In vitro transcription of *Sonchus* yellow net virus RNA by a virus-associated RNA-dependent RNA polymerase

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IN VITRO TRANSCRIPTION OF SONCHUS YELLOW NET VIRUS RNA BY A VIRUS-ASSOCIATED RNA-DEPENDENT RNA POLYMERASE

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C. C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 17 januari 1986
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen.

Opgedragen aan mijn ouders en
natuurlijk aan Anja Derksen

STELLINGEN

1. *Sonchus* yellow net virus bezit een van RNA afhankelijke RNA-polymerase.
   Dit proefschrift.

2. Kawai levert geen overtuigend bewijs dat het L eiwit van rabiesvirus het van RNA afhankelijke RNA-polymerase is.
   Kawai, A. (1977). Journal of Virology 24, 826-835.

3. Mahy and Barrett beweren ten onrechte dat van de negatief-strengige virussen alleen influenzavirus de functionele kern van de gastheercel voor replicatie gebruikt.
   Mahy, B.W.J., and Barrett, T. (1983). In: Replication of viral and cellular genomes. Ed. Y. Becker, Martinus Nijhoff Publishing, Boston, pp. 345-365.

4. Het feit dat Kelly en Lescott geen fosforylering van het polyedereiwit van het *Trichoplusia ni* kernpolyedervirus vinden, terwijl de meeste baculovirusonderzoekers wel fosforylering kunnen aantonen, zou geweten kunnen worden aan comigratie van een gefosforyleerd gastheereiwit en het polyedereiwit.
   Kelly, D.C. en Lescott, T. (1984). Journal of General Virology 65, 1183-1191.
   Maruniak, J.E. en Summers, M.D. (1981). Virology 109, 25-34.

5. Het is onjuist te beweren dat DNA-polymerase α verantwoordelijk is voor de replicatie van baculovirus-DNA in geïnfecteerde insektecellen.
   Miller, L.K., Jewell, J.E., and Browne, D. (1981). Journal of Virology 40, 305-308.

6. Het is nodig een cel-vrij transcriptiesysteem te ontwikkelen waarmee de regulatie van de transcriptie van het baculovirusgenoom in de tijd onderzocht kan worden.

7. Het beschikbaar komen van vaccins gericht tegen virussen die vissen infecteren, is een noodzaak indien commerciële visteelt een betere kans van slagen wil hebben.

8. Het moet mogelijk zijn een vaccin tegen vissenrhabdovirussen te ontwikkelen met behulp van een baculovirus als vector, waarin het gen coderend voor het immuniserend glycoproteïne is gecloneerd.

9. Uit het voorkomen van abnormale hoeveelheden iridium in sedimenten daterend uit het Mesozoïcum, dient niet geconcludeerd te worden dat dinosaurussen uitgestorven zijn ten gevolge van een meteorieteninslag in dat tijdperk.
   Russell, D.A. (1982). Scientific American 246, 58-65.

10. Filosoferen over wetenschap is een empirische en daardoor wetenschappelijke activiteit geworden.

11. Het gen voor het waarderen van de humor van Monty Python is gelocaliseerd op the Y-chromosoom.

P.H. Flore
In vitro transcription of *Sonchus* yellow net virus RNA by a virus-associated RNA-dependent RNA polymerase.
Wageningen, 17 januari 1986.

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ABBREVIATIONS

A 260	Absorbance at 260 nm
ALPA	Antibody-Linked-Polymerase-Assay
AMV	Alfalfa mosaic virus
ATP	Adenosine 5′-triphosphate
BPB	Bromphenol Blue
BSA	Bovine serum albumin
Ci	Curie
CBB R250	Coomassie Brilliant Blue R250
CCMV	Cowpea chlorotic mottle virus
CMV	Cucumber mosaic virus
cpm	Counts per minute
CPMV	Cowpea mosaic virus
CTP	Cytidine 5′-triphosphate
DNase	Deoxyribonuclease
DTE	Dithioerythritol
EDTA	Ethylenediamine tetra-acetic acid
EMDV	Eggplant mottled dwarf virus
ELISA	Enzyme-Linked-Immunosorbent-Assay
Fig	Figure
g	Gravity force
GTP	Guanosine 5′-triphosphate
HMW	High molecular weight
h	Hour(s)
LNYV	Lettuce necrotic yellows virus
M	Molar
mM	Millimolar
mA	Milliampere
min	Minute(s)
mol. wt.	Molecular weight
mRNA	Messenger ribonucleic acid
ND	Not determined
nm	Nanometer
NP-40	Nonidet P-40
NTP	Nucleoside triphosphate
PAS	Periodic acid Schiff staining
PBS	Phosphate buffered saline
PBS-TDS	Phosphate buffered saline-Triton X-100, Na-deoxycholate, SDS
p. i.	Post-inoculation
Poly(A)	Polyriboadenylic acid
Poly(U)	Polyuridylic acid
PYDV	Potato yellow dwarf virus
RNA	Ribonucleic acid

RNase	Ribonuclease
RNP	Ribonucleoprotein
rpm	Revolutions per minute
S	Sedimentation coefficient in Svedberg units
sec	Second(s)
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate buffer
SV	*Sonchus* virus
SYNV	*Sonchus* yellow net virus
SYVV	Sowthistle yellow vein virus
TCA	Trichloro-acetic acid
TMV	Tobacco mosaic virus
Tris	Tris(hydroxymethyl)-aminomethane
tRNA	Transfer ribonucleic acid
TYMV	Turnip yellow mosaic virus
μCi	Microcurie
μg	Microgram
μl	Microliter
μm	Micrometer
UMP	Uridine 5′-monophosphate
UTP	Uridine 5′-triphosphate
V	Volt
VRC	Vanadyl ribonucleoside complex
VSV	Vesicular stomatitis virus
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 RHABDOVIRUSES IN GENERAL

Rhabdoviruses are large, bullet-shaped or bacilliform viruses. Intact virus particles are approximately 70-100 nm in diameter and 180-350 nm long (Thomas *et al.*, 1985). A rhabdovirus particle consists of a nucleocapsid that is a coiled ribonucleoprotein (RNP) strand, and a limiting membrane derived from membranes of the infected host cell (Pal *et al.*, 1985b).
The virus contains a single-stranded RNA, which is not infectious and has a negative polarity.
Until the late 1960's it was assumed that virus particles were too simple to contain any enzyme. However, Kate and McAuslan (1967) demonstrated that vaccinia virus contains a DNA-dependent RNA polymerase. This observation was soon followed by reports on RNA-dependent RNA polymerases in virus particles of double-stranded RNA viruses (Shatkin and Sipe, 1968).
Rhabdoviruses contain an RNA-dependent RNA polymerase (transcriptase), which transcribes the minus-stranded RNA into monocistronic messenger RNA, upon infection of the cell (Baltimore *et al.*, 1970). These mRNAs are translated into rhabdovirus proteins. The minus-stranded RNA is replicated by the same RNA-dependent RNA polymerase into antigenomic RNA, which is used as a template for progeny virus RNA, which is incorporated in the virus particle. Fig. 1.1 represents a schematic version of a productive infection of cells by the type member of the vesicular stomatitis group of the Rhabdoviridae, i.e. vesicular stomatitis virus (VSV).

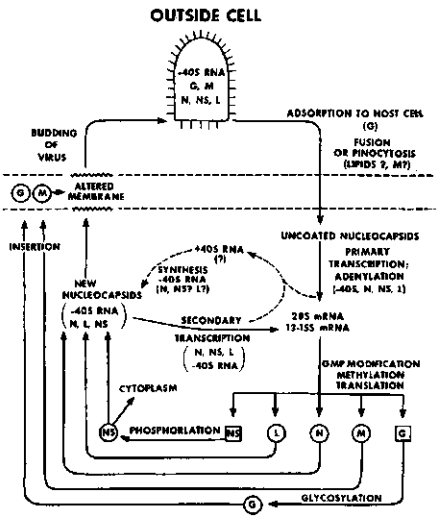


Fig. 1.1.
Schematic representation of the probable infection cycle of vesicular stomatitis virus (VSV). Viral components required at each step are identified in parentheses where possible (Emerson, 1976).

In addition to the RNA-dependent RNA polymerase activity, more enzyme activities have been found associated with rhabdoviruses which appear to be involved in post-transcriptional processing of the mRNAs.
Noted representatives of the Rhabdoviridae are VSV and rabies virus, which infect animal cells; lettuce necrotic yellows virus (LNYV) and potato yellow dwarf (PYDV), which infect plants. The rhabdoviruses are divided into a group, resembling VSV, which replicates in the cytoplasm of the infected cell, and a group, resembling rabies virus, which requires the nucleus for replication.
As most of the research in this field has focussed on VSV, I will discuss the transcription and replication of VSV-RNA in some detail. Rabies virus and rhabdoviruses infecting plants will be discussed briefly, because little is known at the molecular level of the transcriptional and replicational events governing the infectious cycle of these rhabdoviruses.
For an excellent review of the biology of rhabdoviruses, I refer to Rhabdoviruses I-III (Bishop, 1979).

1.2 VESICULAR STOMATITIS VIRUS (VSV)

1.2.1 The structure of VSV

Fig. 1.2 shows a schematic representation of VSV. The VSV envelope consists of an external glycoprotein, designated G, which forms the surface projections, a lipid bilayer and an internal membrane protein, designated M (Cartwright *et al.*, 1969). The envelope surrounds the core structure, which consists of three proteins associated with the single-stranded RNA. These proteins are the nucleocapsid protein (N), which is tightly bound to the RNA, the non-structural protein (NS), which functions with the large protein (L) as an RNA-dependent RNA polymerase. This RNA polymerase transcribes the VSV-RNA of negative polarity into leader RNA and the 5 monocistronic mRNAs (Fig. 1.3).

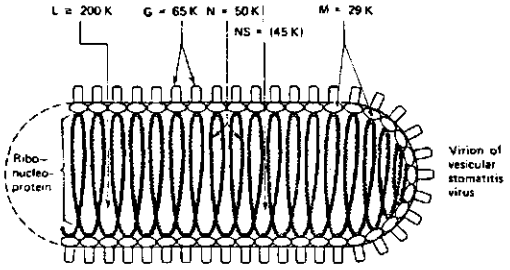


Fig. 1.2.
Schematic representation of the structure of VSV (Luria *et al.*, 1978).

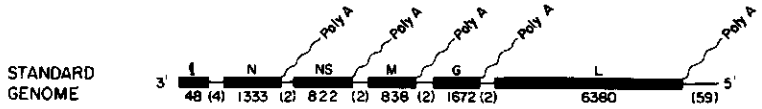


Fig. 1.3.
Diagram of the genome of VSV. The gene order is shown above the genome, while the numbers in parentheses represent the number of nucleotides not found in the transcription products. Transcribed regions are shown as heavy black lines with the length of the transcript in nucleotides given below (Emerson, 1985).

The mol. wt. of these proteins are presented in Table 1.1. The mol. wt. of the RNA is $4x10^6$ with a sedimentation coefficient of 42S (Clewly and Bishop, 1979).
Detergents such as deoxycholate and Triton X-100 disrupt the VSV structure, liberating the nucleocapsid which uncoils, and solubilizing the proteins associated with the envelope. The length of the fully extended ribonucleoprotein strand is 3.5 μm (Nakai and Howatson, 1968; and Thomas *et al.*, 1985).

Table 1.1. Mol. wt. of the proteins associated with VSV (Luria *et al.*, 1977).

Protein	Mol. wt.$x10^3$
L	200§
G	65
N	50
NS	45*
M	29

§: The L gene codes for a protein with a mol. wt. of 241,000 (Schubert *et al.*, 1984).

*: The mol. wt. of the NS protein is actually 25,000 (Marnell and Summers, 1984), but because of the acidic nature of the protein, its mobility in SDS-polyacrylamide gels is anomalous.

1.2.2 G protein of VSV

Results of protease experiments and surface-labeling have shown that the G (glyco) protein of VSV forms the surface projections or spikes, that protrude from the membrane envelope of the virus and is required to infect cells (Taube and Braun, 1982; and Gallione and Rose, 1985). More than 95% of the protein is exposed on the exterior of the virus particle and a hydrophobic domain of 20 amino acids spans the virus membrane. A hydrophilic carboxy-terminal domain of 29 amino acids is inside the virus membrane. It is the only VSV protein, which induces the formation of neutralizing antibodies to VSV (Kelley *et al.*, 1972).
The pathway of biosynthesis and transport of the G protein to the cell surface is complex. Insertion into the rough endoplasmic reticulum, glycosylation and removal of the signal peptide occurs while the G protein is a nascent chain. After synthesis, the G protein is transported to the Golgi apparatus and then to the plasma membrane, where it is incorporated into virus particles (Gallione and Rose, 1985). During the infection cycle the G protein is involved in recognition of receptor sites, adsorption and possibly in the penetration of the cell by the virus particle (Schloemer and Wagner, 1975).

1.2.3 M protein of VSV

The M (matrix) protein of VSV is thought to be located internally to the lipid bilayer,

because the M protein is not susceptible to surface-labeling and mild proteolytic digestion of intact virus particles (Cartwright *et al.*, 1970). Pal *et al.* (1985b) have shown that the M protein is highly resistant to trypsin digestion, which results in an uncleaved polypeptide with an approximate mol. wt. of 22,000. The M protein is antigenic and it is a group-specific antigen. This protein is basic with a pI of 9 and is insoluble in aqueous solutions of Triton X-100 at low ionic strengths, but soluble in guanidine-HCl, SDS and Triton X-100 at high ionic strengths (Emerson and Wagner, 1972). The hydrophobic nature of this protein correlates well with its presumed function during virus maturation, which includes interaction with hydrophobic domains of the N protein and the plasma membrane (Mudd and Swanson, 1978; Knipe *et al.*, 1977; Chatterjee *et al.*, 1984; and Capone and Ghosh, 1984). The M protein plays an important role in the regulation of transcription and replication of VSV-RNA (Huang *et al.*, 1977; and Pal *et al.*, 1985b). This regulation can only be exerted at low ionic strength, because at a high salt concentration the interaction with the ribonucleoprotein strand is too weak (Wilson and Lenard, 1981). The M protein-mediated inhibition of RNA synthesis occurs at the level of leader RNA synthesis and elongation of RNA chains (Pinney and Emerson, 1982b). Initiation of the N gene continues, however (Thornton *et al.*, 1984).
The M protein is phosphorylated in the serine, threonine and tyrosine residues by different protein kinases (Clinton and Huang, 1981). The different phosphorylation strategies may modulate the role of the M protein of VSV in the course of infection. The number of phosphorylated groups in the M protein determines the degree to which the M protein dissociates from the nucleocapsids (Witt and Summers, 1980).
Recently, Rosen *et al.* (1983) have identified a new protein present in VSV-infected cells, which appears to be a specific degradation product of the viral M protein. They speculate that this protein, M′, with a mol. wt. of 17,500 , which accumulates late in infection, plays a role in virus infection, by regulating the amount of M protein present in the cell. Carroll and Wagner (1979), De *et al.* (1982), Pinney and Emerson (1982b), and Pal *et al.* (1985) have implicated the M protein as an inhibitor of transcription *in vitro*, so a mechanism which degrades the protein might allow RNA synthesis to proceed at a higher rate late in infection. The conversion of M to M′ is an enzymatic event. Whether M is the protease itself is unclear. It is uncertain whether M′ is identical to the trypsin resistant part of M, which has been identified *in vitro* by Pal *et al.* (1985b). Ogden *et al.* (1985) showed that the M protein of VSV was cleaved by a protease into a polypeptide M_T, resembling M′, via an intermediate polypeptide M_C.

1.2.4 L protein of VSV

The L (large) protein is associated with the VSV nucleocapsid. The L protein is present in about 30-60 copies per virus particle (Bishop and Roy, 1972; Madore and England, 1977; and Thomas *et al.*, 1985) and has a mol. wt. of approximately 241,000 (Schubert *et al.*, 1984). Several groups have firmly established that the L protein functions as an RNA-dependent RNA polymerase (Emerson, 1976) and forms a transcription complex with the NS protein and RNA, coated with N protein. Recently, Ongradi *et al.* (1985) have shown that L protein on its own is capable of synthesizing RNA using the complex of RNA and N protein (N protein-RNA complex) as template, while the NS protein of VSV exerts a regulatory function in transcription and replication.

1.2.5 NS protein of VSV

The NS (non-structural) protein is synthesized in large quantities in the infected cell (Kawai, 1977) and it occurs free in the cytoplasm and in association with the intracellular core structure (Wagner *et al*,, 1972) in approximately 230-466 copies (Harmon *et al.*, 1985; and Thomas *et al.*, 1985, respectively). Mol. wt. estimates vary because the extremely acidic nature of the protein causes anomalous migration in SDS-polyacrylamide gels. Marnell and Summers (1984), using polyacrylamide gels containing the detergent cetyltrimethylammonium bromide at low pH, have presented a reliable mol. wt. estimate of 23,000, which correlates well with the coding capacity of the NS gene (Gallione *et al.*, 1981). The NS protein is stable at 80°C for 2 min in contrast to the extremely labile L protein (De and Banerjee, 1985).
The NS protein is phosphorylated *in vivo* and *in vitro* by cellular protein kinases and possibly also by the L protein (Sanchez *et al.*, 1985). It has been suggested that phosphorylation of this component in the trancription complex may be involved in the regulation of enzyme function by mediating the switch from transcription to replication (Marnell and Summers, 1984). Hyperphosphorylation would seem to favor transcription of VSV-RNA (Blondel-Maingonnat, 1981) and it is known that early in infection, when transcription is the predominant process, the NS protein is hyperphosphorylated (Sokol and Clark, 1973). Masters and Banerjee (1985) reported equimolar amounts of NS1 and NS2 in intact VSV, which differ in the degree of phosphorylation. In the infected cell NS1 is irreversibly hyperphosphorylated by a cellular protein kinase and is more stable than NS2. NS2 is sensitive to cellular phosphatases, which decreases the level of phosphorylation. This difference is also observed *in vitro*. There appears to be a functional association between the N and the NS protein of VSV (Bell *et al.*, 1984), because serum directed against the NS protein also immuno-precipitates the N protein. The ability to bind to the nucleocapsid is probably related to the degree of phosphorylation of the NS protein (Bell *et al.*, 1984).
For optimal transcription a 1:1 stoichiometric ratio between the L and the NS protein of VSV is required (Emerson and Yu, 1975). De and Banerjee (1984) have reported that, while the L protein of VSV is required for *in vitro* initiation of mRNA synthesis, the NS protein is necessary for elongation. Anti-NS serum terminates transcription of VSV-RNA directly after adding to an *in vitro* transcription system (Imblum and Wagner, 1975). It is thought that the NS protein facilitates the movement of the L protein along the N protein-RNA complex by interacting with both the L and the N protein; its function would be similar to that of an unwinding protein (De and Banerjee, 1985). NS may also be required to stabilize the L protein of VSV (De and Banerjee, 1985).

1.2.6 N protein of VSV

The N (nucleocapsid) protein is the major protein of the nucleocapsid and comprises about 30% of the total protein content (2,100-3,000 copies per virus particle according to Bishop and Roy (1972), although Thomas *et al.* (1985) report only 1258 copies per virus particle). The N protein readily self-assembles in the absence of RNA and it is thought that the association of the cytoplasmic pool of NS protein of VSV with the free cytoplasmic N protein retains the N protein in an active soluble form (Blumberg *et al.*, 1984). The spatial arrangement of the N protein along the virus RNA probably accounts for the ribonuclease

resistance of the RNA in isolated nucleocapsids. The N protein is extremely stable when it is associated with RNA (A.K. Banerjee, personal communication). The function of the N protein is essential in the transcription and replication process, but its function is unknown. Genomic RNA is completely inactive as template, when only the other essential proteins, L and NS, are added to an *in vitro* transcription system (Emerson, 1976). The polymerase does not bind directly to template RNA as was demonstrated by Glass *et al.* (1985). Foreign RNA does not serve as template (Emerson and Wagner, 1972). These observations will be of importance for the study presented in this thesis.

1.3 TRANSCRIPTION AND REPLICATION OF VSV-RNA

The requirements for *in vitro* transcription of VSV-RNA are listed in Table 1.2. When intact VSV is assayed for transcriptase activity, a detergent or mellitin, a substance found in bee-venom (Roy *et al.*, 1982), or pardaxin, a toxin found in Red Sea flatfish (Pal *et al.*, 1981a and b) is required to activate the transcription complex. All four ribonucleoside triphosphates are necessary for transcription to proceed. The presence of a reducing agent, i.e. 2-mercaptoethanol or dithiothreitol is essential for maximum transcription *in vitro*. Mg^{2+}-ions are necessary and cannot be replaced by other metal ions, such as Mn^{2+}. The salt concentration required varies between 0.1 M and 0.15 M NaCl to prevent the M protein from reassociating with the transcribing RNP complex which is the nucleocapsid, freed from the virus by detergent-mediated disruption,infectious and able to perform transcription. The optimum temperature is around 37°C and a pH of 7.8 is usually employed (Massey *et al.*, 1985).

Table 1.2. *In vitro* transcription conditions for VSV (Baltimore *et al.*, 1970).

Detergent 0.08%	Triton N-101
Salt	0.1 M NaCl
Metal ion	3 mM Mg^{2+}
Temperature	37 °C
pH	7.8

Transcription of VSV-RNA *in vitro* requires the presence of the N, L and NS proteins, as has been demonstrated in reconstitution experiments, involving the dissociation of the L and NS proteins from the nucleocapsid and subsequent adding of these proteins to an *in vitro* transcription system, containing only the N protein-RNA complex. Dissociation is achieved by treating the virus particles with 0.72 M NaCl in the presence of Triton N-101 and separating the components by high-speed centrifugation. This treatment solubilizes most of the G, M, L and NS proteins. It has proven difficult to separate the L, N and NS proteins of VSV from the ribonucleoprotein containing fraction completely (Blumberg *et al.*, 1984).

