complex bleek stevig gebonden te zijn aan de membranen en ging niet in oplossing door wassen met een Mg^{2+}-deficiënte buffer, i.t.t. het gastheer-specifieke RNA-afhankelijk RNA polymerase. Het viral replicatie complex kon alleen vrijgemaakt worden uit de membranen met behulp van niet-ionogene detergentia en vervolgens gezuiverd worden door Sepharose 2B kolomchromatografie. Het virale replicatie complex bleef daarbij intakt in de zin dat het nog steeds bestond uit een RNA-afhankelijk RNA polymerase geassocieerd met een endogene virus RNA matrijs. Bovendien was het complex nog steeds in staat om *in vitro* nascente virus RNA ketens te verlengen tot volledige lengte. Sepharose 2B kolomchromatografie scheidde het virale replicatie complex van het grootste gedeelte van de andere eiwitten uit de met detergentia opgeloste membranen.

In hoofdstuk VI wordt dan beschreven hoe we het antiserum gericht tegen het gastheer-specifieke RNA-afhankelijk RNA polymerase (130K eiwit) hebben gebruikt om het met behulp van Sepharose 2B kolomchromatografie gezuiverde virale replicatie complex te onderzoeken op de aanwezigheid van het waardplant-specifieke RNA polymerase. Het bleek dat het 130K RNA-afhankelijk RNA polymerase niet voorkomt in het virale replicatie complex en aangezien dit complex nog wel in staat is om virus RNAs te maken, kan de conclusie niet anders zijn dan dat het gastheer-specifieke RNA-afhankelijk RNA polymerase niet het enzym is dat verantwoordelijk is voor de synthese van virus RNAs in het virale replicatie complex.

In hoofdstuk VI wordt ook beschreven hoe we met behulp van het antiserum tegen het gastheer-specifieke RNA-afhankelijk RNA polymerase het vóórkomen van dit enzym in met CPMV-geïnfecteerde cellen van bladeren en in met CPMV-geïnfecteerde bladcelprotoplasten hebben bestudeerd. Daaruit bleek dat in met CPMV-geïnfecteerde cellen van bladeren de hoeveelheid van dit enzym inderdaad snel toeneemt in vergelijking met gezonde cellen, maar in geïsoleerde bladcelprotoplasten geïnfecteerd met CPMV bleek die toename niet op te treden. Er is dus ook geen sprake van een direkte samenhang tussen de stimulatie van het gastheer-enzym en de replicatie van virus RNA.

Om dan tenslotte de vraag te kunnen beantwoorden wat dan wel het RNA-afhankelijk RNA polymerase in het virale replicatie complex is, is het replicatie complex verder gezuiverd met behulp van dichtheidsgradiëntcentrifugatie in een glycerolgradiënt (hoofdstuk VIII). Het virale replicatie complex bleek vrij klein te zijn (het sedimenteerde met een sedimentatiecoëfficiënt van 20-25S) en nog maar zeer geringe hoeveelheden eiwit te bevatten. Het vermogen

om CPMV RNA's te maken was volledig intakt gebleven. Het zo gezuiverde virale replicatie complex bleek nog drie eiwitten te bevatten, respectievelijk met molekuulgewichten van ongeveer 110,000 (110K), 67,000 (67K) en 57,000 (57K). Met behulp van antisera gericht tegen virus-specifieke eiwitten kon worden vastgesteld dat het 110K eiwit identiek is aan een bekend door CPMV RNA gecodeerd eiwit. De andere eiwitten bleken gastheer eiwitten te zijn.

Op grond van deze gegevens hebben we geconcludeerd, dat het virus-gecodeerd 110K eiwit een functioneel onderdeel vormt van het CPMV RNA replicatie complex Omdat in het virale replicatie complex een RNA-afhankelijk RNA polymerase aanwezig is en dit niet het gastheer enzym is, ligt het voor de hand te veronderstellen dat het 110K eiwit deze functie heeft. Als deze hypothese juist is, betekent dit dat bij de replicatie van CPMV een virus-gecodeerd RNA-afhankelijk RNA polymerase betrokken is. Het lijkt dan aannemelijk dat dit ook het geval zal zijn bij de replicatie van andere plantevirussen met een RNA genoom.

CURRICULUM VITAE

Lambert Dorssers werd op 31 juli 1953 geboren te Sevenum (L). In 1971 deed hij eindexamen Atheneum B en studeerde daarna verder aan de Landbouwhogeschool te Wageningen. In 1978 slaagde hij voor het doctoraal examen in de richting Moleculaire Wetenschappen (N43) met als hoofdvakken Moleculaire Biologie, Biochemie en Moleculaire Fysica en als bijvak Levensmiddelenchemie.

Van 1 oktober 1978 tot 30 september 1982 was hij in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.) - in het kader van de Werkgemeenschap Nucleinezuren van de Stichting voor Scheikundig Onderzoek in Nederland (S.O.N.) - en verbonden aan de vakgroep Moleculaire Biologie van de Landbouwhogeschool, waar hij onderzoek verrichtte aan het mechanisme van de replicatie van cowpea mozaiek virus RNA zoals in dit proefschrift beschreven wordt.

Sinds 1 mei 1983 is hij in tijdelijke dienst van de Stichting Integraal Kankercentrum Rotterdam (I.K.R.) verbonden aan het Radiobiologisch Instituut T.N.O. te Rijswijk.