1.3.1 Synthesis of VSV mRNA in vitro

Hybridization and UV light inactivation studies have demonstrated that transcripion is

sequential along the genome in the order 3′-leader -N-NS-M-G-L-5′ (Ball and White, 1976).

Three general models for the biosynthesis of VSV mRNAs have been proposed to account for the sequential and polar nature of VSV transcription, i.e. the cleavage model, the stop-start model and the multiple initiation model.

In the cleavage model the monocistronic mRNAs are generated via cleavage and processing of a precursor RNA (Herman *et al.*, 1978) (Fig. 1.4).

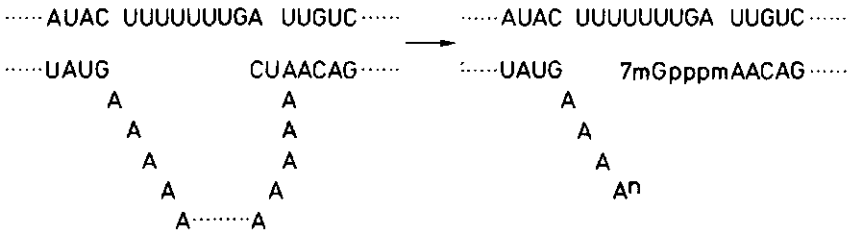


Fig. 1.4.
Cleavage model for the biosynthesis of VSV mRNA (Banerjee, 1979).

The first RNA product synthesized *in vitro* is the leader RNA which is 48 nucleotides long and complementary to the corresponding 3′-terminal portion of the VSV genome. As the transcribing complex completes the synthesis of the leader RNA and enters into the adjacent N-gene, without dissociation, a putative ribonuclease present in the virion cleaves the newly synthesized RNA at a specific site and releases this leader RNA. The 5′-terminal pA-A-C-A-G sequence of the nascent N protein mRNA is then capped with GTP to form the sequence G(5′)ppp(5′)A-A-C-A-G. The transcribing complex continues to move along the genome RNA and the rest of the mRNAs are synthesized sequentially by similar cleavage of the growing RNA molecule, followed by capping of the 5′-termini and polyadenylation of the 3′-termini of the released mRNAs as explained below.

In the stop-start model the mRNA is generated by termination and reinitiation of transcription at each intercistronic region. Emerson (1982) provided evidence that the transcriptase binds exclusively at the leader template and that RNA synthesis occurred sequentially by multiple initiations of the polymerase at putative internal promoter sites for each of the five structural genes, without prior dissociation of the polymerase, but with decreasing probability as the polymerase moves down the template. This stop-start transcription process is therefore polar and attenuated. Pausing between gene junctions is presumably accompanied by a 'chattering' process on a stretch of seven uridylate residues to give rise to mRNA with poly(A) tails (Perrault and McLear, 1984).

The multiple initiation model, which is based on the observation that in addition to the leader RNA at least three promoter-proximal RNAs can be detected almost immediately (one min) after activation of the virus polymerase *in vitro*, suggested that initiation of transcription occurs simultaneously at the beginning of each gene (Testa *et al.*, 1980). The significance of the synthesis of these short fragments is unclear. It is possible that each gene of the genome is spatially looped out, in such a manner that the polymerase may initiate RNA synthesis, but fails to extend beyond this site, due to constraints inherent to the secondary structure of the gene. This constraint can be removed by the leader RNA that acts as an activator. Once the polymerase has read the constrained portion of the gene, the transcription product may similarily activate the next gene and so on. The low-molecular weight RNA species may be precursors of full-length mRNA, which have escaped capping

and are subsequently not extended beyond the putative constrained site.
Further research is needed to establish which if any of the three models is correct.

1.3.2 Modification of VSV mRNAs in vitro

The mRNAs synthesized *in vitro* by purified VSV fall into four distinct classes when analyzed by velocity sedimentation (Moyer and Banerjee, 1975): 31S codes for the L protein; 17S for the G protein; 14.5S for the N protein; and 12S for the M and the NS protein. Almost 90% of the genome is accounted for the production of mRNA. Whether the remaining portion of the genome codes for an unidentified protein or is not transcribed, is unknown. Marks *et al.* (1985) reported 6 complementation groups for VSV and the extra sixth group was postulated to represent a sixth, non-structural virus-encoded protein, involved in the replication pathway. Herman (1985) has obtained evidence that the VSV genome codes for a protein which has not previously been identified and which could be the protein discussed by Marks *et al.* (1985). Kurath *et al.* (1985) have reported a sixth gene, coding for a non-virion protein of infectious hematopoietic necrosis virus, a fish rhabdovirus with a mol. wt. of 12,000.
The mRNAs are modified in several ways:
1.) Polyadenylation of the 3′-termini of the mRNAs during or after transcription and release from the template (Banerjee and Rhodes, 1973). The precise mechanism is unknown. The sequence (3′)AUAC-U7-GA-UUGUC(5′) located at the end of each gene may serve as mRNA termination signals, employed by the polymerase to synthesize the 200 bases of the poly(A) tract by a slippage mechanism (Ehrenfeld and Summers, 1972) (Fig. 1.5). The sequence CU is excised and the new transcript is capped (Herman *et al.*, 1978). Hunt *et al.* (1985) demonstrated that the RNA-dependent RNA polymerase of VSV is a poly(A) polymerase too.

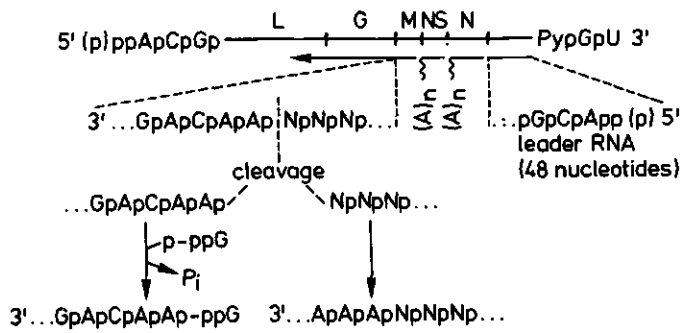


Fig. 1.5.
Model of a slippage mechanism proposed for the processing of VSV mRNA (Jaspars and Bol, 1980).

2.) Capping of the VSV mRNAs synthesized *in vitro*, consists of incorporating the alpha phosphate of ATP and the alpha and beta phosphate of GTP into the blocked structure at the 5′-termini: Gppp + pN.... ....Gpp-pN.... (Banerjee *et al.*, 1984). Cellular mRNAs are capped as follows: Gppp + ppN.... ....Gp-ppN.... (Shatkin, 1976).
3.) In addition to the capping of the mRNAs of VSV, these messengers are methylated in the presence of a methyl donor (S-adenosylmethionine) at the 5′-termini by a methyl transferase, which copurifies with VSV, yielding: 7mG(5′)ppp(5′)mApApCpApGp....

(Abraham *et al.*, 1975).
The processing of VSV mRNAs is carried out by a virus-encoded enzyme presumably and not by cellular enzymes, because mutants of VSV have been described which are defective in methylating and polyadenylating enzymatic activities have been described (Masters and Samuel, 1984). Capping, methylation and polyadenylation of VSV mRNAs are tightly coupled to transcription and cannot occur in its absence (Abraham and Banerjee, 1976).

1.4 REPLICATION OF VSV-RNA IN VITRO

The N, NS and L protein of VSV are all involved in replication of genomic RNA. Replication is dependent on continuous viral protein synthesis since no replication is detected in cells treated with cycloheximide (Huang and Manders, 1972). Transcription does proceed in the presence of the protein synthesis inhibitor.
The replication of VSV can be divided into two phases:
1.) Synthesis of plus-stranded RNA of genome length (complementary RNA replication), and 2.) Synthesis of progeny minus-stranded RNA of genome length.
An intact 42S plus-stranded copy of the VSV genome is a necessary intermediate in replication and has been found *in vivo* (Chang *et al.*, 1974). A full-length copy of the VSV genome can be obtained by omitting the cleavage steps (Banerjee *et al.*, 1977). This would represent an easy switch from transcription to replication. Using a model, involving a stop-start mechanism of transcription, generation of the 42S plus-strand would be possible in the absence of all initiation events, except initiation of the leader RNA.
The N protein of VSV protects the VSV-RNA from degradation by nucleases (Cartwright *et al.*, 1970) and it is tempting to speculate that the N protein is involved in preventing cleavage or initiation of RNA species, other than initiation at the leader RNA stretch. Arnheiter *et al.* (1985) have recently shown that the replication of the VSV genome is controlled by the availability of the N protein, even when the RNA-dependent RNA polymerase has sufficient host factors and multiple phosphorylated forms of the NS protein. The requirement for N protein is continuous during replication. When the N protein of VSV has accumulated sufficiently-later in the infection process-it would bind to sequences of leader RNA of VSV to form a crystallization nucleus for the rapid binding of more N protein in a 5′ to 3′ direction as the nascent chain grows and would protect the RNA from cleavage. The positive copy of the genome RNA with its full complement of N protein is then ready to serve as template for the synthesis of negative-stranded VSV-RNA. The negative strand is then packaged into progeny virus.
The L protein of VSV is the putative RNA-dependent RNA polymerase involved in the replication as well as in transcription of VSV-RNA as discussed above (Johnson and Lazzarini, 1977). A different state of phosphorylation of the NS protein of VSV would confer a different affinity for the VSV N protein-RNA complex which in turn can influence the switch from transcription to replication.
It is thought that the M protein of VSV also plays an important role in the switch from transcription to replication by inhibiting transcription later in infection when enough M protein has accumulated, signaling the need for virus replication (Carroll and Wagner, 1979). The mechanism by which the transcription is inhibited by this protein is unknown, but Pal *et al.* (1985a) have provided evidence that exposure of a single epitope on the surface of the M protein may be involved in the regulation of *in vitro* viral transcription.

1.5 RABIES VIRUS

Little is known about the role of the various proteins of rabies virus in the transcription and replication of its RNA. They are designated L, G, N, M1 and M2 partially in accordance with the symbols given to VSV proteins (Kawai, 1977 and Dietzschold *et al.*, 1979). Wunner *et al.* (1985) reported two forms of the glycoprotein GI and GII which differ in their degree of glycosylation. Two undefined minor components, designated P40 and P43 were also detected in rabies virus preparations (Kawai, 1977). Cox *et al.* (1981) and Wunner *et al.* (1985) suggested that the M1 protein of rabies virus is equivalent to the NS protein of VSV, because it is associated with the transcribing RNP complex and phosphorylated to different degrees (NS1 and NS2). Tuffereau *et al.* (1985) reported that the M1 protein is probably involved in transcription and replication of the virus. The M2 protein is exposed on the outer surface of the virus membrane and possibly serves as a bridge between the G protein and the nucleocapsid (Cox *et al.*, 1981). Table 1.3 lists the mol. wt. of these proteins.

Table 1.3. Mol. wt. of rabies virus proteins (Kawai, 1977).

Rabies virus proteins	Mol. wt.x10^3
L	190
G	67* (GI) 62 (GII)
N	54
P43	43
P40	40
M1	37
M2	24

*: According to Wunner *et al.* (1985).

The single-stranded RNA of a negative polarity has a mol. wt. of $4.6x10^6$. Rabies virus replication requires the presence of the cell nucleus in contrast to VSV replication (Wiktor and Koprowski, 1974).
Based on data that [^{3}H]GMP-incorporating activity was found associated with the rabies virus nucleocapsid, that the products were RNase-sensitive and that the product RNA contained sequences complementary to rabies virus RNA, Kawai (1977) concluded that rabies virus contains an RNA-dependent RNA polymerase. Harsh biochemical conditions were employed to induce the transcriptase activity. Four percent NP-40 was necessary to activate the enzyme, reflecting the stability of the envelope of rabies virus.
Kawai (1977) could not demonstrate an inhibitory effect of the matrix protein on *in vitro* transcription of rabies virus RNA, as has been proven for VSV-RNA transcription (Carroll and Wagner, 1979). One would expect an inhibition by this protein in the light of the difficulty to remove this protein from the nucleocapsid of rabies virus and of the low activities reported by Kawai (1977) and Flamand *et al.* (1978) after long periods of

incubation.
Flamand *et al.* (1978) found evidence for a rabies virus associated transcriptase with a requirement for a bivalent cation (Mg^{2+} or Mn^{2+}) and a temperature optimum of 31°C. The efficiency of the incorporation of radioactive ribonucleotide precursors was low and the pH optimum of 8.9 was very high when compared to the pH optimum reported by Kawai (1977). However, Flamand *et al.* (1978) used a much lower detergent concentration (0.01%), because the high pH was sufficient for disruption of the membrane. The high pH also provided protection against RNase activity, present in purified preparations of rabies virus. Under these circumstances Flamand *et al.* (1978) reported a linear rate of incorporation of radioactive precursor for 7 h, before reaching a plateau. Flamand *et al.* (1978) mentioned a contaminating nucleoside triphosphatase activity present in all rabies virus preparations, and this would decrease the ribonucleoside triphosphate concentration and the ribonucleoside monophosphates would inhibit transcription. Protein kinase and phosphotransferase activity have also been reported to be associated with rabies virus particles (Sokol and Clark, 1973).
Kawai (1977) reported that the transcriptase activity was fairly proportional to the amount of L protein present in the virus particle, although he did not provide evidence that the L protein has an identical function as the L protein of VSV. Dietzschold *et al.* (1979) provided some evidence that the amount of L protein in rabies virus particles can be correlated with virulence *in vivo* and with transcriptase activity *in vitro*, indicating that the L protein of rabies virus is an RNA-dependent RNA polymerase.
The absence of the sequence (3′)AAUAAA(5′), present in eucaryotic polyadenylated mRNAs, in the non-coding region of rabies virus mRNA presumably reflects the role of virus-associated proteins in the polyadenylation process instead of utilizing host enzymes (Anilionis *et al.*, 1981).
The gene order in rabies virus is (3′)leader-N-M1-M2-L(5′), while the correct position of the gene coding for the G protein is not exactly known.

1.6 RHABDOVIRUSES INFECTING PLANTS

Some plant rhabdoviruses are of economic importance, because these viruses can devastate entire crops (Jackson *et al.*, in press). More insight into the biology of these viruses is therefore desirable.
The rhabdoviruses infecting plants can be divided into two groups (Peters,1981):
1.) Subgroup A with type member lettuce necrotic yellows virus (LNYV), which replicates in the cytoplasm and has a protein pattern on SDS-polyacrylamide gels resembling VSV. The requirements for *in vitro* transcription of the RNA are similar to those for VSV-RNA transcription.
2.) Subgroup B with type member potato yellow dwarf virus (PYDV). *Sonchus* yellow net virus (SYNV) also belongs to this group, which resembles the rabies virus group in its protein pattern on SDS-polyacrylamide gels. The nucleus is required for replication (Van Beek *et al.*, 1985c) and as in rabies virus Mn^{2+} or Mg^{2+} are required for *in vitro* transcription.
Plant rhabdoviruses are transmitted by arthropods and replicate in their vector (Jackson *et al.*, in press) and may have bridged the gap between animal and plant viruses through the insect vectors.

Plant rhabdoviruses are bacilliform or bullet-shaped particles (depending on fixation methods), 200-350 nm long and 70-95 nm in diameter with 5-12 nm spike. The particles contain 70% protein, 25% lipids, 4% polysaccharids and 1% RNA of negative-stranded polarity. They consist of a nucleocapsid, surrounded by a membrane. The nucleocapsid is shaped like a hollow bullet, 130-300 nm long and 45-65 nm in diameter and is formed by a helically wound ribonucleoprotein strand. The tubular part of the condensed ribonucleoprotein strand consists of 40 turns of the helix with a pitch of 4-4.5 nm (Peters, 1981). Table 1.4 lists the mol. wt. of several plant rhabdovirus proteins (Dale and Peters, 1981; and Peters, 1981).

Table 1.4. Mol. wt. of proteins of rhabdoviruses, infecting plants (Peters, 1981).

Subgroup	Virus	Mol. wt.x10^3 of the proteins						
		L	G	N	NS	M	M1	M2
A	LNYV	170	71	56	38	19		
A	SV	170	72	55	38	19		
A	BNYV		93	60		18		
B	PYDV		78	56			33	22
B	EMDV#		83	61			27	21
B	SYVV		83	60			44	36
B	SYNV		77	64			45	39

#: Eggplant mottled dwarf virus.

G and N protein are found in all rhabdoviruses examined, and L and NS are detected in some plant rhabdoviruses, where they appear to have the same function as the L and NS proteins of VSV, i.e. they constitute the polymerase complex associated with the transcribing RNP complex (Toriyama and Peters, 1980 and 1981).
In VSV the M protein is located at the inner surface of the membrane and bridges the G and N protein as described above. This is also the case for *Sonchus* virus (SV), broccoli necrotic yellows virus (BNYV) and LNYV. PYDV, SYNV and sowthistle yellow vein virus (SYVV) appear to have two membrane proteins, although no definite function has been assigned to them. Dale and Peters (1981) have detected a high molecular weight protein (HMW) in LNYV, which is released together with G and M after treatment with 1% to 2% NP-40. This HMW protein is believed to be a dimer of the G protein, because the mol. wt. is double that of the G protein of LNYV and the HMW protein stains with a stain specific for carbohydrates (PAS). Ziemiecki and Peters (1976a) observed a similar protein in SYVV, which like the G protein could be removed by trypsin.
The RNAs of plant rhabdoviruses have a mol. wt. of approximately $4x10^6$ and are not infectious, whereas the transcribing RNP complexes are.
Considering the fact that LNYV and BNYV are similar to VSV, it is to be expected that these viruses possess an RNA-dependent RNA polymerase activity. Francki and Randles (1972) and Toriyama and Peters (1980 and 1981) have indeed shown that LNYV and

BNYV contain transcriptase activity.
Progress in understanding the transcription and replication process of plant rhabdoviruses has been hampered by a lack of a protoplast system in which synchronous infection can be achieved. Infection of plant protoplasts with PYDV has been reported (Riesterer and Adams, 1981), but only 8% of the cells were infected. PYDV can also infect plant leafhopper cell monolayers and although it does not induce any clear cytopathic effects, infection can readily be detected by immunofluorescent techniques (Reddy, 1977). However, nobody has pursued this line of research.
Van Beek *et al.* (1985a) have succeeded in infecting cowpea protoplasts with SYNV at high efficiencies, so detailed research on the infection process of plant rhabdoviruses at the molecular level should now be feasible.

1.7 SYNV

SYNV infects several dicotyledonous plants, *Sonchus oleraceus* L. and *Bidens pilosa* (Christie *et al.*, 1974). SYNV is maintained in greenhouses in a tobacco hybrid produced by S.R. Christie. This hybrid (*N. christii*) is a cross between *Nicotiana clevelandii*, a species of high virus susceptibility, and *N. glutinosa* L., which harbours a gene for resistance to tobacco mosaic virus (TMV).
SYNV is transmitted by an aphid (*Aphis coreopsidis* Thomas) and can be mechanically transmitted to *Capsicum frutescens* L., *Gomphrena globosa* L., *Chenopodium quinoa* Willd, *Lactuca sativa* L., *N. clevelandii* Gray and *N. glutinosa* L.
The purified virions of SYNV are bacilliform (248 x 94 nm) with surface projections of 6 nm (Jackson and Christie, 1977) and have a nucleocapsid with periodic cross-striations like all rhabdoviruses (periodicity is 4.1 nm), which encapsidates the viral genome RNA (mol. wt. is 4.4×10^6).
SYNV is difficult to purify free from host plant material and aggregates easily in the presence of plant proteins (Jackson *et al.*, in press). Yields of virus varies greatly with the growth conditions of the plant and hence on the season. Spring and autumn appear to be the most optimal for purification of SYNV (D. Zuidema, personal communication).
SYNV is purified from plants by Celite filtration and sucrose density gradient centrifugation and sediments at 1044S in linear log gradients and bands at 1 18 g/ml in sucrose equilibrium gradients. The mol. wt. of the virus particle is estimated to be 9×10^8. The nucleic acid from SDS-disrupted virus particles is suceptible to RNase activity and sediments at 44S (Jackson and Christie, 1977). The mol. wt. of the proteins of SYNV have been listed in Table 1.4. The minor proteins of SYNV include two to three high mol. wt. proteins (possibly analogous to the L protein of VSV and dimers of G protein) and a protein, analogous to the NS protein of VSV, which migrated slightly ahead of the N protein of SYNV on a 12.5% SDS-polyacrylamide gel (Jackson, 1978).
Upon treatment of SYNV with a non-ionic detergent and separation on sucrose gradients, the RNP complex is still infectious, but the infectivity is considerably less than that of intact virus (Jackson, 1978).
Methods in which centrifugation and ^{125}I-labeling techniques were combined, identified and localized the virus proteins of SYNV. Both M2 and G protein appear to be exposed on the surface of the virus particle, because they are heavily iodinated when the virus is intact. M1 then, is apparently partially buried beneath the membrane, because it is less reactive

with ^{125}I (Jackson, 1978). Jackson *et al.* (in press) suggest that the M1 protein is analogous to the NS protein of VSV and rabies virus. The N protein is tightly bound to the RNA and is not labeled with ^{125}I in intact virus. Unexpectedly, the putative L protein of SYNV behaves like a surface protein as judged with the above-mentioned techniques. This could reflect an artifact of the method used. It is not clear whether this high mol. wt. protein is indeed equivalent to the L protein of VSV. Likewise it cannot be ruled out that the position of M2 is also mistaken by the same cause.

Milner and Jackson (1983) reported the existence of polyadenylated mRNAs in association with membrane-bound polyribosomes with mol. wt. of 0.83×10^6 and 0.46×10^6, which are consistent with the expected sizes of the mRNAs coding for G, and M1 and M2 respectively, so there appears to be a link at the level of mRNA, because the other SYNV proteins are not synthesized on membrane-bound ribosomes, confirming the iodination experiments.

Evidence that SYNV contains a virus-coded transcriptase comes from the work done by the group of Jackson (Rezaian *et al.*, 1983) and from Flore and Peters (1981). Rezaian *et al.* (1983) show that isolated RNA from free-and membrane-bound polyribosomes of SYNV-infected tobacco leaves could be hybridized to SYNV-RNA. RNA from free ribosomes was protected for 100% against RNase activity, whereas RNA from membrane-bound polyribosomes was only protected for 40% (Milner and Jackson, 1979 and 1983; Milner *et al.*, 1979).

Recently Heaton *et al.* (1985) and Zuidema *et al.* (1985) have reported the sequence of the gene coding for the N protein and the leader RNA of SYNV, respectively. The leader RNA was 147 nucleotides long which is approximately three times as long as the leader RNA of VSV. A consensus sequence for polyadenylation was found in the N gene, which has not been reported for VSV or rabies virus. Several internal stopsites in the N gene were postulated, which are not detected in the VSV and rabies virus N gene. The significance of these sequences in the genome of SYNV is not yet understood, but may shed light on the interaction of SYNV with the plant host cell and also on the relation of SYNV with other rhabdoviruses.

The gene order of SYNV is probably (3′)-leader-N-M2-M1-G-L-(5′). If we asssume that M1 of SYNV is equivalent to NS of VSV we would have expected the gene order to be (3′)-leader-N-M1-M2-G-L-(5′). More sequence data on SYNV need to be accumulated, before this difference can be addressed.

Our laboratory has demonstrated transcriptase activity in crude extracts of SYNV-infected plants and in purified preparations of SYNV (Peters, personal communication; and Flore and Peters, 1981).

The aim of the research, culminating in this thesis, was to determine which protein(s) of SYNV has (have) an RNA polymerase function in the transcription of SYNV-RNA *in vitro*. The effect of other SYNV proteins on transcription *in vitro* was also examined.

CHAPTER 2

PURIFICATION AND CHARACTERIZATION OF SONCHUS YELLOW NET VIRUS (SYNV)

2.1 INTRODUCTION

This chapter discusses the development of SYNV in infected plants and evaluates different plant species as hosts for purification studies. Optimal conditions were established for the purification of SYNV. The protein and RNA composition was determined and compared with data found in the literature on SYNV.
Four major and at least two minor proteins have been resolved by polyacrylamide gel electrophoresis of purified SYNV denatured with SDS (Jackson and Christie, 1977). The characteristics and location of these polypeptides have been described by Jackson (1977). The identity and location of the structural proteins must be known to elucidate the involvement of virus-specific proteins in the transcription and replication of SYNV-RNA *in vitro* and ultimately *in vivo*.
The transcribing ribonucleoprotein (RNP) complex has been defined (Chapter 1) as that part of the virus that remains infectious after the virus envelope has been solubilized by a neutral detergent in the presence of salt and which transcribes RNA *in vitro*. The complex which remains after the transcription machinery has been removed and which is no longer infectious, has been termed the non-transcribing N protein-RNA complex.

2.2 MATERIALS AND METHODS

2.2.1 Plant and virus material

An isolate of *Sonchus* yellow net virus (SYNV) was kindly provided by Dr. A.O. Jackson (Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana, USA). The virus was maintained on either *Nicotiana glutinosa* or a *N. clevelandii* x *N. glutinosa* hybrid henceforth referred to as *N. christii*, in climate rooms and greenhouses.
Inocula were prepared by grinding infected leaves with a small volume of 0.5% Na_2SO_3 in sterile water. Leaves of plants sown one month before were inoculated mechanically with carborundum powder and rinsed with tapwater immediately thereafter.
To test the infectivity of SYNV preparations *N. christii* and *Chenopodium quinoa* plants were routinely used. *N. christii* developed a systemic infection 8-12 days post-inoculation and *C. quinoa* developed local lesions after approximately 12 days.

2.2.2 Purification of SYNV

Leaves with characteristic symptoms were harvested 8 to 12 days post-inoculation. Fifty gram was ground with or without the mid-ribs in a Waring Blender in 3 volumes (w/w) of buffer A (0.01 M Tris-HCl, pH 8.0, containing 0.01 M Na_2SO_3). The homogenate was strained through two layers of cheese-cloth and the extract was centrifuged for 15 min at

8,500 rpm in a MSE 18 centrifuge. The supernatant was mixed with Celite (1 g Celite/2 g leaf material). The slurry was filtered through a cushion of 12.5 g Celite on a paper filter in a Buchner-funnel and washed with buffer B (0.01 M Tris-HCl, pH 7.4) until the filtrate remained clear.
The virus in the filtrate was pelleted at 12,500 rpm for 1 h in the MSE 18 centrifuge. The pellet was resuspended in 4 ml buffer B and layered onto a 3-30% linear sucrose gradient in a SW28 tube and centrifuged for 23 min at 28,000 rpm in a Beckman L5-65 centrifuge. The virus banded at 1/3 from the bottom of the tube and was easily detected with a lightbeam from above. The virus in the band was removed with a needle and diluted with 20 ml buffer B. Virus particles were collected by centrifugation in a Sorvall RC-2 centrifuge at 12,500 rpm for 60 min. The pellet was resuspended in buffer B at a concentration of 250 μl/10 g starting material. All manipulations were carried out at 4 °C.
Celite was obtained from Brunschwig Chemie in two grades of purity. One batch consisted of very coarse and brownish material. The other batch had been purified to a white powder. The crude batch of Celite retained more plant material than the more purified batch. Although the crude batch also retained more virus we used this crude grade because we were interested in obtaining pure virus.
The virus yield from infected *N. christii* plants was lower than from *N. glutinosa* (results not shown) and SYNV was therefore routinely purified from *N. glutinosa*. In the course of this study we noticed that the virus yield decreased gradually upon repeated inoculation of SYNV on *N. glutinosa*, independent of the season. We did not further investigate this phenomenon, but SYNV was propagated once every few months in *N. christii* to circumvent this problem.

2.2.3 Purification of transcribing RNP complexes

The RNP complexes were extracted from purified SYNV preparations by a method developed in our laboratory. To a purified SYNV preparation in buffer B, one volume of 2% NP-40 containing 2 M NaCl was added. After incubation for 60 min at room temperature this suspension was layered onto a 30% sucrose cushion of 2 ml, which contained 0.1 M NaCl and 0.1% NP-40 in buffer B. The RNP complexes were pelleted at 40,000 rpm in a SW50.1 rotor in a Beckman L5-65 centrifuge for 90 min at 4 °C and resuspended in buffer B. The solubilized proteins in the supernatant were precipitated with ethanol at -20 °C overnight and collected by centrifugation at 12,500 rpm for 30 min into a pellet. This pellet was also resuspended in buffer B. The proteins in both pellets were analyzed on a 10% SDS-polyacrylamide gel (Laemmli, 1970).

2.2.4 Preparation of SYNV-RNA

SYNV-RNA was extracted at room temperature with a mixture of phenol, hydroxyquinoline and m-cresol (90:10:0.01) after solubilization of the virus proteins with 1% SDS, 0.1 M NaCl and 0.001 M EDTA in 0.01 M Tris-HCl buffer, pH 7.0. The RNA was ethanol precipitated in the presence of 0.3 M Na-acetate, pH 5.5, and the concentration was determined spectrophotometrically at 260 nm.

2.2.5 RNA gel electrophoresis

RNA was analyzed by flat-bed electrophoresis under denaturing conditions in 0.8% agarose gels using 5 mM methylmercury hydroxide (Bailey and Davidson, 1977) as denaturing agent. Electrophoresis was carried out at 150 V for 3h. The agarose gels were stained for 30 min with 1 μg/ml ethidium bromide in 0.5 M NH_4-acetate.
RNA bands could be visualized under UV-light. Cowpea mosaic virus (CPMV) RNA, tobacco mosaic virus (TMV) RNA and cowpea chlorotic mottle virus (CCMV) RNA were used as mol. wt. markers.

2.2.6 Electron microscopy

SYNV preparations were mixed with an equal volume of 2% glutaraldehyde and applied to a carbon-coated grid for 2 min, washed twice with H_2O and stained with phosphotungstate acid or uranylacetate for 15 sec.
RNP strands were prepared by treatment of purified SYNV with 0.5% Zwittergent TM 312 or 1% NP-40 in the presence of 0.5 M NaCl. The strands were pelleted and resuspended in 1x PBS (1x PBS is 30 mM NaCl in 2 mM Na-phosphate buffer, pH 7.2) plus 0.4 M NaCl and mixed with an equal volume of a solution of 0.1 mg/ml cytochrome C in a 0.02 M Tris-HCl buffer, pH 8.5, containing 2 mM EDTA. A droplet of this suspension was applied to a carbon-coated grid for one min and rinsed once with distilled water. The specimen was then shadowed rotationally with 90% platinum and 10% iridium under a 10 degrees angle at a distance of 1.5 cm and examined in a Siemens Elmiskop-101 electron microscope.

2.2.7 Polyacrylamide gel electrophoresis of virus proteins under denaturing conditions

Proteins were separated on 10% SDS-polyacrylamide gels as described by Laemmli (1970). Phosphorylase A (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and RNase A (14,000) were used as mol. wt. markers.
The proteins in the gels were stained with Coomassie Brilliant Blue R250 (CBB R250) or with the silver-stain technique described by Morrissey (1981). Periodic acid Schiff (PAS) staining, which is specific for glycoproteins, was performed as described by Zacharius *et al.* (1969).

2.2.8 Iodination of SYNV proteins

To label the SYNV proteins with ^{125}I the procedure of Bolton and Hunter (1973) was modified. Purified SYNV (50 μl) was first disrupted in 100 μl 5% Triton X-100 in H_2O. Then 100 μl 0.1M borate buffer, pH 8.5 and the Bolton Hunter reagent (1 mCi/ml, New England Nuclear) in 200 μl 0.1 M borate buffer, pH 8.5 was added to the virus proteins and the reaction was allowed to proceed for 15 min at 0 °C. The reaction was terminated by adding 0.5 ml 0.2 M glycine-borate buffer, pH 8.5 at 0 °C. Unincorporated ^{125}I was removed from the reaction mixture by dialysis against antibody buffer (50 mM Tris-HCl, pH 7.4 containing 4.5 mM EDTA, 150 mM NaCl, 0.25% gelatin and 0.5% NP-40), or by Sephadex G-50 column chromatography as described by Bolton and Hunter (1973). The

virus proteins were labeled to a specific activity of 4-5 μCi/μg of protein.

2.2.9 V8 protease mapping of SYNV proteins

To characterize the SYNV proteins by V8 protease digestion and mapping of the peptides, we used the method, described by Cleveland *et al.* (1977) and modified by Reavy and Moore (1981). The SYNV proteins were separated on a 10% SDS-polyacrylamide gel (Laemmli, 1970) and an appropriate lane was excised and applied to a 16% denaturing polyacrylamide gel, on top of which a 3% stacking gel had been cast. Over this excised lane 3 ml of a solution containing 180 μg V8 protease in 125 mM Tris-HCl, pH 7.2 containing 0.1% SDS, 1 mM EDTA, 20% glycerol and 0.003% BPB was layered. Electrophoresis was first carried out at 150 V, until the dye front had moved into the 3% spacer gel. The proteolytic reaction was allowed to proceed at room temperature for 30 min in the spacergel. After digestion of the SYNV proteins, the electrophoresis run was continued for 7 h at 150 V, or until the dye had migrated far enough. The peptides in the gel were silver-stained, or exposed to Kodak RP Royal-X-Omat film at -70 °C using a DuPont Cronex Lightning Plus intensifying screen, when ^{125}I-labeled proteins were analyzed.

2.3 RESULTS AND DISCUSSION

2.3.1 Purification of SYNV

N. glutinosa or *N. christii* plants showed symptoms of vein clearing and leaf cupping in the upper growing leaves approximately 8 to 12 days after inoculation. The growth of the infected plants was retarded to 12 days when compared to mock-inoculated plants.
The development of SYNV in the infected plant was monitored by ELISA (Clark and Adams, 1977). The first viral antigens were detected 8 days after inoculation in young expanding leaves and increased in concentration until 16 days post-inoculation (Fig. 2.1). The appearance of virus symptoms was retarded in this experiment, which was done in winter, as compared to the 8 days necessary for virus symptoms to appear in summer.

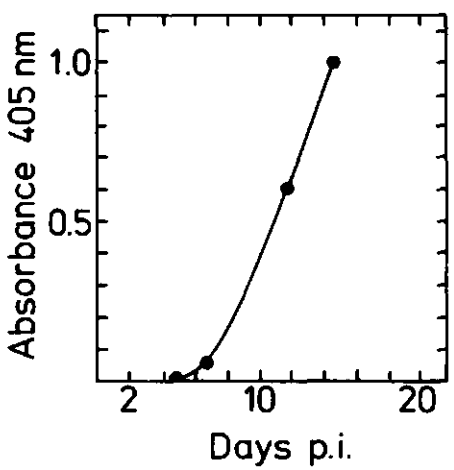


Fig. 2.1.
The development of SYNV in leaves of *N. christii* as demonstrated by ELISA. Young expanding leaves were harvested at the indicated times post-inoculation and crude extracts were prepared for ELISA according to Francki and Peters (1978). The extracts were stored at -20 °C and used in an ELISA. Values obtained in the ELISA with extracts of mock-inoculated plants are subtracted.

It became apparent in the course of this study that the time of appearance of virus symptoms and virus yield was dependent on plant growth conditions and hence on the season. Yields of 200 μg of virus protein from 10 g of leaf material were obtained using optimum conditions for purification of SYNV which were determined to be those described in Chapter 2.2.2. Jackson *et al.* (in press) reported yields of 500 μg of virus protein from 10 g of leaf material under optimal growth conditions.

A study was made to determine which host plants and what conditions would give higher yields of virus. Yields were low, when SYNV was extracted from *N. tabacum* cv. Xanthi-nc, *N. clevelandii*, *N. rustica* or *Sonchus oleraceus*. Different buffers were studied in the purification procedure of SYNV. The use of Mg-glycine buffer, phosphate buffer and citrate buffer did not improve virus yields. The addition of reducing agents or BSA to the buffers studied to stabilize the virus, did not improve yields (results not shown).

Purified preparations of SYNV were examined by the electron microscopy and the particles were measured. The SYNV particle measured 235 x 100 nm (Fig. 2.2). Jackson and Christie (1979) reported 250 x 94 nm after fixation in glutaraldehyde and negative staining of the virus particles. The size difference could be a result of the different fixation methods used.

The cross-striations in some virus particles are clearly visible (Fig. 2.2), suggesting damage of the virus envelope so that uranyl acetate can penetrate into the internal virus particle. Fig. 2.2 also shows rounded particles, which could result from a complete disintegration of the nucleocapsid structure during the processing of the virus samples for EM. Milne (1984) discussed on the difficulty of preparing intact plant rhabdoviruses for examination by the EM.

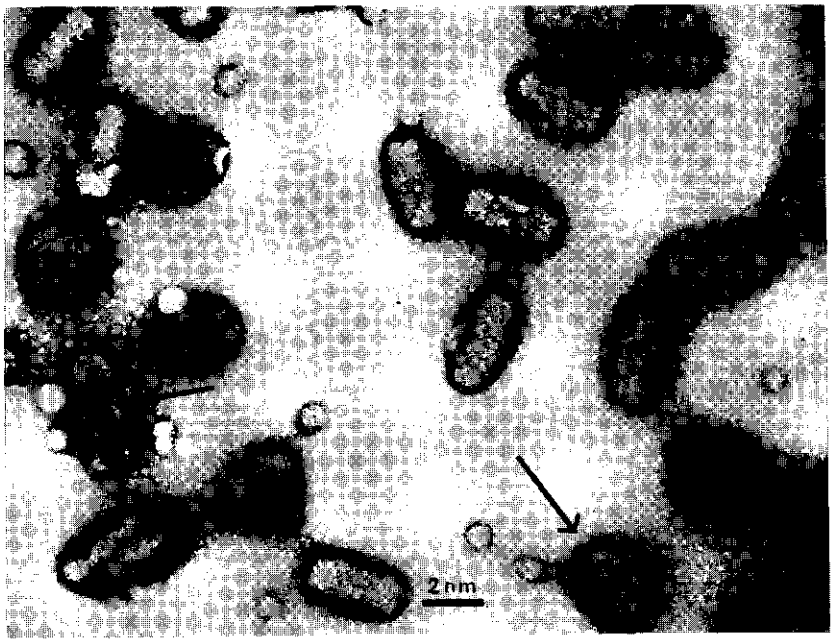


Fig. 2.2.
Electron micrograph of purified SYNV. Virus particle with internal cross-striations (—►); rounded virus particles (→).

2.3.2 Characterization of SYNV proteins

Proteins of purified SYNV were analyzed on a 10% SDS-polyacrylamide gel. The four major proteins of SYNV, denoted G, N, M1 and M2 as described by Jackson and Christie (1978) are shown in Fig. 2.3. In addition two proteins with a high mol. wt., designated HMW1 and HMW2, were often detected. Silverstaining of the proteins of purified SYNV, separated on a 10% SDS-polyacrylamide, also revealed the presence of two proteins with lower mol. wt., designated X and Y. Protein Y was not consistently present in purified preparations of SYNV.

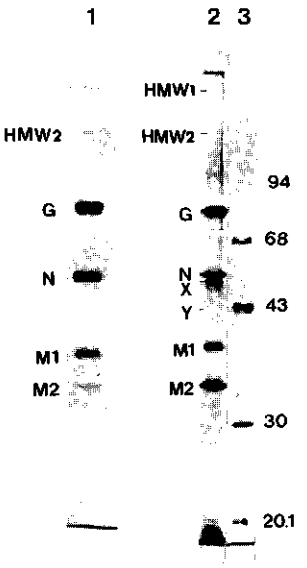


Fig. 2.3.
Analysis of SYNV proteins on a 10% SDS-polyacrylamide gel. (1) Purified SYNV after staining with CBB R250 and (2) after staining with silver. (3) Mol. wt. marker proteins are as described in Materials and Methods and were stained with silver. The mol. wt. are presented at the right side of the gel. The proteins of SYNV are designated at the left side of the gel.

The estimates of the mol. wt. of the SYNV proteins are given in Table 2.1 and compared to values reported in the literature. The mol. wt. estimates of SYNV proteins presented in this study, are the mean of ten separate analyses. They differ from those reported by Jackson and Christie (1977). This may simply reflect differences in the gelsystems used, because the mol. wt. are in general agreement with those reported by Dale and Peters (1981) and Van Beek *et al.* (1985b).

Table 2.1. Mol. wt.x10^3 of SYNV proteins.

	HMW1	HMW2	G	N	X	Y	M1	M2
Present study	184	140	80	56	51	42	38	34
Jackson and Christie (1979)			77	64			45	39
Dale and Peters (1981)			82	59			34	31
Van Beek *et al.* (1985b)			82	56			41	35

To determine whether HMW1, HMW2, X and Y proteins are related to the major SYNV proteins, V8 protease mapping was used (Reavy and Moore, 1981). The V8 protease digest revealed that all four major proteins gave rise to a distinct peptide pattern in the second dimension (Fig. 2.4). Fig. 2.4 shows that the peptides found in protein HMW2 and Y of SYNV resemble those of the G protein. The HMW2 and Y protein also stained with the PAS method (not shown), indicating the presence of carbohydrates in these proteins (Zacharius *et al.*, 1969). We conclude that the Y protein is a specific degradation product of the G protein of SYNV, which was present in variable amounts indicating that the degradation varied between preparations of SYNV. The HMW2 protein which has a mol. wt. of approximately twice that of the G protein is a dimer of G. Mol. wt. estimates of large proteins on a 10% SDS-polyacrylamide gel are inaccurate, however. This HMW2 protein has also been reported by Dale an Peters (1981). Dietzschold *et al.* (1978) reported a glycosylated protein with a mol. wt. of 50,000 (gp50) associated with rabies virus which is a specific degradation product of the glycoprotein. The Y protein is possibly analogous to gp50.
We could not establish a relation between HMW1, X and the structural proteins of SYNV due to the low amounts of HMW1 and X in the virus particles.

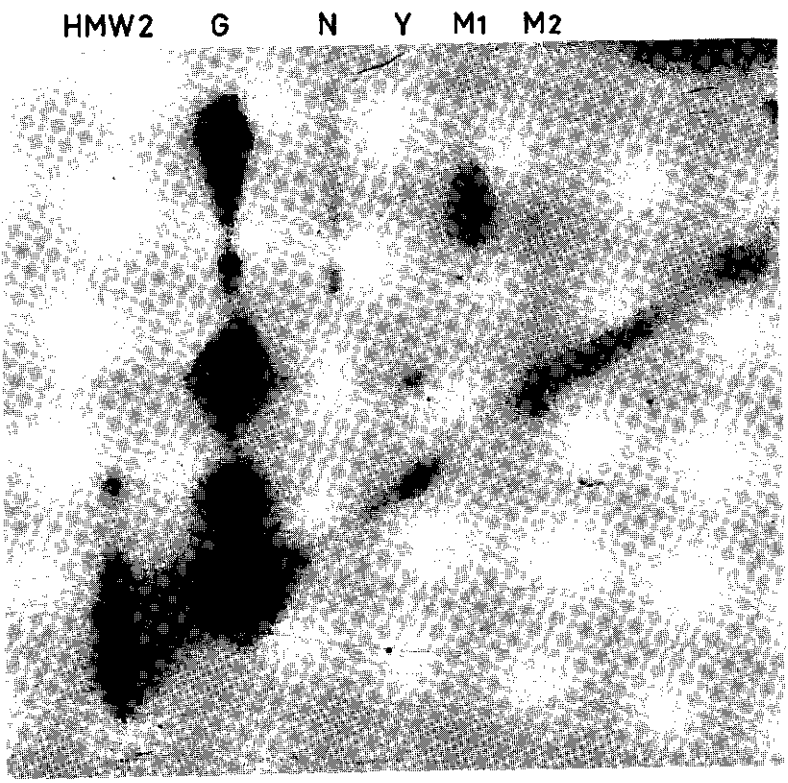


Fig. 2.4.
Peptide analysis of SYNV proteins, which have been separated on a 10% SDS-polyacrylamide gel in the first dimension. The V8 protease digests were analyzed on a 16% SDS-polyacrylamide gel in the second dimension. SYNV proteins were labeled with ^{125}I. The positions of the proteins in the first dimension are indicated.

2.3.3 Solubilization of SYNV proteins

Because we do not know which proteins of SYNV are necessary for infection, we decided to disrupt the virus in a controlled manner, while assaying the resulting fraction containing the RNP complexes for infectivity. The virus was treated with detergent and fractionated by centrifugation into an RNP fraction and a soluble protein fraction as described in Chapter 2.2.3. The pellet and supernatant fractions were analyzed on a 10% SDS-polyacrylamide gel.

Detergents such as Lubrol, dodecyl-ß-D-maltoside (Bujarski *et al.*, 1982), Triton X-100, Zwittergent TM 312 (Gonenne and Ernst, 1978) and NP-40 which were tested for their efficacy to disrupt virus particles under different experimental conditions. Mellitin, a substance found in bee venom, was used in an attempt to permeabilize membranes (Pal *et al.*, 1981a). Temperature and incubation time were optimized and the effect of the addition of salt was tested. SDS-polyacrylamide gel electrophoretic analysis indicated that 0.5% Zwittergent TM 312 in the presence of 0.5 M NaCl solubilized the matrix proteins more effectively than NP-40, although not completely (Fig. 2.5). The amount of X protein increased in the pellet fraction and a protein, migrating slightly ahead of the X protein on the 10% SDS-polyacrylamide gel, appeared. The nature of this protein is unknown. The resulting RNP containing fractions were not infectious when tested on *N. glutinosa*, so Zwittergent TM 312 could not be employed to prepare infectious RNP complexes of SYNV. The other compounds were not as effective as Zwittergent TM 312 and NP-40 in removing proteins from the complex (results not shown). Treatment of SYNV particles with 1.0% NP-40 in the presence of 1 M NaCl resulted in the partial release of the G, M1 and M2 proteins (Fig. 2.6, lane 2).

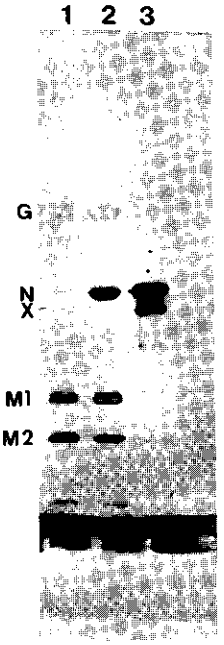


Fig. 2.5.
SDS-polyacrylamide gel electrophoretic analysis of SYNV proteins after solubilization of the virus with 0.5% Zwittergent TM 312 in 0.5 M NaCl. The components were separated by centrifugation. Lane 1: proteins present in the supernatant. Lane 2: proteins prior to dissociation. Lane 3: proteins present in the pellet fraction. The gel was stained with silver.

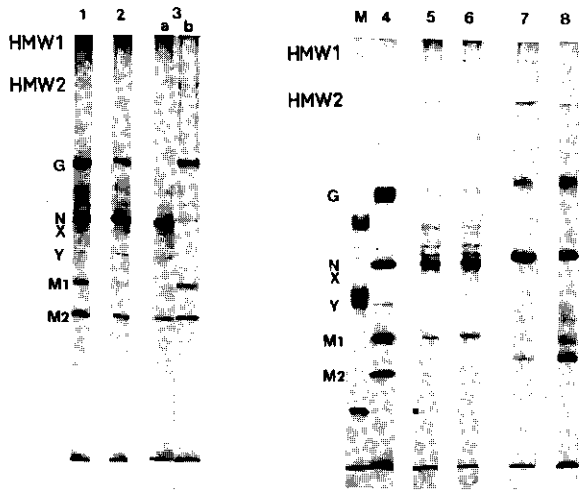


Fig. 2.6.
SDS-polyacrylamide gel electrophoresis of SYNV proteins associated with the RNP complex after disruption of the virus particle. The components were separated by centrifugation through a 30% sucrose cushion. SYNV proteins present in purified preparations (lane 1 and 4). This disruption was studied with 1% NP-40 in the presence of 1 M NaCl (lane 2); 5% NP-40 with 1 M NaCl (lane 3a: proteins present in the pellet fraction; lane 3b: proteins remaining in the supernatant); 1% NP-40 with 8 M urea (lane 5); 1% NP-40 with 8 M urea and 0.5 M 2-mercaptoethanol (lane 6); 1% NP-40 at pH 9.4 (lane 7); 1% NP-40 at pH 6.0 (lane 8). Mol. wt. markers are shown in lane M. The gels were stained with silver.

In an attempt to improve dissociation with NP-40 the concentration was increased to 5% in the presence of 1 M NaCl. Minor improvements were detected (Fig. 2.6, lane 3a). In the supernatant HMW2, G, M1, M2 and trace amounts of N protein were detected (Fig. 2.6, lane 3b). This result is not in agreement with Dale and Peters (1981), who completely removed the matrix proteins from the RNP complex. They stained their gel with CBB R250, while we used the more sensitive silver stain which could detect residual M1 and M2 proteins associated with the RNP complex. Urea was tested at different concentrations up to 8 M in combination with 1% NP-40 and in the presence and absence of 0.1 M and 0.5 M 2-mercaptoethanol. Even at the highest concentration urea (8 M) dissociation of M1 and M2 was not complete (Fig. 2.6, lane 5). The addition of 2-mercaptoethanol gave no appreciable difference in the degree of dissociation (Fig. 2.6, lane 6). To investigate the possibility that the dissociated proteins of SYNV aggregate and subsequently coprecipitate with the RNP complex, we varied the pH in this buffer between 6.0 and 9.4. Again no consistent difference in solubilization was detected (Fig. 2.6, lane 7 and 8).
In conclusion, I have shown that a complete solubilization of SYNV M1 and M2 proteins is difficult to achieve. Zwittergent TM 312 gave the best results, but the resulting RNP complexes were not infectious any more. Of the non-ionic detergents, NP-40 appeared to solubilize the SYNV proteins most effectively, but not completely. Addition of urea and 2-mercaptoethanol did not improve solubilization significantly. The influence of the pH of the solubilization buffer on the dissociation of proteins from the RNP complex was negligible. Dietzschold *et al.* (1978) reported that the M1 protein of rabies virus is not a part of the virus envelope and that M2 is associated with the RNP complex and the envelope. Our results suggests that the M1 and M2 proteins of SYNV do not bind exclusively to the virus envelope either. We were not able to demonstrate in these experiments that the

HMW1 protein is associated with the RNP complex. The X protein appears to be tightly associated with the RNP complex and we shall present more evidence about this association in Chapter 4.

2.3.4 Electron microscopy of ribonucleoprotein strands

To determine whether RNP complexes which had been solubilized with 0.5% Zwittergent TM 312 were completely uncoiled in the absence of M1 and M2, they were examined by electron microscopy and compared to complexes which had been treated with 1% NP-40. The RNP strands were spread onto electron microscope grids using the cytochrome C method (Kleinschmidt, 1968) and their length was measured.
Zwittergent TM 312 treated nucleocapsids (for their protein composition see Fig. 2.5) were fully uncoiled (Fig. 2.7). The NP-40 treated nucleocapsids were never detected in a fully extended state (Fig. 2.7). The presence of M1 and/or M2 proteins (Fig. 2.6) may prevent complete uncoiling of the nucleocapsids (Newcomb *et al.*, 1982).
The length of extended RNP strands was 4.48 μm and the width 5 nm (Fig. 2.7), as determined in 10 independent measurements. This is slightly longer than that reported for the length of the VSV-RNP strand which was 3.5 μm (Nakai and Howatson, 1968).

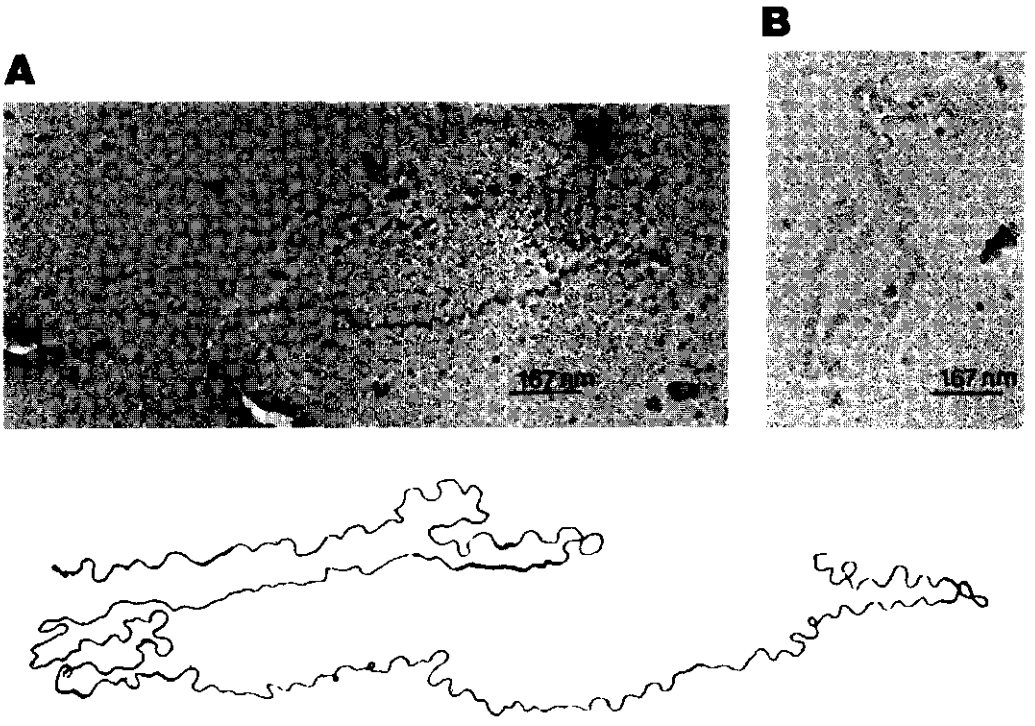


Fig. 2.7.
Electron micrographs of ribonucleoprotein strands treated with either Zwittergent TM 312 (A) or NP-40 (B)). The specimens were prepared for electron microscopy, using cytochrome C. An interpretive figure of A is presented below the micrograph.

2.3.5 Determination of the mol. wt. of SYNV-RNA

To determine the mol. wt. of SYNV-RNA, the RNA was phenol-extracted and electrophoresed on a 0.8% agarose gel containing methylmercury hydroxide (Bailey and Davidson, 1976). In addition to the SYNV-RNA band two other bands were detected in most experiments (Fig. 2.8). These bands comigrated with 28S and 18S ribosomal RNAs, purified from healthy plants. The presence of ribosomal RNA in SYNV-RNA preparations indicates that the virus preparations are still contaminated with ribosomal material. RNA preparations of other plant rhabdovirus tested sofar also contained the two ribosomal RNA species (not shown). The mol.wt. of the RNA was approximately 4.4×10^6 using the RNAs of VSV, TMV, CPMV and CCMV as markers (Reijnders *et al.*, 1974). This value has also been reported by Jackson and Christie (1979) and is slightly higher than that obtained for VSV-RNA.

The mol. wt. of SYNV-RNA indicates the presence of approximately 13,000 nucleotides, which is sufficient to code for the four major proteins of SYNV (G, N, M1 and M2), the HMW1 protein and for the X protein (see also Rezaian *et al.*, 1983).

It is important for future reference, to note that SYNV-RNA, prepared by phenol extraction of purified SYNV, is extremely sensitive to degradation. It is unclear whether this degradation is mechanical or caused by a nuclease, present in purified SYNV preparations. In the N protein-RNA complex, the integrity of the RNA is preserved by the N protein against nucleases, as is the case with VSV-RNA (Blumberg *et al.*, 1984).

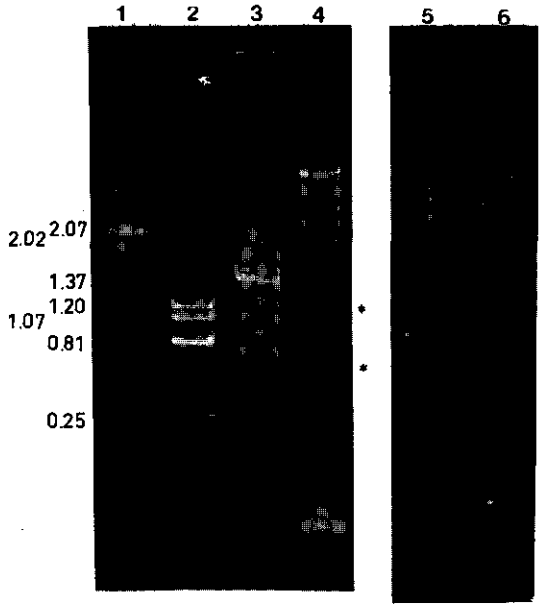


Fig. 2.8.
SYNV-RNA (lane 4 and 6) was analyzed under denaturing conditions on a 0.8% agarose gel containing 5 mM methylmercury hydroxide. RNA bands were visualized by staining with ethidium bromide. The black dots indicate RNAs comigrating with purified plant ribosomal RNAs. Transfer RNA runs at the bottom of the gel. Marker RNAs are: TMV-RNA (lane 1); CCMV-RNA (lane 2); CPMV-RNA (lane 3). To compare SYNV-RNA with VSV-RNA they were run on a separate gel: VSV-RNA (lane 5) and SYNV-RNA (lane 6). Mol. wt.x10^6 are shown at the left side of the gel.

CHAPTER 3

IN VITRO TRANSCRIPTION AND TRANSLATION OF SYNV-RNA

3.1 INTRODUCTION

The main theme of this chapter comprises the detection of the transcriptase activity in SYNV, which resembles rabies virus in its protein pattern on a SDS-polyacrylamide gel. The presence of an RNA-dependent RNA polymerase, associated with rabies virus, has been demonstrated by two research groups, but the experimental procedures used, differed remarkably.
Kawai (1977) employed a concentration of 4% Triton X-100 to activate the RNA-dependent RNA polymerase of rabies virus. The enzyme incorporates ribonucleoside monophosphates at a low rate and continues to do so for 8 h with a slight initial lag phase. The product was single-stranded RNA, which was complementary to rabies virus RNA. The synthesized product RNA sedimented at 6 to 12S. This author reported the presence of a protein with a high molecular weight (L protein) associated with the rabies virus nucleocapsid which was assumed to be the RNA-dependent RNA polymerase, in analogy with the L protein of VSV. It is worth noting, that the RNA-dependent RNA polymerase activity in rabies virus preparations found by Kawai (1977) was only a hundredth of the polymerase activity associated with VSV, Indiana serotype, and it is tempting to correlate the relatively low activity of the enzyme with the low rate of replication of rabies virus in infected cells. Dietzschold *et al.* (1979) suggest a positive correlation between the amount of L protein present in rabies virus and the polymerase activity and virulence of the virus.
Flamand *et al.* (1978) used a milder condition to dissociate rabies virus. They used 0.01% Triton X-100, but the pH of their reaction mixture was 8.9. This is high, compared to pH 7.4 employed by Kawai (1977) for rabies virus *in vitro* transcription assays. Flamand *et al.* (1978) used a high pH to compensate for the low detergent content in their assay mixture, for it has been reported that solutions with a high pH effectively disrupt virus particles with a membrane (Fiszman *et al.*, 1974). The alkaline pH also inhibits RNases and thus prevents degradation of product RNA.
Few results have been reported in the literature on the requirements of *in vitro* transcription of plant rhabdovirus RNA, and then only for viruses belonging to subgroup A which resemble VSV in their protein pattern on a SDS-polyacrylamide gel (Toriyama and Peters, 1980 and 1981).
Flore and Peters (1981) reported transcriptase activity associated with SYNV. This chapter describes experiments to optimize this transcription activity *in vitro* and to characterize the product RNA by translation in an *in vitro* system.

3.2 MATERIALS AND METHODS

3.2.1 In vitro transcription of SYNV-RNA

The standard transcription reaction mixture (125 μl was used) contained 40 mM Tris-HCl,

pH 7.4, 4 mM Mg-acetate, 80 mM NaCl, 2.56 mM 2-mercaptoethanol, 0.8% (v/v) NP-40, 2 μg actinomycin D, 0.8 mM each of ATP, GTP, and CTP, 0.08 mM UTP, and 1.0 μCi [^{3}H]UTP (38.5 Ci/mmole, New England Nuclear) and 25 μl SYNV. The reaction was incubated at 25 °C and stopped by the addition of 200 μl 80 mM Na-pyrophosphate as an RNA-dependent RNA polymerase inhibitor, and 200 μl 25% TCA to precipitate the newly synthesized RNA in the presence of 25 μg transfer RNA. After being kept on ice for 10 min 1 ml ice-cold ethanol was added to disperse the NP-40. The samples were transferred to Whatman GF/C filters after 2 h at -20 °C and counted in Lipo-Luma in a Packard liquid scintillation counter.
As the quantity of purified SYNV obtained was low, it was often considered wasteful to determine the protein concentration, using the Lowry method (Lowry *et al.*, 1951). The transcription data will be presented in this study in cpm/assay. The activity of the enzyme is calculated per gram infected leaf material.

3.2.2 A coupled transcription and translation system for SYNV-RNA

To study the transcription of SYNV-RNA and protein synthesis, directed by SYNV-RNA, a wheat germ cell-free extract was used (Marcu and Dudock, 1974 and Verkleij, 1982). The reaction mixture (25 μl was used) contained 10 μl of wheat germ cell-free extract, 2.5 mM ATP, 225 μM GTP, 7 mM creatine phosphate, 10 μg/ml creatine phosphate kinase, 35 μM amino acids, except methionine, 20 mM Hepes-KOH, pH 7.6, 0.2 mM spermidine, 110 mM K-acetate, 3.25 mM Mg-acetate, 7 μCi [^{35}S]methionine (800 Ci/mmol, New England Nuclear), 3 μCi [^{3}H]UTP and 0.8% NP-40, and 4 μl of a solution containing the four ribonucleoside triphosphates, was added to adjust their concentrations to those used in an *in vitro* transcription mixture. Five μg SYNV-RNA or TMV-RNA in 5 μl or 5 μl purified SYNV was added to the 25 μl of reaction mixture to initiate transcription and translation.
After incubation of this transcription-translation mixture at 30 °C for 1 h or 22 h, samples of 2.5 μl were assayed for incorporation of [^{35}S]methionine into hot TCA-precipitable radioactively labeled product (Moorman *et al.*, 1976). Samples of 5 μl were assayed for incorporation of [^{3}H]UMP into TCA-precipitable radioactively labeled material as described above.

3.2.3 Antiserum production

Rabbits were injected subcutaneously with 1 ml purified SYNV (0.6 mg/ml) after mixing with an equal volume of Freund's incomplete adjuvant. This injection was repeated two weeks later and two to three weeks thereafter the rabbits were bled from the ear. The titer of the serum, raised against purified SYNV was 1/64 as determined in Ouchterlony agar gel diffusion test after solubilization of the virus with 1% Triton X-100 to facilitate diffusion of SYNV antigens.

3.2.4 Detection of SYNV-specific proteins by immuno-precipitation

To the rest of the transcription and translation mixture, 10 μl 5x PBS-TDS (5x PBS-TDS is 10 mM Na-phosphate buffer, pH 7.2, containing 150 mM NaCl, 1% Triton X-100, 0.5%

Na-deoxycholate and 0.1% SDS), 5 μl 100 mM EDTA, pH 7.0 and 12.5 μl distilled water was added. The dissociated ribosomes were removed by centrifugation at 100,000 *g* for 20 min in a Beckman air-fuge. The supernatant was divided into two samples of equal size. To one sample 5 μl anti-SYNV serum was added; to the other sample 5 μl of a solution pre-immune serum. To both mixtures 10 μl 5x PBS-TDS was added and distilled water to a final volume of 50 μl. The mixtures were incubated overnight at 4 °C. In order to precipitate the reaction products, 10 mg protein A-Sepharose (Pharmacia), preswollen in 10 mM Na-phosphate buffer, pH 7.2 containing 0.9% NaCl, and washed with 1x PBS-TDS, was added and incubated for 45 min at room temperature with occasional shaking. After pelleting the Sepharose-protein complexes at 8,000 *g* for 1 min in an Eppendorf centrifuge, the pellets were washed three times with 1x PBS-TDS, resuspended in Laemmli buffer and boiled for 3 min to dissociate the Sepharose beads from the proteins. Proteins were electrophoresed on a 10% SDS-polyacrylamide gel (Laemmli, 1970). The proteins in the supernatant were precipitated with ice-cold ethanol and also analyzed by electrophoresis. The gels were processed by fluorography (Bonner and Laskey, 1974) and dried. Bands were visualized by exposure of the dried gel to Kodak RP Royal-X-Omat film at -80 °C using a DuPont Cronex Lightning Plus intensifying screen.

3.2.5 In vitro hybridization experiments

Samples of product RNA (about 4,000 cpm) were hybridized in 2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M Na-citrate, pH 7.0) to 4 μg SYNV-RNA or incubated without RNA to measure the amount of selfannealing. The mixture was heat-denatured at 100 °C and then either quickly cooled on ethanol-ice (background) or incubated at 62 °C for two h or overnight. The hybridization mixtures were divided into two samples; one was digested with 50 μg RNase A and 2 μg RNase T1 per ml of sample for 30 min at 37 °C. The other sample was made up to the same volume with distilled water and also incubated at 37 °C. After the incubation 50 μg of transfer RNA and 300 μl 10% TCA was added to both samples. The samples were transferred to Whatman GF/C filters, washed and assayed for radioactivity.

3.3 RESULTS

To demonstrate the association of transcriptase activity with SYNV, the incorporation of [^{3}H]UMP into TCA-precipitable radioactively labeled material by SYNV preparations was studied by incubation at 25 °C under standard reaction conditions for 60 min. The standard reaction mixture, determined to be optimal, contained 40 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 2.56 mM 2-mercaptoethanol, 80 mM NaCl, 0.8% NP-40, 0.8 mM ATP, GTP, CTP and 0.08 mM UTP. Actinomycin D (16 μg/ml) was added to inhibit DNA-dependent RNA polymerase which is present in host plant cells.
Incorporation of [^{3}H]UMP into TCA-precipitable counts was proportional to the concentration of the labeled precursor. Mn-ions were as efficient as Mg-ions in stimulating *in vitro* transcription of SYNV-RNA. This is also the case for *in vitro* transcription of rabies virus RNA, but not for VSV-RNA transcription *in vitro* (Kawai, 1977).
The results obtained show that SYNV-RNA can only be transcribed *in vitro* at a low rate. Maximum incorporation was only 656 cpm in a typical reaction containing purified SYNV

and 75 cpm in a reaction containing mock-inoculated plant material.
It is conceivable that the SYNV associated transcriptase is either a very inefficient enzyme, or that it is present in low amounts or that it is inhibited *in vitro* by a structural protein, as has been demonstrated for the M protein of VSV when it is not dissociated from the transcribing RNP complex of VSV (Carroll and Wagner, 1979).
To ascertain that the reaction mixture contained the essential components for the *in vitro* transcription of SYNV-RNA, transcription of VSV (kindly supplied by Dr. B.A.M. van der Zeijst, Department of Virology, Faculty of Veterinary Medicine, University of Utrecht) was tested under the standard reaction conditions described above, and an incorporation of 8,150 cpm of [^{3}H]UMP was detected after 60 min. The amount of VSV added to the reaction mixture was comparable to that of SYNV. The low efficiency of [^{3}H]UMP-incorporation with SYNV preparations was not due to a deficiency in the transcription assay mixture. Kawai (1977) reported a 100-fold difference in incorporation of radioactively labeled precursors by rabies virus when compared to VSV.
To demonstrate that virus preparations used in *in vitro* transcription assays contained infectious particles, aliquots of these preparations were inoculated onto *N. glutinosa*. Even preparations that exhibited low levels of transcriptase activity contained infectious particles, indicating that the RNA polymerase of SYNV was capable of exerting activity *in vivo*.

3.3.1 Effect of prolonged incubation on in vitro transcription of SYNV-RNA

The incorporation of TCA-precipitable counts was vastly enhanced upon prolonged incubation in a standard reaction mixture containing SYNV (Fig. 3.1). The reaction is characterized by a considerable lagtime of 12 h, before the incorporation starts to increase rapidly and reaches a high level of incorporation of [^{3}H]UMP (45,000 cpm) after approximately 30 h. The period in which maximum levels of incorporation were obtained, varied from preparation to preparation of SYNV.
To determine whether the incorporation of [^{3}H]UMP in long incubation periods was due to SYNV transcriptase activity, bacterial growth or host plant specific RNA polymerase activity, various inhibitors of polymerases and bacterial growth were tested for their effect on the amount of TCA-precipitable radioactivity during prolonged incubation periods (Table 3.1).

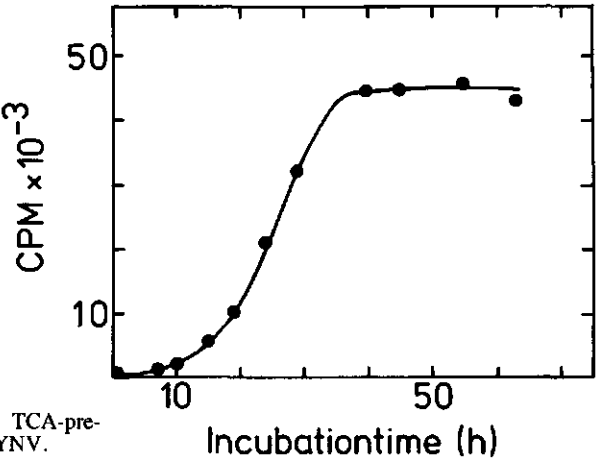


Fig. 3.1.
Time-course of [^{3}H]UMP-incorporation into TCA-precipitable material by preparations of purified SYNV.

Table 3.1. The effect of inhibitors and incubation conditions on the transcriptase activity associated with purified SYNV.

Reaction mixture containing	[^{3}H]UMP-incorporation (cpm) in 20 h
Purified SYNV (standard)	10,800
No virus	52
Purified SYNV minus ribonucleoside triphosphates	150
Purified SYNV plus 20 μg/ml RNase A	8,530
Purified SYNV plus 20 μg/ml DNase I	11,000
Purified SYNV minus actinomycin D	11,150
Purified SYNV plus 32 mM Na-pyrophosphate	169
TMV-RNA	130
SYNV-RNA	54
Purified SYNV plus 40 μg/ml chloramphenicol	8,650
Purified SYNV plus 2.5 mg/ml penicillin	8,100

Table 3.1 shows that no [^{3}H]UMP incorporation was obtained after a prolonged incubation period when SYNV was omitted from the reaction mixture, whereas in the presence of purified SYNV 10,800 cpm were found. The synthesis of TCA-precipitable radioactively labeled material occurred also when 20 μg/ml DNase I was present in the reaction mixture. No degradation of product RNA was detected in the presence of RNase A. The four ribonucleoside triphosphates were required for the synthesis of acid-precipitable product. Omission of actinomycin D did not result in a dramatic increase of [^{3}H]UMP incorporation. The reaction is strongly inhibited by Na-pyrophosphate. No incorporation of [^{3}H]UMP into TCA-precipitable product was detected when deproteinized SYNV- and TMV-RNA were incubated in the reaction mixture instead of SYNV (Table 3.1). Bacterial growth inhibitors (chloramphenicol and penicillin) failed to abolish the incorporation of [^{3}H]UMP, so bacterial incorporation of ribonucleoside triphosphates probably did not occur.
Our results show that SYNV preparations do not contain a DNA-dependent RNA polymerase activity and that we are measuring an RNA-dependent RNA polymerase which is apparently activated by prolonged incubation in the transcription mixture.

3.3.2 Characterization of the protein composition of SYNV at different time intervals of a transcription reaction

To determine the effect of prolonged incubation on the structural proteins of SYNV we examined the protein composition of the reaction mixture at different time intervals by SDS-polyacrylamide gel electrophoresis (Fig. 3.2). Migrating slightly ahead of the N protein a protein, designated X, appears after approximately 2 h of incubation. The concentration of this protein increases in time, while the amount of M1 protein decreases. We do not know if this decrease is in one way or another associated with the increase of X protein, because the CBB R250 staining is not quantitative enough.
No degradation products of SYNV proteins were detected on the gel before 7 h. Between 10 h and 15 h the amount of N protein decreased slightly, but remained constant until 45 h.

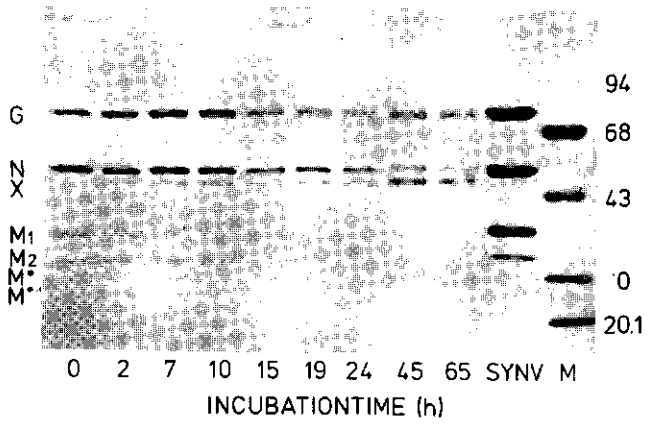


Fig. 3.2.
Analysis of the proteins occurring in a transcriptase assay mixture during an incubation of 65 h. Samples were removed at the indicated times. The proteins were precipitated with ethanol and the resulting pellets were resuspended in Laemmli buffer and analyzed by SDS-polyacrylamide gel electrophoresis. Purified SYNV proteins were run in the lane marked SYNV. Mol. wt. marker proteins are shown on the right side of the gel.

A. K. Banerjee (personal communication) has demonstrated that the N protein of VSV is extremely stable when bound to VSV-RNA. Based on these observations we assume that the degradation of the N protein is of little significance for *in vitro* transcription. After 15 h, when a slight increase in [^{3}H]UMP incorporating activity was found, the M1 protein was completely degraded. M2 decreased in concentration at a lower rate than M1 and was nearly completely degraded after 19 h, corresponding with a dramatic increase in RNA polymerase activity. Novel bands migrating faster in the polyacrylamide gel than M2 could be discrete degradation products. These proteins, migrating slightly ahead of M2 and designated M* and M**, were found 7 h after incubation. By a process of elimination we assume that M* and M** are degradation products of M2. The nature of these proteins will be further discussed in Chapter 4, where we shall argue that they are similar to a discrete degradation protein (M′) of the M protein of VSV (Rosen *et al.*, 1983). Ogden *et al.* (1985) showed that the M protein of VSV was cleaved by a protease into a polypeptide M_T, resembling M′, via an intermediate M_C.
The G protein of SYNV is stable throughout the incubation period and is almost certainly not involved in *in vitro* transcription of SYNV-RNA.
Pre-incubating purified SYNV for 20 h in the transcription mixture, with NP-40 and without the ribonucleoside triphosphates, reduces the time required for efficient incorporation of [^{3}H]UMP to 2 h after adding the ribonucleoside triphosphates. As shown in Table 3.2 an incubation of a complete mixture for 22 h resulted in 17344 cpm, whereas 14456 cpm were obtained in 2 h when the ribonucleoside triphosphates were omitted in the first 20 h of incubation. The changes in protein composition during the pre-incubation period were identical to those shown in Fig. 3.2. The high incorporation achieved in 2 h after pre-incubation has to be attributed to the availability of activated protein and the removal of the inhibition at the moment that the ribonucleoside triphosphates were added.
In this section we have shown a direct correlation between the disappearance of M2 protein from the transcription reaction mixture after approximately 15 h and initiation of high levels of transcription *in vitro*. The degradation of M2 to the polypeptides M* and M**

Table 3.2. Effect of a 20 h pre-incubation of purified SYNV in a standard transcription assay mixture without ribonucleoside triphosphates on the incorporation of [^{3}H]UMP.

Incubation time	No pre-incubation	Pre-incubation
0-2	653*	-
0-20	12,578	-
0-22	17,344	-
20-22	4,766	14,456

*: Transcriptase activity was assayed as described in Materials and Methods and recorded in cpm.

apparently terminates the inhibitory role of M2 which we assume to be the same as that of M in VSV-RNA transcription *in vitro*. The appearance of X and the disappearance of M1 after 2 h to 7 h may be essential to transcribe SYNV-RNA. The presence of a protein, presumably M2 which inhibits the transcription, precludes the unraveling of the precise role of X and M1 in the transcription of SYNV-RNA.

3.3.3 Properties of the in vitro synthesized product RNA

To characterize the RNA synthesized *in vitro* by SYNV in a prolonged transcription assay, this RNA was phenol-extracted and hybridized to SYNV-RNA. After hybridization at 68 °C for 2 h in 2x SSC the product RNA was 60% resistant (above the background) to degradation by RNase A and RNase T1 whereas 42% of the RNA selfannealed. This 60% was rather low when the selfannealing is taken into account. The high level of selfannealing can be explained by a snap-back of RNA after denaturing of the newly-synthesized RNA. It is also possible that product RNA remains associated with the transcribing complex during the transcription. Phenol extraction may then result in the formation of double-stranded RNA which is resistant to RNase and which can only be denatured at high temperatures. The samples were therefore denatured at 120 °C. Using this approach selfannealing was reduced to 5%. This result indicates that the product RNA is complementary to SYNV-RNA.

3.3.4 Coupled in vitro transcription and translation of SYNV-RNA in a cell-free extract

The incorporation of [^{3}H]UMP into TCA-precipitable material by VSV associated RNA-dependent RNA polymerase is enhanced by adding cell-free extracts to the transcription mixture (Breindl and Holland, 1976; Ball and White, 1978). These extracts provide crucial factors which are apparently lost during purification of VSV and which are necessary for correct initiation and termination of RNA transcription by the VSV-RNA polymerase (Hill and Summers, 1982).
Because the polymerase associated with SYNV was not very active in *in vitro* transcription assays, even though the virus particles were infectious, we decided to supplement the transcription reaction mixture with a cell-free extract to stimulate transcription by the

SYNV associated polymerase and to couple this reaction with translation of product RNA. In such a coupled transcription and translation system for SYNV-RNA, to which a wheat germ cell-free extract was added (Breindl and Holland, 1976), 411,000 cpm of [^{3}H]UMP was incorporated into TCA-precipitable material after a 22 h incubation (Table 3.3). When SYNV was assayed in the absence of the wheat germ extract, only 20,900 cpm were incorporated. It is clear from these results that the wheat germ extract stimulated the RNA synthesis and/or provided protection for the newly-synthesized RNA. No incorporation of [^{3}H]UMP into TCA-precipitable material was detected in the presence of SYNV-RNA, TMV-RNA or in the absence of template RNA.

Table 3.4 shows that a high incorporation of [^{35}S]methionine into polypeptides was found after incubating SYNV for 22 h. The products were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3.3A). Incubation of this coupled transcription-translation system with SYNV resulted in a stimulation of polypeptide synthesis (Fig. 3.3A, lane 3 and 4). Analyzing these products by immuno-precipitation with serum raised against SYNV and complexed to protein A-Sepharose, showed that virus-specific polypeptides were synthesized (Fig. 3.3B, lane 1). These polypeptides were not found when pre-immune serum or serum raised against tomato spotted wilt virus was used. The G, N and only one of the matrix (M1) proteins of SYNV were found. The G protein, migrated slower in a 10% SDS-polyacrylamide gel than the G protein labeled with ^{125}I. This behaviour was not expected since *in vitro* synthesized proteins are not glycosylated and would therefore migrate faster than the glycosylated form. However, it may be possible that the signal peptide is not removed from the synthesized G protein in the cell-free extract, which results in a lower electrophoretic mobility of this protein. From immunological experiments (see Chapter 4) we concluded that anti-SYNV serum reacted to a higher extent with protein M1 than with M2. So it may be possible that M2 was not detected in Fig. 3.3B due to a lower titer of the antiserum for this protein. The HMW1 protein of SYNV was not detected and this can be explained by low levels of translation of this large protein. Mammalian cell-free extracts also failed to support the synthesis of the large L protein of VSV (Ball and White, 1978).

Table 3.4 also shows that a high [^{35}S]methionine incorporation into polypeptides directed by TMV-RNA was obtained after 1 h. The amount of label incorporated into polypeptides

Table 3.3. *In vitro* transcription of different RNA species in a coupled transcription-translation cell-free system derived from wheat germ. Incubation was at 30 °C for the indicated time. Incorporation of [^{3}H]UMP was measured as described in Materials and Methods.

Reaction system containing	[^{3}H]UMP-incorporation (cpm)		
	0	1	22 h
No RNA	60	178	233
5 μl SYNV	55	678	411,000
5 μg TMV-RNA	78	212	301
5 μg SYNV-RNA	51	154	103
5 μl SYNV in the absence of the wheat germ system	75	558	20,980

Table 3.4. *In vitro* translation of different RNA species in a coupled transcription-translation cell-free system from wheat germ. Incubation was at 30 °C for the indicated time. Incorporation of [^{35}S]-methionine was assayed as described in the text.

Reaction system containing	[^{35}S]-methionine incorporation (cpm)		
	0	1	22 h
No RNA	2,100	3,400	2,700
5 μl SYNV	2,880	4,100	130,200
5 μg SYNV-RNA	2,300	5,200	58,500
5 μg TMV-RNA	2,450	33,000	8,000

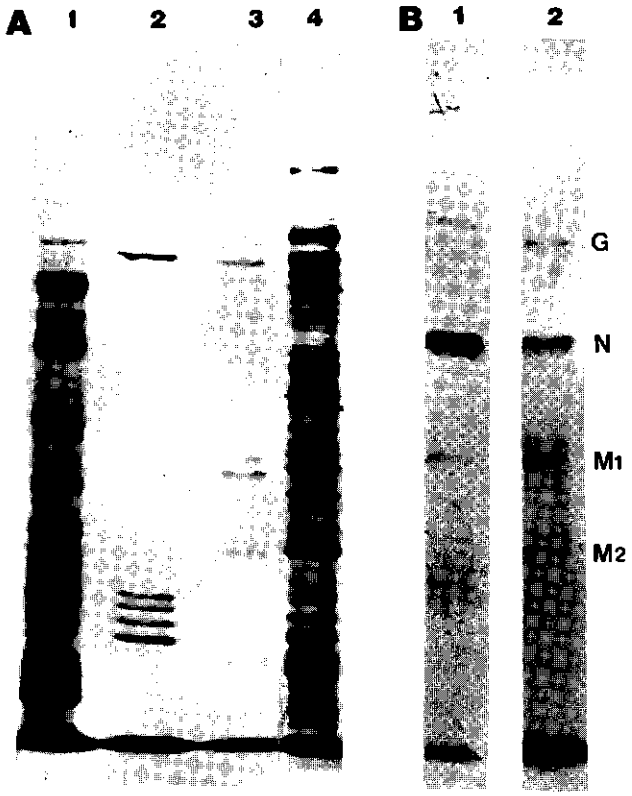


Fig. 3.3.
(A) Analysis of the *in vitro* translation products of TMV-RNA (lane 1), SYNV-RNA (lane 2), in the absence of template RNA (lane 3), and SYNV (lane 4). The proteins, synthesized after 1 h (TMV-RNA) or 22 h (control, SYNV-RNA and SYNV) in a coupled transcription-translation system using a wheat germ cell-free extract, were analyzed on a 10% SDS-polyacrylamide gel. Lane 1 to 3 were exposed to X-ray film for a longer period than lane 4. (B) SDS-polyacrylamide gel electrophoresis of the immuno-precipitates of the translation products, synthesized under the direction of SYNV in a coupled transcription-translation system using a wheat germ cell-free extract during 22 h (lane 1). ^{125}I-labeled SYNV was run in lane 2. Virus proteins are designated at the left side.

decreased upon prolonged incubation, possibly due to degradation of the TMV-RNA and protein. The pattern of polypeptides synthesized under the direction of TMV-RNA after 1 h differs from that obtained with SYNV-RNA, or in the absence of RNA, or with SYNV. [^{35}S]methionine was incorporated when SYNV-RNA was incubated for 22 h in this coupled transcription-translation assay (Table 3.4). But no virus-specific proteins were detected after analysis on a SDS-polyacrylamide gel (Fig. 3.4A) and no proteins could be immunoprecipitated, although some low mol. wt. polypeptides were found. We were not able to establish the nature of these polypeptides.

3.4 DISCUSSION

In this chapter we demonstrated RNA polymerase activity in purified SYNV preparations. Incorporation of ribonucleoside monophosphates into RNA was optimal at 25 °C in the presence of Mg- or Mn-ions in a Tris buffer at pH 7.4. ATP, GTP, CTP and UTP were required. NaCl and 0.8% NP-40 were used to solubilize the virus envelope, resulting in an activation of the associated transcriptase. Incorporation of [^{3}H]UMP was low, but prolonged incubation of SYNV resulted in high levels of incorporation after a lag-time. We provided evidence that this incorporation was virus-specific. The product RNA was resistant to RNase A, presumably because of the double-stranded nature of the RNA. The high percentage of selfannealing during hybridization of radioactively labeled RNA to SYNV template RNA is in agreement with this assumption. The product RNA showed 60% homology with template RNA.

It seems amazing that the transcriptase is only activated in a long incubation, but this finding is not without precedent. Yamakawa *et al.* (1981) retained transcriptase activity associated with reovirus after 16 h in the presence of chymotrypsin. Kawai (1977) incubated rabies virus for 16 h and VSV for more than 12 h at 30 °C.

We have shown that a protein with an electrophoretic mobility similar to that of protein X appeared at approximately 2 h after incubation of the reaction mixture. The nature of this new protein is unclear. The simultaneous decrease in the amount of M1 protein and increase of protein X suggests that the latter is derived from M1 possibly by changing the degree of phosphorylation of the M1 protein which may cause that the newly formed protein has a lower electrophoretic mobility. Cox *et al.* (1981) have shown that rabies virus M1 protein occurs in two phosphorylated forms in the nucleocapsid like NS of VSV. These authors have now designated the M1 protein of rabies virus as NS, while M2 has been renamed M. The M1 protein has been implicated in the transcription and replication of rabies virus RNA (Tuffereau *et al.*, 1985). Van Beek *et al.* (1985b) have recently shown that the M1 protein of SYNV is phosphorylated in cowpea protoplasts infected with SYNV. In some experiments these authors detected a phosphorylated protein with the same mol. wt. as the X protein (personal communication). The observations made suggest that for an efficient SYNV-associated transcriptase activity M1 protein has to be modified with respect to the degree of phosphorylation. The requirement of NS proteins with different degrees of phosphorylation for an efficient transcription of VSV-RNA has been shown by Bell *et al.* (1984).

Although the explanation of the origin of X is plausible, we cannot exclude the possibility that X is a degradation product of another SYNV protein. No evidence for this assumption was obtained so far.

The M2 protein decreases gradually after 7 h with a concurrent increase in the amount of two new proteins designated M* and M**. The timing of these events coincides with an increase of transcriptase activity at approximately 19 h after initiation of the reaction. It is of interest to note that Ziemicki and Peters (1976a) reported a proteolytic activity associated with SYVV, which selectively degrades the matrix protein of this rhabdovirus. Rosen *et al.* (1983) reported that the M protein of VSV autocatalytically dissociates to M' in the course of infection and this degradation is thought to decrease inhibition of transcription by the M protein.

Pal *et al.* (1981a) report that the envelope of VSV must be solubilized, before the ribonucleoside triphosphates become available for the RNA-dependent RNA polymerase of VSV in an *in vitro* transcription assay, but it is not clear whether the M protein has an additional, enzymatic inhibitory role. It has been shown that *in vitro* solubilization of the M protein of VSV is required for transcription to proceed (Rosen *et al.*, 1983), but its inhibitory mechanism is poorly understood (Thornton *et al.*, 1984). Pal *et al.* (1985a) reported that exposure of only one epitope on the surface of the M protein of VSV was involved in the inhibition of VSV-RNA transcription *in vitro* by the M protein. The lag-time shown in Fig. 3.1 may therefore be correlated to the time required for solubilization of the M2 protein and degradation of one or more epitopes of the M2 protein of SYNV, yielding M* and M**.

No dramatic decrease in the amount of G protein was detected in the prolonged incubation. The G protein has never been implicated to have a role in the transcription of any rhabdovirus.

In conclusion we suggest a mechanism whereby a virus-specific protein, presumably M1 has to be activated, presumably by changing the degree of phosphorylation which results in a protein X with a lower electrophoretic mobility in a 10% SDS-polyacrylamide gel, and that the M2 protein presumably inhibits transcription *in vitro* until it is modified.

It is not known whether protein synthesis is required for the synthesis of full-length mRNAs from the SYNV template. We do see a 20-fold increase in [^{3}H]UMP-incorporated material in a coupled transcription-translation system, using a wheat germ cell-free extract. The synthesis of mature virus proteins in this system suggests that full-length mRNAs are being transcribed from the SYNV negative-stranded RNA. The increase found can be explained by a protection of synthesized RNA *in vitro*; a requirement of protein synthesis in the transcription of mRNA cannot be deduced from this observation.

CHAPTER 4

PURIFICATION OF TRANSCRIBING RIBONUCLEOPROTEIN COMPLEXES BY PHOSPHOCELLULOSE COLUMN CHROMATOGRAPHY

4.1 INTRODUCTION

The results discussed in Chapter 3 suggest that the M2 protein of SYNV may inhibit the transcription of SYNV-RNA *in vitro* and that the proteins X and M1 may also play an important role in the regulation of transcription. To test these ideas, it is necessary to separate selectively these proteins from the RNP complex and to determine the effect of these proteins on transcription *in vitro* and on infectivity *in vivo* after each step. Experiments to dissociate these proteins from the virus have been described in Chapter 2.
To obtain pure actively transcribing RNP complexes we attempted to separate them from the solubilized proteins by phosphocellulose column chromatography after dissociation of the virus.
The M protein of VSV has a high affinity for phosphocellulose. The G protein of VSV elutes at low salt concentrations (0.1 M NaCl), while the M protein elutes at 1.0 M NaCl (Carroll and Wagner, 1979). They could subsequently demonstrate the inhibitory action of the column-purified M protein on *in vitro* transcription of VSV-RNA. The L protein of VSV can be eluted at 1.0 M NaCl and the NS protein at 0.2 M NaCl (De and Banerjee, 1984; and Isle and Emerson, 1982).

4.2 MATERIALS AND METHODS

The purification and dissociation of SYNV, the isolation of transcribing RNP complexes and the analysis of virus proteins by SDS-polyacrylamide gel electrophoresis have been described in previous chapters. *In vitro* transcription of SYNV-RNA was performed as described in Chapter 3.

4.2.1 Phosphocellulose column chromatography

SYNV was extracted from 50 g *N. christii* leaves by filtering over Celite and concentrated by centrifugation at 35,000 rpm in a R35 rotor (MSE 18) for 45 min. The pellet was resuspended in 5 ml 0.01 M Na-phosphate buffer, pH 7.4. NaCl and NP-40 were added to a final concentration of 1.0 M and 1%, respectively. After incubating at room temperature for 30 min the suspension was diluted twice and applied to a phosphocellulose column (Whatman P11 phosphocellulose, 9x2.1 cm), equilibrated with elution buffer 'C', consisting of 0.01 M Tris-HCl, pH 8.1, 0.1 mM EDTA, 25 mM NH_4Cl and 5 mM 2-mercaptoethanol (Dorssers *et al.*, 1983). The elution rate was 0.4 ml per min. The proteins were eluted stepwise from the column at room temperature with 30 ml 0.08 M, 15 ml 0.22 M, 15 ml 0.5 M, 20 ml 1 M and 20 ml 2 M KCl or K-acetate in buffer 'C'. The first 10 ml was discarded. Fractions of 5 ml were collected and centrifuged at 55,000 rpm in a SW55 Ti rotor (Beckman L5-65) for 1 h. The pellets were resuspended in a small volume of 0.01 M

Na-phosphate buffer, pH 7.4. Alternatively, the collected samples were dialyzed against 50 mM Na-phosphate buffer, pH 7.4, containing 1 mM EDTA, 10 mM 2-mercaptoethanol and a saturating amount of $(NH_4)_2SO_4$ (767 g/l). After 1 h at room temperature the dialysis-bags were reduced in size and the samples were dialyzed against the same buffer without $(NH_4)_2SO_4$ overnight at 4 °C.
The proteins in the samples were separated on a 10% SDS-polyacrylamide gel (Laemmli, 1970) and visualized by silver staining or transferred to nitrocellulose as described below for immunological detection of the proteins with ^{125}I-labeled protein A.
The fractions were assayed for infectivity using *N. glutinosa*, or *C. quinoa* plants and for transcriptase activity *in vitro*.

4.2.2 Electroblotting of virus proteins

After separating SYNV proteins on a 10% SDS-polyacrylamide gel the gel is sandwiched together with a sheet of nitrocellulose (BA 85, 0.45 μm, Schleicher and Schuell) between two layers of Whatman 3MM paper, two pads of Scotch Bright and two plastic perforated holders. The nitrocellulose, Whatman 3 MM paper and the Scotch Bright pads were immersed in transfer buffer consisting of 25 mM Tris-HCl, pH 8.3, 192 mM glycine and 20% methanol. Blotting took place in a Bio-Rad Trans-Blot electrophoretic apparatus for 5 h at room temperature and at 230 mA. These parameters were established experimentally. The blots were either used for the immunological detection of proteins, using ^{125}I-labeled protein A or stained with a solution of 0.1% aniline blue-black in 43% methanol and 10% acetic acid for 15 min and destained in a solution of 90% methanol and 2% acetic acid.

4.2.3 Immunological detection of proteins on protein blots, using ^{125}I-labeled protein A

Nitrocellulose filters with bound proteins were incubated in antibody buffer, consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% gelatin and 0.05% NP-40 in sealed plastic bags at room temperature for 4 h. Following this incubation serum against SYNV was added in a dilution of 1:100 or 1:1,000, depending on the titer of the antiserum and on the requirements of the particular experiment, and incubated at room temperature overnight. The blots were then washed with antibody buffer to remove unbound antibodies. Subsequently $5x10^5$ cpm/ml ^{125}I-labeled protein A in antibody buffer was added and incubated overnight at 37 °C with vigorous shaking. Protein A (1 mg/ml) was labeled with ^{125}I as described in Chapter 2 for SYNV proteins with omission of a treatment with detergent. The blots were washed extensively with a 50 mM Tris-HCl, pH 7.4 buffer containing 5 mM EDTA, 1.0 M NaCl, 0.25% gelatin and 0.4% sarkosyl. The dried filter was exposed to Kodak RP Royal-X-Omat film at -80 °C, using a DuPont Cronex Lightning Plus intensifying screen.

4.2.4 Dotblot hybridization

Nitrocellulose filters (1 cm^2) were incubated for 30 min in 20x SSC (1x SSC is 0.15 M NaCl plus 0.015 M Na-citrate, pH 7.0) and dried. SYNV-RNA or control RNA (0.5 μg in both cases) was denatured in the presence of 1 M glyoxal and 50% DMSO in 10 mM Na-phosphate buffer, pH 7.0 for 1 h at 50 °C. After cooling on ice the RNA was applied to the

nitrocellulose filters and the RNA was baked at 80 °C for 6 h. The filters were then incubated overnight at 50 °C in 0.2 ml prehybridization buffer consisting of 50% deionized formamide, 5x SSC, 0.3% SDS and 0.5 mg/ml transfer RNA in 50 mM sodium phosphate buffer, pH 7.0. ^{3}H-labeled RNA in prehybridization buffer was denatured at 100 °C for 10 min, rapidly cooled and added to the filters. Hybridization was carried out for 20 h at 50 °C. The filters were washed in 4 changes of 2x SSC, 0.1% SDS at room temperature and with 2 changes of 0.1x SSC, 0.1% SDS at 50°C. The filters were dried and exposed to Kodak RP Royal-X-Omat film at -80 °C.

4.3 RESULTS

4.3.1 Purification of transcribing ribonucleoprotein complexes on phosphocellulose columns

A phosphocellulose column was loaded with a virus sample, which had been treated with 1% NP-40 and 1.0 M NaCl and eluted step-wise with 0.08 M, 0.12 M, 0.22 M, 0.5 M, 1.0 M and 2.0 M KCl or K-acetate. SYNV-specific proteins were eluted with 0.5 M to 1.0 M KCl and 1.0 M to 2.0 M K-acetate as judged by an immunological detection method using ^{125}I-labeled protein A (Fig. 4.1A/B). Two bands, eluting with 0.08 M KCl and with 0.08 M K-acetate were detected at the approximate position of the G protein on a 10% SDS-polyacrylamide gel. Because these bands react with serum against purified SYNV; one of these will be formed by the G protein whereas the second one, migrating faster, may be a partial degraded form of G (Ziemiecki and Peters, 1976b). A protein which reacted aspecifically with anti-SYNV serum was eluted with 0.08 M KCl and with 0.08 M K-acetate (Fig. 4.1A, denoted with an arrow). This band is also found when extracts from mock-inoculated plants were analyzed (see below).
The proteins M1, M2 and X elute together with the N protein from the phosphocellulose column, when the proteins were eluted with either KCl or with K-acetate (Fig. 4.1 A and B). The interaction between the RNP complex and the phosphocellulose column is stronger in the presence of K-acetate (Fig. 4.1B) than with KCl (Fig. 4.1A). In the presence of KCl the RNP complex elutes with 0.5 M and with K-acetate with 1.0 M to 2.0 M. The presence of G and Y protein in fractions eluting from the column with 0.5 M KCl together with the RNP complex suggest that solubilization of G and Y was not fully achieved in the presence of KCl (Fig. 4.1A). A protein with a higher electrophoretic mobility (designated M*) is detected in the fractions 9 to 11 eluted with KCl (Fig. 4.1A) and in the fractions 14 to 16 eluted with K-acetate (Fig. 4.1B). This protein was assumed to be a degradation product of the M2 protein of SYNV as discussed in the previous chapter. It has to be pointed out that M2 appears to be more antigenic in the fractions, eluting from the phosphocellulose column than in purified preparations of SYNV (Fig. 4.1A and B). We assume that by a conformational change in M2, e.g. by exposing more epitopes on the surface of the protein, M2 becomes more antigenic. In Chapter 3 we have argued that the disappearance of an epitope on the surface of the M2 protein may reduce inhibition of *in vitro* transcription of SYNV-RNA.
We could not detect HMW1 protein in any of the fractions, possibly because of the low concentration of this protein in purified preparations of SYNV.
After dialysis to remove salt and detergent, the fractions were inoculated onto *N. glutinosa*

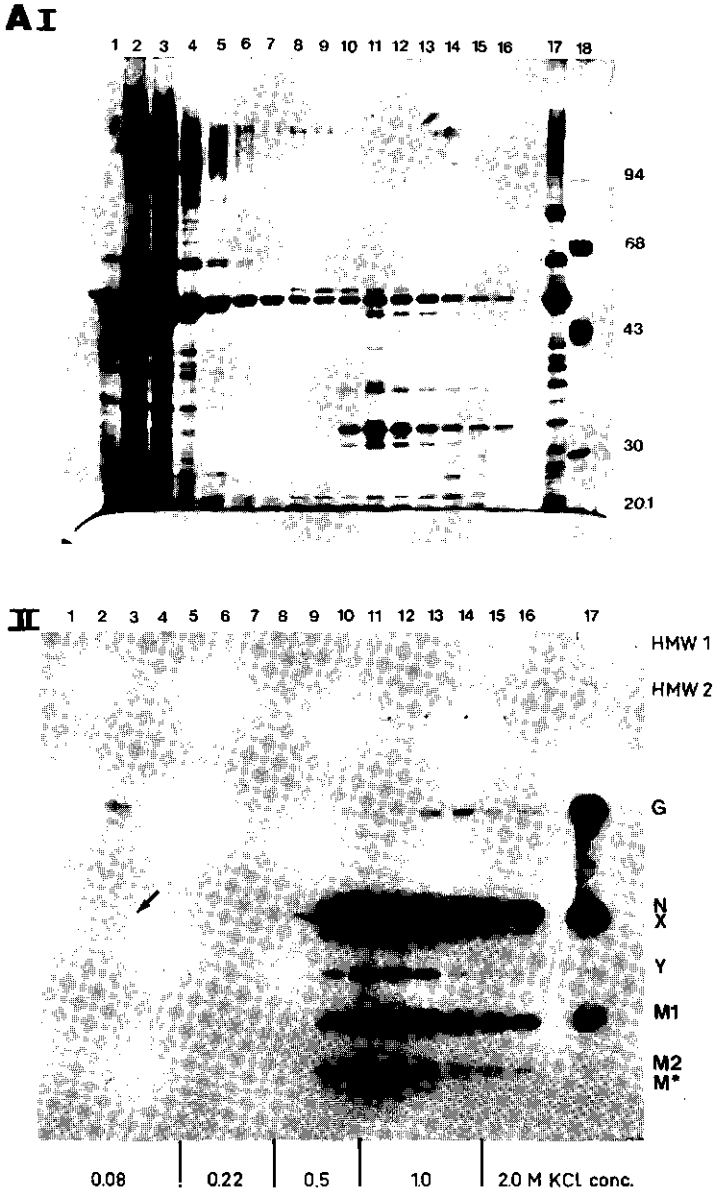


Fig. 4.1. (A)
Analysis of SYNV proteins eluted step-wise from a phosphocellulose column with (A) KCl or (B) K-acetate. SYNV was solubilized with 1% NP-40 and 1.0 M NaCl after Celite filtration and concentration by centrifugation. Proteins were eluted from the phosphocellulose column as described in the text and analyzed on a 10% SDS-polyacrylamide gel. Proteins were visualized with silver (I), or transferred to nitrocellulose filter and incubated with anti-SYNV serum and finally with ^{125}I-labeled protein A. The protein bands were visualized by exposing the filters to X-ray film using an intensifying screen at -80 °C (II).
Lane 1-16 (A I and II; B I and II): proteins present in the various fractions eluted from the phosphocellulose column. Lane 17 (A and B): SYNV before applying to the column. Lane 18: mol. wt. marker proteins. The mol. wt. are presented at the right side of the gel.

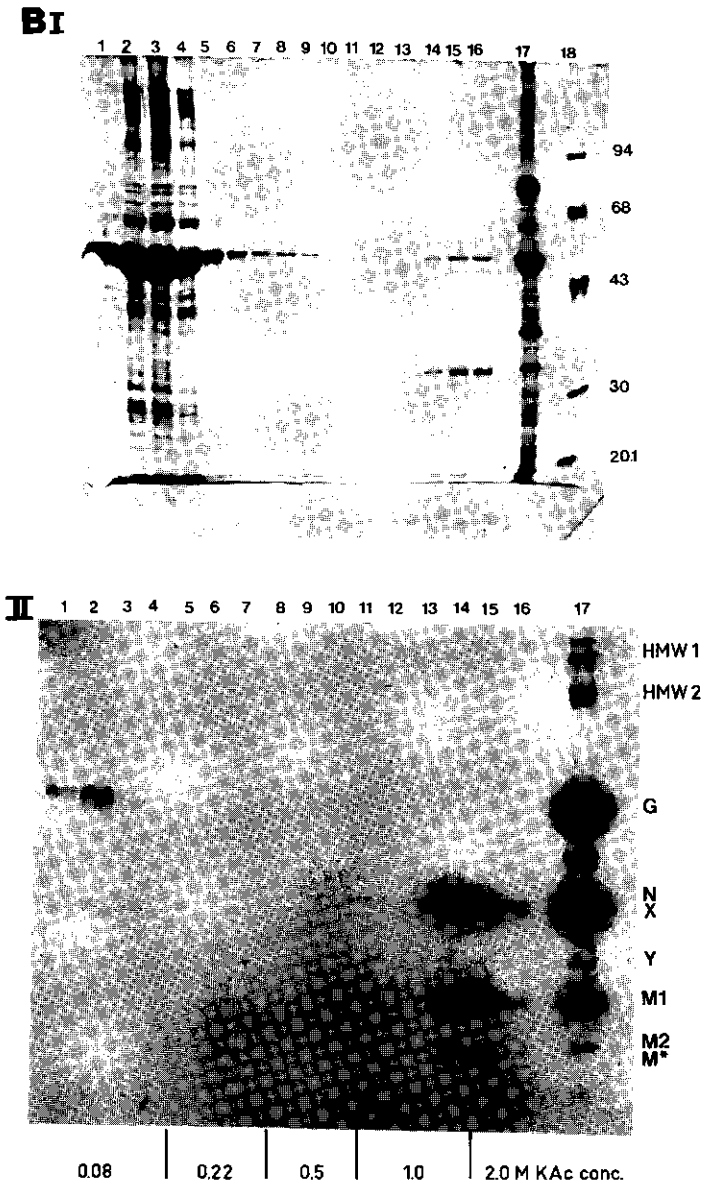


Fig. 4.1. (B)
Analysis of SYNV proteins eluted step-wise from a phosphocellulose column with (A) KCl or (B) K-acetate. SYNV was solubilized with 1% NP-40 and 1.0 M NaCl after Celite filtration and concentration by centrifugation. Proteins were eluted from the phosphocellulose column as described in the text and analyzed on a 10% SDS-polyacrylamide gel. Proteins were visualized with silver (I), or transferred to nitrocellulose filter and incubated with anti-SYNV serum and finally with ^{125}I-labeled protein A. The protein bands were visualized by exposing the filters to X-ray film using an intensifying screen at -80 °C (II).
Lane 1-16 (A I and II; B I and II): proteins present in the various fractions eluted from the phosphocellulose column. Lane 17 (A and B): SYNV before applying to the column. Lane 18: mol. wt. marker proteins. The mol. wt. are presented at the right side of the gel.

plants. Plants inoculated with fractions eluting with low salt concentrations from the phosphocellulose column (fraction 1 to 3) failed to develop the characteristic symptoms of a SYNV infection, while the RNP-containing fractions 10 to 16 (Fig. 4.1A) and 14 to 16 (Fig. 4.1B)-even in a 100-fold dilution-caused 100% infection, suggesting that these fractions contained the transcriptase. Therefore, it is difficult to explain that the HMW1 protein could not be detected in the transcribing RNP fractions.
In a control experiment extracts were preparad from mock-inoculated plants in a similar manner as preparations of purified SYNV and applied to a phosphocellulose column. Proteins were eluted as described for infected material. Fig. 4.2 shows that plant proteins eluted only with 0.08 M KCl. Only the aspecific band, also shown in Fig. 4.1A and B, was detected immunologically with anti-SYNV serum complexed to ^{125}I-labeled protein A, when the gel shown in Fig. 4.2 was transferred to nitrocellulose filters (not shown).
As demonstrated above (Fig. 4.1) a good separation of the M2 and G proteins from the other proteins of SYNV is not achieved. In these experiments SYNV was dissociated with NP-40 prior to the column chromatography. As described in Chapter 2 Zwittergent TM 312 in combination with salt was more efficient in solubilizing the proteins of SYNV. However, the resulting complexes were not infectious and did not exhibit transcriptase activity *in vitro*. Therefore, the separation of SYNV proteins by Zwittergent TM 312 was not studied in using phosphocellulose column chromatography.

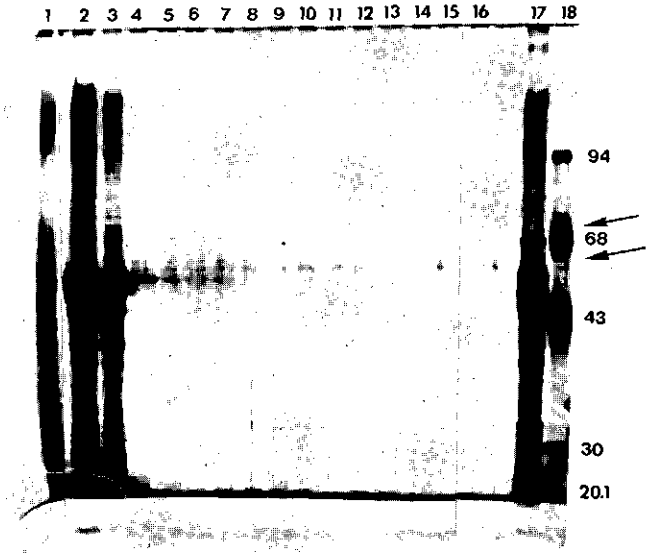


Fig. 4.2.
Analysis of plant proteins eluted from a phosphocellulose column. Material from mock-inoculated *N. glutinosa* plants was filtered through Celite as described for SYNV purification, concentrated by centrifugation and applied to the phosphocellulose column. The proteins were eluted step-wise with 0.08 M to 2.0 M KCl in buffer 'C'. After electrophoresis on a 10% SDS-polyacrylamide gel the proteins were stained with silver. Lane 1-16: proteins present in fractions eluted from the phosphocellulose column at different salt concentrations. Lane 17: proteins present in the starting material. Lane 18: mol.wt. marker proteins. Mol. wt. are presented at the right side of the gel.
The two sets of bands (arrows) in the 68,000-53,000 dalton region are probably artifacts caused by the interaction of 2-mercaptoethanol (present in the Laemmli buffer (Laemmli, 1970)) and skinproteins (Ochs, 1983; and Tasheva and Dessev, 1983).

4.3.2 Properties of the transcribing ribonucleoprotein complex after purification by phosphocellulose column chromatography

Fractions eluting from a phosphocellulose column loaded with solubilized SYNV were tested for host plant and SYNV-RNA polymerase activity. The fractions were assayed for transcriptase activity in the presence or in the absence of plant RNA, respectively (Fig. 4.3). A similar experiment was done with extracts from mock-inoculated plants. [^{3}H]UMP-incorporating activity was mainly detected in fractions, eluted with 0.08 M or with 0.22 M K-acetate and incubated for 60 min with exogenously added plant RNA. This activity was found in fractions from columns loaded with solubilized SYNV as well as in extracts from mock-inoculated plants. The distribution of [^{3}H]UMP-incorporating activity was very similar for extracts from mock-inoculated plants and from solubilized virus, although the amount of TCA-precipitable material differed. In the absence of plant RNA no incorporation was detected in the first 10 fractions. [^{3}H]UMP-incorporating activity was detected in SYNV-infected material eluted with 1.0 M or with 2.0 M K-acetate in the absence of plant RNA. Adding of this RNA slightly decreased the TCA-precipitable counts. No activity was detected in fractions from mock-inoculated plant extracts eluted with 1.0 M or 2.0 M K-acetate. These results show that the [^{3}H]UMP-incorporating activity found in the fractions eluting with 0.08 M, 0.22 M or 0.5 M K-acetate was not SYNV-specific and appeared to be of host origin. The incorporating activity in the fractions eluting with 1.0 M or 2.0 M K-acetate can be considered to be virus-specific, which could be expected from the presence in these fractions of RNP complexes and proteins which may have a function in the transcription (Fig. 4.1B).

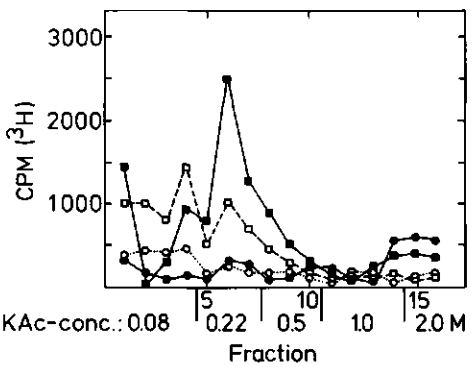
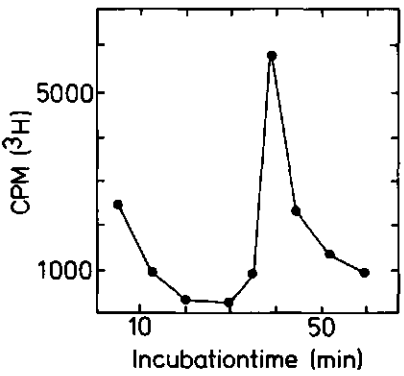


Fig. 4.3.
[^{3}H]UMP-incorporating activity associated with fractions eluted from a phosphocellulose column. Partially purified SYNV was dissociated with 1% NP-40 and applied to a phosphocellulose column, which was eluted step-wise with 0.08 M to 2.0 M K-acetate in buffer 'C'. Mock-inoculated plant material was treated similarly. Fractions of 5 ml were collected, concentrated by dialyzing against a solution saturated with $(NH_4)_2SO_4$ and subsequently dialyzed against 50 mM Na-phosphate buffer, pH 7.4 containing 1 mM EDTA and 10 mM 2-mercaptoethanol. Each fraction (10 μl) was assayed for RNA polymerase activity in the presence of 4 mg/ml plant RNA.
Fractions from a phosphocellulose column, loaded with solubulized SYNV were assayed for transcriptase activity in the presence (■—■) or absence (□—□) of plant RNA for 60 min at 25 °C.
Fractions from a phosphocellulose column, loaded with extracts from mock-inoculated plants were assayed for transcriptase activity in the presence (●—●) or absence (○—○) of plant RNA for 60 min.

The fractions, eluting with 1.0 M and 2.0 M K-acetate from a phosphocellulose column, loaded with solubilized SYNV must contain a virus-associated RNA polymerase. The fractions 14 to 16 which contained virus-specific RNA polymerase activity were pooled and further characterized. These pooled fractions were assayed for transcriptase activity in the absence of plant RNA as a function of the incubation time. An optimal incorporation was obtained within a time-interval of 40 min (Fig. 4.4). The time needed for optimal incorporation varied from one experiment to the other but was consistent for a given

Fig. 4.4.
Time-course of [^{3}H]UMP-incorporating activity. For this experiment the transcribing RNP fractions eluting with 1.0 M and 2.0 M K-acetate from a phosphocellulose column loaded with solubilized SYNV proteins (see Fig. 4.3) were pooled. *In vitro* transcription of the pooled sample (10 μl) was assayed as described in Chapter 3.



preparation. The amount of incorporation found also varied from preparation to preparation, but this may be a reflection of the time of sampling, because incorporation and degradation of [^{3}H]UMP alternate rapidly.
The pooled fractions 14 to 16 were assayed at different temperatures to study the kinetics of incorporation by the RNA polymerase present in this sample (Fig. 4.5). The optimal temperature is 25 °C, which is in agreement with the value found for preparations of purified SYNV (Chapter 3). The graph shows a remarkable, but reproducible fluctuation of [^{3}H]UMP-incorporation as a function of the incubation time. The products synthesized after 5 min are degraded and after 40 min other RNA chains are synthesized which are then rapidly degraded. This fluctuation is not understood, but it might reflect the presence of RNase actvity in these fractions. Linthorst (1982) claims that RNases are tightly bound to phosphocellulose and are not eluted with 0.5 M KCl. Possibly they elute at 1.0 M and 2.0 M K-acetate.
To explain this course of RNA synthesis it is also conceivable that RNA chains occurring in the transcribing RNP complexes, whose synthesis has been initiated *in vivo* are rapidly elongated to a certain length after incubation in the *in vitro* system. A new round of RNA synthesis is then initiated after 25 to 35 min in the *in vitro* transcription reaction, when the

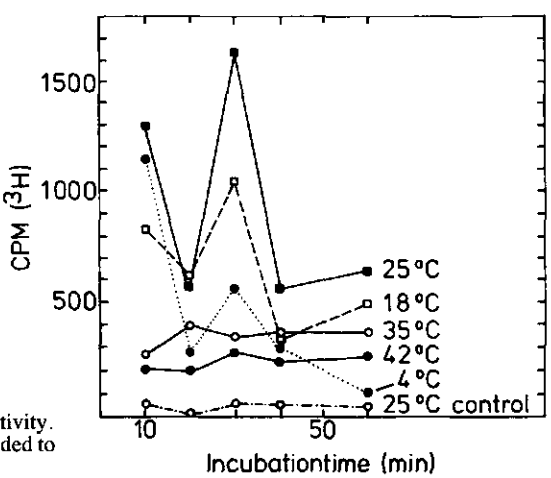


Fig. 4.5.
Effect of temperature on [^{3}H]UMP-incorporating activity. See legend to Fig. 4.4. In the control no virus was added to the *in vitro* transcription assay.

original RNA chain has fallen off from the transcribing RNP complex and will then be degraded by RNases. This mechanism has been proposed for other virus polymerases *in vitro* systems too (Jaspars *et al.*, 1985).
To investigate the occurrence of RNase in these fractions, vanadyl ribonucleoside complex (VRC) was added to inhibit its activity. Fig. 4.6 shows similar fluctuations as those discussed above. The addition of VRC prevents a rapid degradation of product which does occur in the absence of VRC, indicating that RNase contamination is a likely cause for this degradation. This result provides additional evidence that RNA is synthesized in our *in vitro* system. However, VRC did not completely prevent degradation of product which was synthesized at 5 min. As a side effect VRC slows down the transcription reaction. Talib and Hearst (1983) and Perrault and McLear (1984) demonstrated that a longer incubation time was needed for optimal transcription of VSV-RNA *in vitro* in the presence of VRC.
To determine whether the synthesized RNA was specific for SYNV, aliquots from an *in vitro* transcriptase reaction (plus VRC) were removed at different time intervals, extracted with phenol and hybridized to dotblots as described in Chapter 4.2.4. Fig. 4.7 demonstrates that after 5 min the *in vitro* synthesized product hybridizes to SYNV-RNA on the nitrocellulose filter. The degree of hybridization decreased after 15 min and increased again after 50 min. This time scale correlates well with that shown in Fig. 4.6.

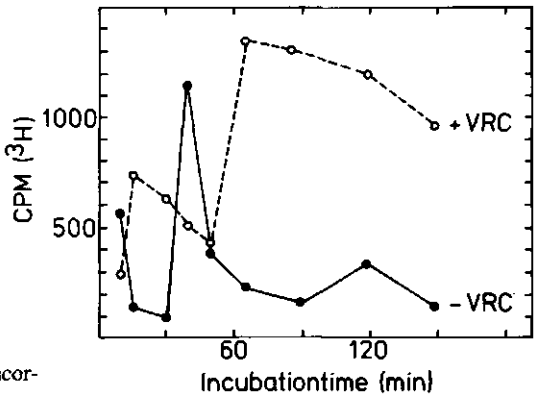


Fig. 4.6.
Effect of the RNase inhibitor VRC on [^{3}H]UMP-incorporating activity. See legend to Fig. 4.4.

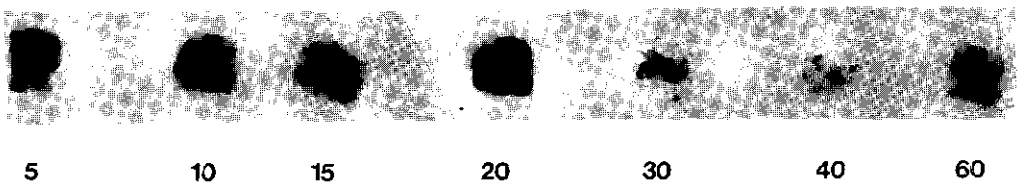


Fig. 4.7.
Dotblot hybridizations of ^{3}H-labeled product to immobilized SYNV-RNA. The transcribing RNP fractions eluting with 1.0 M and 2.0 M K-acetate from a phosphocellulose column loaded with solubilized SYNV proteins were pooled and incubated for the indicated time in a standard transcription assay. Aliquots were removed, deproteinized with phenol and hybridized to 0.5 μg SYNV-RNA as described in Materials and Methods.

4.4 DISCUSSION

SYNV solubilized with 1% NP-40 can be separated on a phosphocellulose column by elution with K-acetate into a fraction containing glycoprotein and an infectious fraction that contains mainly N, X, M1 and M2 proteins and that synthesizes RNA specific for SYNV upon incubation with ribonucleoside triphosphates and Mg-ions. When the M1, M2 and maybe the HMW1 proteins are completely removed as can be achieved with Zwittergent TM 312, no transcriptase activity and infectivity could be detected in the N protein-RNA complex (Chapter 2 and 3). We have previously suggested that M2 is not necessary for transcription and infection, but indeed inhibits transcription *in vitro*. We have not been able to detect the HMW1 protein eluting from the column, possibly because of its low concentrations in the virus. According to the literature on VSV, the L protein (the RNA-dependent RNA polymerase) elutes at 1.0 M salt (De and Banerjee, 1984). However, HMW1 ought to be present in the transcribing RNP fraction since these RNP complexes are infectious. We, therefore, suggest that at least the HMW1, N and X proteins are necessary for transcription of SYNV-RNA.

The preparations used in the transcription experiments described in this chapter differ from the disrupted virus particles, used in transcription experiments described in Chapter 3. It was no longer necessary to incubate the fraction with RNA polymerase activity for prolonged periods of time to obtain incorporation of [^{3}H]UMP. It is of interest to note that M2 protein, which was implied to inhibit transcription of unfractionated SYNV, was changed in some way after elution from the phosphocellulose column. The serum against SYNV reacted strongly with M2 and a protein migrating slightly ahead of M2 (M*), when M2 was chromatographed on a phosphocellulose column, but very weakly when the virus was only disrupted. We suggested that a conformational change resulted in the removal of a fragment which carries an epitope inhibiting transcription of SYNV-RNA. Pal *et al.* (1985a) have suggested this mechanism for the M protein in VSV-RNA transcription *in vitro*.

The kinetics of the incorporation of [^{3}H]UMP in the presence or absence of RNase inhibitor (VRC) at different temperatures, coupled with hybridization data, provided evidence that the fractions eluting at 1.0 M and 2.0 M K-acetate from a phosphocellulose column loaded with dissociated SYNV contained a virus-specific RNA polymerase. An RNase activity appeared to be present in these fractions. In transcription experiments described in Chapter 3 this RNase could not degrade the product RNA, synthesized after 22 h, because the product was either double-stranded. The RNP complexes, however, did not protect the product RNA as shown in this chapter.

CHAPTER 5

ANTIBODY-LINKED-POLYMERASE-ASSAY (ALPA) ON PROTEIN BLOTS TO IDENTIFY THE TRANSCRIPTASE ASSOCIATED WITH SYNV

5.1 INTRODUCTION

A crucial step in the identification of the RNA-dependent RNA polymerase of SYNV is to correlate the enzymatic activity with one or more particular proteins of SYNV.
Methods to identify the L and NS proteins of VSV as components of the transcriptase complex, make use of the fact that the N protein-RNA complex of VSV exhibits no transcriptase activity. By adding purified L and NS proteins, the transcriptase activity can be restored (Emerson and Wagner, 1972). Recently, these results have been confirmed by Thornton *et al.* (1983) and De and Banerjee (1984 and 1985). The transcriptase of VSV was also analyzed by incubating VSV in a transcription mixture in the presence of antiserum against the some VSV proteins. Anti-NS serum immediately terminates the transcription directly after addition to an *in vitro* incubation mixture (Imblum and Wagner, 1975).
Enzyme activity has successfully been correlated with certain proteins by combining the high resolving power of SDS-polyacrylamide gel electrophoresis with an assay *in situ*, following renaturation of the proteins within the gel (Rosenthal and Lacks, 1978; Chang *et al.*, 1982; Spanos and Hubscher, 1983). Alternatively, enzyme activity has been restored after SDS-polyacrylamide gel electrophoresis by renaturing the protein of interest after elution from the gel (Hager and Burgess, 1980). The crucial point in these methods of detecting catalytic activity lies in the successful renaturation of the enzyme that has been subjected to electrophoresis.
A method was developed by Van der Meer *et al.* (1983) in which the product RNAs attached to the transcriptase complex, are visualized (Fig. 5.1). The method is based on a solid-phase 'sandwich' enzyme-immunoassay, recently developed by Muilerman *et al.* (1982) and avoids problems associated with renaturation of a SDS-denatured enzyme prior to the enzymatic reaction and with the spatial separation of subunits which need to be complexed to form an active enzyme. This technique, designated antibody-linked-polymerase-assay (ALPA) has successfully been applied by Van der Meer *et al.* (1983) to identify a 130,000 dalton RNA-dependent RNA polymerase from infected cowpea leaves infected with CPMV.

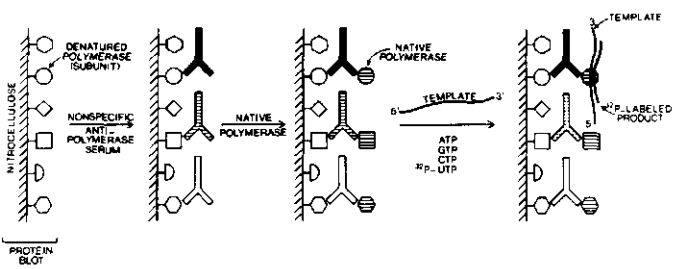


Fig. 5.1.
Schematic representation of the antibody-linked-polymerase-assay (ALPA) (Van der Meer *et al.*, 1983).

Applied to SYNV-RNA-dependent RNA polymerase, the principle of the method is as follows: SYNV proteins are denatured with SDS and separated by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose and then incubated with an excess of poly-specific antiserum raised against SYNV proteins. High antibody concentrations ensure that statistically only one Fab site per IgG molecule is bound to a SYNV protein in the first binding reaction. In the second binding reaction the native, transcribing RNP complexes are attached to the other Fab sites of the IgG molecules, when IgG-specific antigens occur in the RNP complex. Subsequent incubation of the IgG-bound RNP complexes with a complete transcription mixture containing [^{32}P]UTP, precipitation of the nascent ^{32}P-labeled RNA *in situ* with TCA and autoradiography of the nitrocelluose paper strips visualize those proteins which are immunologically related to the RNA polymerase.

5.2 MATERIALS AND METHODS

5.2.1 ALPA

Purified SYNV or a sample of the pooled transcribing RNP fractions eluting from a phosphocellulose column with 1.0 M and 2.0 M K-acetate were denatured in SDS and subjected to electrophoresis on a 10% SDS-polyacrylamide gel at 150 V for approximately 6 h (Laemmli, 1970). After the separation, the proteins were blotted onto nitrocellulose filters, which were processed as described in Chapter 4. The nitrocellulose filters were subsequently incubated with either anti-SYNV or with pre-immune serum, diluted 100-fold in antibody buffer (consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% gelatin and 0.05% NP-40). The filters were then washed extensively with antibody buffer. Strips (0.2 cm wide) of these filters were either incubated with ^{125}I-labeled protein A to detect SYNV-specific proteins or used in ALPA. In the latter case the nitrocellulose strips were equilibrated with ALPA-buffer (containing 40 mM Tris-HCl, pH 7.4, 0.1% NP-40, 2.56 mM 2-mercaptoethanol, 80 mM NaCl and 4 mM $MgCl_2$) in two washings with 25 ml for 30 min each at room temperature. The nitrocellulose strips were then incubated overnight at room temperature in 2.5 ml ALPA-buffer with 10 μg RNP complexes. In an experiment using 10 μg purified SYNV the concentration of the NP-40 in the ALPA-buffer was raised to 1.0% to solubilize the virus.
The strips were washed extensively with ALPA buffer and assayed for antibody-linked transcriptase activity by incubating them in 250 μl of ALPA-buffer, containing 4 μg actinomycin D, 3.2 mM ATP, GTP, CTP and 0.3 mM UTP. Three μCi [alpha-^{32}P]UTP (600 Ci/mmol, New England Nuclear) was used per assay. The reaction was done in sealed plastic bags at 30 °C for appropriate times.
The reaction was terminated by rinsing the nitrocellulose filters with 800 μl ice-cold 12.5% TCA, containing 0.04 M Na-pyrophosphate and 10 μg transfer RNA to precipitate the synthesized RNA *in situ*. The strips were then washed twice with 10% TCA, containing 1% Na-pyrophosphate and five times with 5% TCA, containing 1% Na-pyrophosphate. The supernatant of the reaction to which the stop-solution had been added, was spotted onto Whatman GF/C filters. The nitrocellulose filters were dried at 70 °C and exposed to Kodak RP Royal-X-Omat film at -80 °C, using a DuPont Cronex Lightning Plus intensifying screen.

5.2.2 Antiserum production

Proteins were coupled to dansylchloride (Tijssen and Kurstak, 1979) before SDS-polyacrylamide gel electrophoresis. The protein bands were visualized under UV-light after electrophoresis. The bands were excised from the gel, macerated in a small volume of 0.01 M Tris-HCl buffer, pH 7.4 and left overnight at 4 °C. The polyacrylamide was then removed by centrifugation. The proteins, extracted from the gels, were injected subcutaneously in rabbits after mixing with an equal volume of Freund's incomplete adjuvant. The protein concentration was 50-100 μg. Two weeks later the rabbits were again injected and two to three weeks thereafter bled from the ear.

5.3 RESULTS AND DISCUSSION

Since attempts to renature the putative RNA-dependent RNA polymerase of SYNV *in situ* after SDS-polyacrylamide gel electrophoresis were unsuccessful (unpublished results) we applied the ALPA technique.
In an ALPA experiment using pooled transcribing RNP fractions, eluted from a phosphocellulose column with 1.0 M and 2.0 M K-acetate, two bands were revealed. One at the position of the HMW1 protein and one at the position of the N protein of SYNV, respectively (Fig. 5.2, lane 2). The position of these proteins on the nitrocellulose strip is

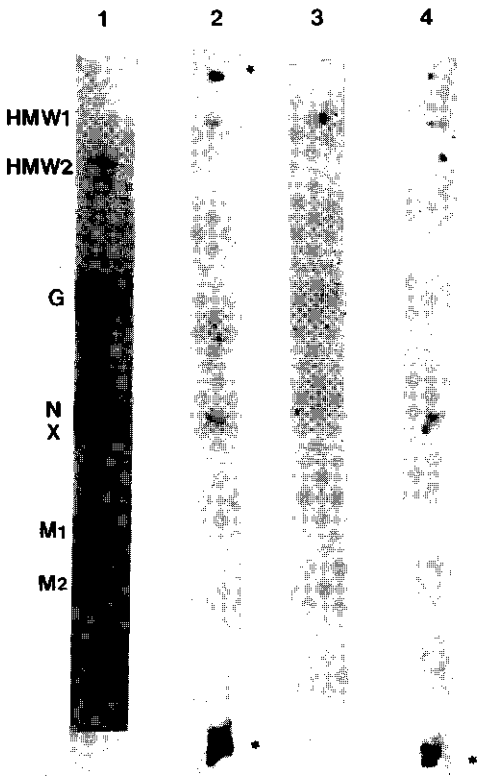


Fig. 5.2.
Detection of SYNV RNA-dependent RNA polymerase by ALPA. Purified SYNV was electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose filter which was cut into strips. The strips were assayed as indicated and exposed to X-ray film. Lane 1 was incubated with anti-SYNV serum and ^{125}I-labeled protein A. Lane 2, 3 and 4 were incubated with anti-SYNV serum, followed by incubation with the pooled transcribing RNP fractions from a phosphocellulose column which were eluted with 1.0 M and 2.0 M K-acetate (see Fig. 4.3) (lane 2) or with material, purified as SYNV from mock-inoculated plants (lane 3) or with purified SYNV, which had been dissociated with 1% NP-40 (lane 4). Lane 2 and 3 were then assayed for antibody-linked polymerase activity for 60 min and lane 4 for 24 h. Lane 3 was incubated in the presence of plant RNA. Dots denote aggregates or fragments of proteins with RNA polymerase activity.

identical to their position on a SYNV protein blot incubated with anti-SYNV serum and subsequently with ^{125}I-labeled protein A (Fig. 5.2, lane 1). In addition two other bands, which are not detected with ^{125}I-labeled protein A, are found in the ALPA; one at the top and one at the bottom as marked by dots in Fig. 5.2, lane 2. The one at the top may be composed of aggregated proteins and the other of protein fragments. Once the IgGs have attached to these fragments or aggregates, ALPA cannot discriminate between them and SYNV proteins which are functional in the transcription. Therefore, we have to keep in mind that a band consisting of radioactive product can also appear at the site of otherwise functionally inactive protein fragments or aggregates (Miassod and Got, 1984).
These results show that ALPA can be used to detect proteins which play a role in the transcription of SYNV-RNA, since the N and HMW1 protein are assumed to have a function in infectious and transcribing RNP complexes.
We have shown in Chapter 3 that prolonged incubation is necessary to activate the transcriptase associated with purified preparations of SYNV while in Chapter 4 we have demonstrated transcriptase activity within 60 min using transcribing RNP complexes purified on phosphocellulose columns after solubilization of SYNV. To examine the effect of prolonged incubations of purified preparations of SYNV on transcriptase activity, an ALPA was done utilizing a protein blot which had been incubated with anti-SYNV serum, and subsequently with purified and solubilized SYNV, instead of transcribing RNP complexes obtained from phosphocellulose columns. After precipitating the product RNA *in situ* with TCA we could not detect a band after a 60 min incubation. However, after incubating the strip for 24 h we could detect bands at the position of the HMW1 and N proteins (Fig. 5.2, lane 4). This again shows that an inhibitory activity has to be removed and is in agreement with the observation made earlier. As explained, M2 may remain associated with the RNP complex at the HMW1 and N protein position, thereby inhibiting transcription at these positions until the M2 protein has been degraded (see also Chapter 3). Using transcribing RNP complexes instead of intact SYNV, the M2 protein has already been modified during the phosphocellulose column chromatography at room temperature, so the transcription reaction is not delayed and we demonstrated bands at the positions of the HMW1 and N proteins after 60 min incubation. Again the results demonstrate the difference in transcription rate between SYNV with M2 protein and RNP complexes with modified M2.
No bands at the position of the G, X, M1 and M2 proteins were detected (Fig. 5.2, lane 4). We have already shown that the G protein is not involved in transcription of SYNV-RNA. The degradation or conformational changes of the M2 protein may cause that the transcribing RNP complex dissociates from the M2 protein and so also from the IgG at the M2 protein position on the filter; therefore no band becomes visible at the M2 position after prolonged incubation. We cannot explain the absence of bands at the position of the X and M1 proteins. It is possible that the IgGs at the position of the X and M1 proteins inhibit transcription of the RNP complex attached to those IgGs, analogous to the inhibition of transcription of VSV-RNA by anti-NS serum (Imblum and Wagner, 1975). When the ALPA was executed with pre-immune serum no bands were detected under the conditions described above for SYNV or transcribing RNP complexes derived from SYNV, indicating that virus-specific IgGs were required to visualize the bands discussed above (results not shown).
In an experiment designed to detect the presence of host plant polymerase activity,

exogenous RNA was added in the final step of the ALPA with material, extracted in a similar manner as SYNV from mock-inoculated plants (Fig. 5.2, lane 3). However, no activity of any polymerase could be detected. This negative result also excludes the possibility that any of the 'ALPA-positive' bands merely reflect adventitious binding of [^{32}P]UTP to protein.

To establish more firmly that the N protein of SYNV was not the transcriptase itself, we attempted to mask the N protein site on the nitrocellulose strip. After incubating a strip with anti-SYNV serum and with transcribing RNP complexes, the strip was incubated for 4 h at room temperature in antibody buffer containing an excess of mono-specific serum directed against the N protein. Fig. 5.3, lane 2 shows a band at the position of the N protein which is enhanced after treatment with anti-N serum (Fig. 5.3, lane 3). The stimulation of transcriptase activity found after application of the anti-N serum can be explained by a change in the conformation of N protein. This may cause that the N protein can be detached more easily from the template. We cannot exclude the possibility that the excess of anti-N serum itself can cause the detachment of the N protein from the template. In both explanations this allows precursor ribonucleoside triphosphates and the transcriptase a better access to the template. The result of the experiment shown in Fig. 5.3 is in agreement with data obtained by adding anti-N serum to a transcription assay containing purified SYNV. Anti-N but not anti-G serum stimulated the reaction 6-fold (from 5,500 cpm of [^{32}P]UMP incorporation to 31,100 cpm after a 20 h incubation of purified SYNV).

TCA-precipitable material was also present at the position of the X protein (Fig. 5.3, lane 3). Anti-N serum would promote the action of X in the transcription by lifting the N protein from the template. This process compensates for the apparent inhibition of the X protein by

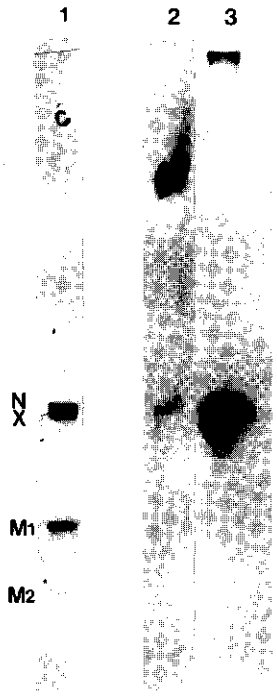


Fig. 5.3.
Detection of RNA-dependent RNA polymerase by ALPA. In this experiment a sample of the pooled transcribing RNP fractions which were eluted from a phosphocellulose column with 1.0 M and 2.0 M K-acetate was electrophoresed on a SDS-polyacrylamide gel instead of complete SYNV. Nitrocellulose strips were treated as described in Fig. 5.2. Lane 1 was incubated with anti-SYNV serum and ^{125}I-labeled protein A. Lane 2 and 3 were incubated with anti-SYNV serum, followed by incubation with the phosphocellulose column purified transcribing SYNV RNP complex. The strips were then assayed for antibody-linked polymerase activity for 60 min. Lane 3 was preincubated with anti-N serum for 4 h at 25 °C before transcription was assayed *in vitro*.

IgGs specific for the X protein and thereby activates the transcriptase of SYNV (HMW1 protein). When the transcribing RNP complexes bind to the X protein, it is conceivable that these complexes also bind to M1 protein. We have suggested in previous chapters that M1 and X are two forms of a protein, analogous to the NS1 and NS2 form of the NS protein of VSV, which differ in the degree of phosphorylation. Because the X protein is phosphorylated to a higher degree than M1 it would have more affinity for the RNP complex than M1 under the conditions of *in vitro* transcription (Bell *et al.*, 1984). Because M1 has less affinity for the RNP complex compared with the X protein no band was detected at the position of the M1 protein, apparently due to the detachment of the transcribing RNP complex from this position during the 4 h incubation with anti-N serum in antibody buffer.
Incubation of the strips with anti-G serum after application of the transcribing RNP complexes did not change the results already obtained with ALPA. We did not have mono-specific anti-HMW1, anti-M1 or anti-M2 serum to test for their role in the transcription of SYNV-RNA.
The results of these experiments suggest that the N protein is not a transcriptase, because anti-N serum would inhibit the N protein. We do not understand why no band was detected at the position of the HMW1 protein in this experiment. Van der Meer *et al.* (1983) reported that the newly synthesized RNA in ALPA dissociates from the template into the incubation mixture. We tested this by precipitating ^{32}P-labeled material from the incubation mixture after removing the nitrocellulose filter and we could indeed precipitate radioactive material from this incubation mixture. This indicates that we had not terminated the transcription reaction early enough. However, we were not able to demonstrate from which position on the filter this ^{32}P-labeled material was released.
In conclusion our results have provided evidence that the HMW1, N, and X proteins are necessary for transcription of SYNV-RNA. The HMW1 protein of SYNV is therefore the equivalent of the L protein of VSV.

SUMMARY AND GENERAL DISCUSSION

The aim of the investigation presented in this thesis was to elucidate the nature of the RNA-dependent RNA polymerase, thought to be associated with *Sonchus* yellow net virus (SYNV), a rhabdovirus infecting plants. This research was initiated to shed light on the transcription activity in rhabdoviruses with similarities to rabies virus. It has been difficult to detect RNA polymerase activity in rabies virus particles in contrast to VSV with its highly active RNA-dependent RNA polymerase.

The proteins of SYNV and their location in the virus particle are discussed in Chapter 2. We detected four major proteins, designated G, N, M1 and M2 in purified preparations of SYNV, which were separated on a 10% SDS-polyacrylamide gel. Minor proteins HMW1, HMW2, X and Y were usually also detected. To understand more about the interactions of SYNV and the host plant cell, it is important to determine which virus proteins are associated with the transcribing ribonucleoprotein (RNP) complex, which is infectious.

We propose a model for the transcription of SYNV-RNA in which the single-stranded RNA with a negative polarity forms the transcribing RNP complex with HMW1 (RNA-dependent RNA polymerase), N, X and M1 proteins. The N protein-RNA complex serves as a template for the virus-associated RNA-dependent RNA polymerase. The X protein is assumed to be a form of the M1 protein which is phosphorylated to a different degree than M1. The M2 protein which forms a bridge between the G protein in the envelope and the RNP complex, may inhibit transcription.

In Chapter 3 we showed, that an enzyme is associated with purified SYNV, which has the ability to catalyze the polymerization of ribonucleoside triphosphates.

The RNA polymerase showed low activity after a 60 min incubation of purified SYNV. Prolonged incubation of purified SYNV considerably enhanced SYNV-specific [^{3}H]UMP-incorporation. The results obtained by analysis of the protein composition of the reaction mixture during these prolonged incubations shows that the M1 and M2 proteins require modification before transcription could proceed.

It is tempting to hypothesize that phosphorylation of one of the SYNV proteins, equivalent in function to the NS protein of VSV is necessary for efficient transcription of SYNV-RNA. In Chapter 3 we showed that the decrease in M1 protein corresponds to an increase in X protein. During chromatography of disrupted viruses on phosphocellulose columns, discussed in Chapter 4, the RNP complex was enriched in X protein when compared with the amount of X protein in purified preparations of SYNV. The lagtime in transcription was considerably reduced after eluting solubilized SYNV from a phosphocellulose column with KCl or K-acetate. The results of an experiment discussed in Chapter 5 has been interpreted as evidence that the X protein is analogous to the NS protein of VSV. The X protein is specifically inhibited by its own IgGs in an ALPA and this inhibition can be eliminated by incubating with anti-N serum which apparently mimicks the action of X by lifting the N protein from the template RNA, thereby activating the RNA polymerase. Kingsford and Emerson (1980) have shown that phosphorylation of the NS protein of VSV is necessary for transcription of VSV-RNA. This phosphorylation takes place in our SYNV system during incubation and activates a protein. Changes in degree of phosphorylation is reflected in the increasing amounts of X1 during the prolonged incubations as shown by SDS-polyacrylamide gel electrophoresis (Chapter 3). Van Beek *et al.* (1985) reported that the M1 protein and sometimes a protein with a mol. wt. similar to the X protein of SYNV

are phosphorylated in cowpea protoplasts infected with SYNV.
Our results indicated an inhibitory role of the M2 protein. A regulatory role may be ascribed to the M2 protein, because we have shown in Chapter 3, that M2 protein is degraded to discrete products (M* and M**), concomitant with an increase in transcriptase activity *in vitro*. In experiments using phosphocellulose column purified RNP complexes the M2 protein of SYNV appeared to be modified to a more antigenic form and no longer inhibited transcription *in vitro*. Inhibition of transcription by the M protein of VSV has been reported by Carroll and Wagner (1979) and is now widely accepted for VSV. Pal *et al.* (1985a) recently showed that the exposure of only one epitope on the surface of the M protein of VSV may be important for the inhibition of *in vitro* transcription of VSV-RNA. In this thesis the importance of the M2 protein of SYNV in regulating transcription *in vitro* is stressed. The regulatory role of the M protein of VSV in *in vivo* and *in vitro* transcription was further investigated by Rosen *et al.* (1983). The M protein of VSV can be cleaved *in vivo* and *in vitro* by a protease to a discrete product (M'). The M' protein could be involved in regulating the transcription of VSV in a positive manner, while the M protein inhibits transcription. Whether the products of M2 had any role in the transcription of SYNV-RNA was not studied.
To establish that the HMW1 protein of SYNV is the RNA-dependent RNA polymerase associated with SYNV, an ALPA was used in which transcribing RNP complexes are immobilized on nitrocellulose via an IgG bridge between SDS-denatured, electrophoretically separated SYNV proteins and the RNP complex. The polymerase activity is detected by incubating the nitrocellulose filters in a transcription mixture containing the ribonucleoside triphosphates. The newly synthesized RNA is then precipitated onto the nitrocellulose and visualized by autoradiography. Using this approach we provided evidence that the HMW1 protein of SYNV exhibits polymerase activity.
To prove that the *in vitro* synthesized RNA was specific for SYNV, a direct and an indirect approach was used. Dotblot hybridizations demonstrated that SYNV-RNA was transcribed into complementary RNA. In a coupled *in vitro* transcription-translation experiment it was shown that the SYNV-associated polymerase was capable of elongating RNA into full-length mRNAs, which were translated into the major SYNV proteins in a wheat germ cell-free system.
It proved difficult to visualize the product RNAs on denaturing gels. Many bands were found (not shown), indicating random initiation or premature termination of the polymerase on the SYNV template. Perrault and McLear (1984) have demonstrated that the VSV RNA polymerase often aborts the transcription of VSV-RNA *in vitro*. It is of interest to note that Thornton *et al.* (1984) reported that the M protein of VSV can abolish the transcription of the leader RNA whereas increasing amounts of fragments with 11 to 14 nucleotides transcribed from the 3' end of the N-mRNA gene are found. This has been explained by a M protein-mediated inhibition at the level of leader RNA synthesis and elongation.
One of the major problems in studying plant virus replication is the occurrence of a host-encoded RNA-dependent RNA polymerase in healthy plants which is usually enhanced upon infection of the plant (Dorssers, 1983; Dorssers *et al.*, 1982 and 1983; and Hall *et al.*, 1982). The results obtained with the phosphocellulose-purified RNP particle of SYNV did not indicate the presence of contaminating host polymerases in that fraction, but rather in fractions eluting at lower salt concentrations, which is in agreement with the results obtained by Mouches *et al.* (1984), who eluted the host RNA polymerase at low salt

concentrations, while the turnip yellow mosaic virus-induced RNA polymerase elutes at higher salt concentrations. Furthermore the polymerase of rhabdoviruses requires an N protein-RNA complex as template and it is inconceivable that a host plant RNA-dependent RNA polymerase would also accept this template. We conclude therefore that a host RNA-dependent RNA polymerase did not play a significant role in our investigations concerning the *in vitro* transcription of SYNV-RNA.

In conclusion, we have provided evidence that the HMW1 protein of SYNV in possible combination with the M1 protein, phosphorylated to a different degree, can transcribe protein-RNA complexes *in vitro* when the inhibitory M2 protein has been modified.

SAMENVATTING

In deze samenvatting zal ik proberen de inhoud van dit proefschrift uit te leggen op een manier die ook voor niet-ingewijden te begrijpen is.
Het *Sonchus* yellow net virus (SYNV) is een virus dat in de Verenigde Staten van Amerika in de natuur op melkdistel gevonden kan worden. Na besmetting van de plant door een bladluis, die in Europa niet voorkomt, krijgen de nerven op de bladeren een gele kleur, waar de Engelse naam op duidt. Het virus hebben we geen Nederlandse naam gegeven omdat het niet in Nederland voorkomt.
Het virus behoort tot de groep van de rhabdovirussen die of dieren of planten kunnen besmetten. Een bekend virus dat dieren kan besmetten en erg gevaarlijk is voor de mens, is het hondsdolheidsvirus (rabies virus).
Rhabdovirussen hebben een bacilvormig uiterlijk en bezitten een gekompliceerde struktuur. Bij bestudering van het virus met de elektronenmikroskoop zien wij in het virusdeeltje een kogelvormige struktuur (nucleocapside), die uit een opgewonden draad van ribonucleinezuur (RNA) en eiwit bestaat. Dit RNA is het erfelijk materiaal van het virus en het voornaamste eiwit in dat nucleocapside (N eiwit) beschermt het RNA. Behalve dit N eiwit bevat de nucleocapside nog een of twee eiwitten. Deze eiwitten komen in kleine hoeveelheden in het virus voor en hebben een funktie bij de vermeerdering van het RNA na de infektie in de cel. De nucleocapside wordt door een membraan omgeven waarop aan de buitenkant eiwitten (G eiwit) zijn ingeplant. Aan de binnenkant bevindt zich op het membraan nog een eiwit (M2 eiwit) dat een verbinding vormt tussen het G eiwit en het N eiwit en waardoor het virus zijn vorm houdt.
De rhabdovirussen kunnen in principe in twee groepen verdeeld worden. De vermeerdering van virussen in de ene groep, met name vesicular stomatitis virus, is uitvoerig bestudeerd, terwijl studies over de vermeerdering van virussen uit de andere groep (rabies virus bijvoorbeeld) schaars zijn vanwege de moeilijkheden bij deze virussen, die bij de bestudering van de vermeerdering ontmoet worden en waarover hieronder meer verteld zal worden. SYNV behoort namelijk tot die tweede groep. Omdat dit virus relatief gemakkelijk op gezonde tabaksplanten is over te brengen en eenvoudig te zuiveren is, besloten wij een studie te maken over de eerste stap van de vermeerdering van het RNA (transkriptie geheten) en een analyse te maken van de virus eiwitten die bij deze transkriptie betrokken waren. De resultaten van dit onderzoek, dat uiteindelijk veel moeilijker bleek te zijn dan verwacht, zijn in dit proefschrift beschreven.
In hoofdstuk 2 behandel ik de zuivering van het virus uit besmette planten en worden er een aantal eigenschappen van de virusdeeltjes, de eiwitten en het RNA beschreven. Deze eigenschappen worden bovendien vergeleken met die, die in de literatuur al bekend waren. Deze eigenschappen bleken namelijk van belang te zijn voor het verdere onderzoek.
In hoofdstuk 3 staat beschreven dat bij gebruik van gezuiverd virus slechts weinig transkriptie van RNA gemeten kon worden indien het reactie mengsel voor een korte periode (1 uur) geïnkubeerd werd. Daarentegen werd er veel transkriptase aktiviteit gevonden wanneer het mengsel lange tijd (22 uur) werd geïnkubeerd. Het nieuw gemaakte RNA bleek alleen door het virus gemaakt te worden. In experimenten werd aangetoond dat bepaalde eiwitten veranderd moesten worden, alvorens het virus met de transkriptie van RNA in de reageerbuis kon beginnen. Het bleek namelijk dat een van de eiwitten (M2 eiwit) de transkriptie sterk remde en dat dit eiwit eerst afgebroken moest worden. Een tweede eiwit (M1 eiwit)

moest echter, ongeveer tegelijkertijd, geaktiveerd worden en wel vermoedelijk door het toevoegen van fosfaat groepen, alvorens het transkriptie proces door kon gaan.
In het vierde hoodstuk worden pogingen beschreven om het eiwit dat de transkriptie remt te verwijderen van de nucleocapsiden. Dit gebeurde door het virus eerst uit elkaar te laten vallen met zepen en zouten, en daarna te fraktioneren met behulp van kolomchromatografie. Er werd verwacht dat bij de chromatografie stap het remmende eiwit (M2 eiwit) gescheiden zou worden van de nucleocapsiden, die de transkriptie uitvoeren. Dit werd echter slechts ten dele bereikt, want slechts een gedeelte van het M2 eiwit bleek verwijderd te zijn. Wel werd enige transkriptie gevonden na een korte inkubatie (1 uur) in fracties die het nucleocapside bevatten. Dit wees erop dat de remming enigermate opgeheven was, bij het verwijderen van M2 eiwit.
Hoofdstuk 5 beschrijft een methode, die onlangs door andere onderzoekers is ontwikkeld om de eiwitten aan te wijzen, die een rol spelen bij de transkriptie van RNA. Ik heb dit gedaan met behulp van antilichamen die in een konijn tegen het virus zijn gemaakt. In deze techniek worden de eiwitten na scheiding op een gel overgebracht op nitrocellulose papier, dat daarna behandeld wordt met een overmaat aan antilichamen, zodat de nucleocapsiden kunnen reageren met die antilichamen die korresponderen met de eiwitten op de nucleocapside. Na opnieuw toevoegen van nucleocapsiden en inkuberen met de benodigde komponenten in het reaktiemengsel, kon daarna bepaald worden welke eiwitten aan het transkriptie proces meedoen. Op deze wijze heb ik inderdaad kunnen bepalen dat een eiwit met een hoog molekuul gewicht (L eiwit), het N eiwit en het eiwit dat waarschijnlijk van fosfaat groepen voorzien moest worden (M1 eiwit), aan de transkriptie meedoen.

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CURRICULUM VITAE

Ik ben geboren op 20 maart 1953 in Palembang (Indonesie). In 1972 behaalde ik het HBS-B diploma aan de Van Oldenbarnevelt Scholengemeenschap te Rotterdam en begon ik mijn studie aan de Landbouwhogeschool te Wageningen in de richting Biologie. Na een aantal jaren specialiseerde ik mij in de celbiologische richting. In 1978 behaalde ik mijn Kandidaats-examen en in 1979 volgde het doktoraal examen in de Biologie met als hoofdvak Moleculaire Biologie bij Prof. Dr. A. Van Kammen en de bijvakken Virologie bij Dr. J. M. Vlak, Plantenfysiologie bij Dr. L. C. Van Loon, Wijsbegeerte bij Prof. A. Meyer.
Vanaf 13 december 1979 tot juni 1983 was ik als wetenschappelijk assistent in dienst van de Landbouwhogeschool te Wageningen. Mijn promotie onderzoek bij de Vakgroep Virologie mondde uit in dit proefschrift.
Vanaf januari 1984 ben ik werkzaam op het Boyce Thompson Institute for Plant Research at Cornell University in Ithaca, New York, waar ik bij Dr. H. A. Wood onderzoek doe naar de moleculaire interactie van baculovirussen met insektecellen.