ON THE EXPRESSION STRATEGY OF THE TOSPOVIRAL GENOME

Frank van Poelwijk

Promotor: Dr. R.W. Goldbach
Hoogleraar in de Virologie

Co-promotor: Dr. D. Peters
Universitair hoofddocent
Vakgroep Virologie

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Frank van Poelwijk

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1. Tospovirussen en tenuivirussen zijn via onafhankelijke wegen het plantenrijk binnengedrongen.

   Dit proefschrift.

2. De conclusie van Yamada *et al.* dat de ribozym activiteit versterkt wordt door fusie van het ribozyme met het Rev Responsive Element wordt niet onderbouwd door experimentele gegevens.

   Yamada *et al.* (1996). A chimeric human imunnodeficiency virus type 1 (HIV-1) minimal Rev Response Element-ribozyme molecule exhibits dual antiviral function and inhibits cell-cell transmission of HIV-1. Journal of Virology 70, 1596-1601.

3. Het gebruik van de term "coat protein-mediated resistance" voor bescherming van planten op basis van het nucleocapside-gen van het tomatebronsvlekkenvirus is onjuist.

   Pang *et al.* (1996). Post-transcriptional silencing and consequent tospovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. Plant Journal 9, 899-909.

4. De bewering van Faretra *et al.* dat het voorkomen van beide "mating" type allelen in isolaten van *Botrytis cinerea* wordt veroorzaakt door heterokaryose dient ondersteund te worden door experimenten met moleculaire merkers.

   Faretra *et al.* (1996). Genetic studies of the phytopathogenic fungus *Botryotina fuckeliana* (Botrytis cinerea) by analysis of ordered tetrads. Mycological Research 100, 620-6240.

5. Het is niet duidelijk op grond van welke criteria de International Committee on Taxonomy of Viruses nucleopolyhedrovirussen heeft onderverdeeld in soorten (species) en mogelijke soorten (tentative species)

   Sixth Report of the International Committee on Taxonomy of Viruses (Murphy *et al.*) 1995. Springer Verlag, Wenen, p. 104-113 en p. 526-527.

6. De bewering van Windisch *et al.* dat zij als eersten een uitgebreide biochemische karakterisering van de RNase-activiteit van het envelop eiwit E^{rns} van het varkenspestvirus beschrijven geeft blijk van onvoldoende kennis van de vakliteratuur.

   Windisch *et al.* (1996). RNase of classical swine fever virus: biochemical characterization and inhibition by virus-neutralizing monoclonal antibodies. Journal of Virology 70, 352-358.

Hulst *et al.* (1994). Glycoprotein E2 of classical swine fever virus: Expression in insect cells and identification as a ribonuclease. Virology 200, 558-565.

7. De toegevoegde waarde van een "marker vaccin" bij een gerichte eradicatie van infectieziekten komt alleen tot zijn recht indien een bij het vaccin behorende diagnostische test kan worden ontwikkeld die specifiek de geïnfecteerde dieren detecteert in een met het marker vaccin gevaccineerde populatie.

8. Het grote aantal "kits" waarover men tegenwoordig kan beschikken geeft al aan dat niet iedere zogeheten standaard moleculair biologische techniek ook standaard werkt.

9. Het op grote schaal vernietigen van het regenwoud leidt tot het openen van een nieuwe (virologische) doos van Pandora.

10. De uitdrukking "dromen zijn bedrog" moet, na het behalen van de gouden olympische medaille door de Nederlandse herenvolleybalploeg in Atlanta, grondig worden herzien.

11. Het is te hopen dat kabelexploitant A2000 zich bij de programmering van het pakket, dat naast Sport 7 ook soft porno zal bevatten, zal laten leiden door het motto: "geen sex voor de wedstrijd"!

12. Het groene boekje is zo groen niet meer.
NRC Handelsblad, 8 augustus 1996.

13. Wat veelbelovend lijkt moet je veelbelovend laten.

Stellingen behorend bij het proefschrift:

On the expression of the tospoviral genome

Wageningen, 20 september 1996 Frank van Poelwijk

Voor mijn ouders

Voorwoord

Niemand schrijft zijn eigen proefschrift alleen..... Dat dit waar is moge duidelijk worden uit de onderstaande lijst van personen die ik wil bedanken voor hun bijdrage aan de totstandkoming van dit proefschrift. Allereerst wil ik Rob Goldbach en Dick Peters bedanken voor hun begeleiding en kritische reflectie. Van onschatbare waarde waren natuurlijk de Tospovirologen: Alice, Antonio, Axel, Cor, Erwin, Fennet, Hanke, Kit, Marc, Marcel (De Kroonprins), Marjolein, Peter, Renato, Richard en Tatsuya. Ook wil ik de (ex) studenten Bart v/d Beek, Kees Frijters, Marco Gielkens, Joost Kolkman, Martijn Nawijn, Rob Oosterling, Theo Prins, Inge Tas en Sebastiaan Verduin bedanken.

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Anita en Nicole zou ik op een meer gepaste wijze hebben willen bedanken ware het niet dat dit proefschrift in de weg zat.

Mijn paranimfen Harold "love" en Marcel "Al", die (nog!) tot mijn beste vrienden behoren, wil ik bedanken "for being there" toen ik ze nodig had.

Daarnaast gaat mijn dank uit naar mijn ouders omdat ik daar altijd terecht kon (de VN kan wat dat betreft aan het begrip "safe haven" nog een puntje zuigen) en vooral ook naar mijn "grote" zus Anna voor de morele steun (en dead-line herinnering) tijdens de dipjes.

Last but not least natuurlijk Ineke, die mij alle tijd en rust heeft gegeven om mijn proefschrift af te ronden toen ik het zo nodig had.....

CONTENTS

CHAPTER 1

Introduction

Tomato spotted wilt virus

Based on molecular analysis previously performed by De Haan (1991) and Kormelink (1994) tomato spotted wilt virus (TSWV) has now been recognized as a plant-infecting member of the family *Bunyaviridae* (Murphy *et al.*, 1995). The virus has a wide host range spanning over 650 plant species belonging to 72 botanical families (Cho *et al.*, 1987; Matthews, 1982; Goldbach and Peters, 1994). TSWV is exclusively transmitted by thrips (*Thysanoptera: Thripidae*) in a propagative manner (Wijkamp *et al.*, 1993).

TSWV consists of spherical enveloped particles, ranging in diameter from 70-110 nm, covered with spikes. Purified virus preparations contain four structural proteins, i.e. a large protein of more than 200 kDa, present in minor amounts, two (envelope) glycoproteins of 78 kDa (G1) and 58 kDa (G2a) and the nucleocapsid (N) protein of 29 kDa (Mohamed *et al.*, 1973; Tas *et al.*, 1977a). Often a fifth protein of 52 kDa is observed which is believed to be derived from the 58 kDa (G2a) protein and therefore dubbed G2b (Tas *et al.*, 1977b).

The genome of TSWV consists of three single stranded RNA segments, denoted S (small) RNA, M (medium) RNA and L (large) RNA, which are tightly wrapped with the N protein, forming pseudo-circular nucleocapsid structures (Van den Hurk *et al.*, 1977; Mohamed, 1981; De Haan *et al.*, 1989). Isolated RNA of TSWV is not infectious and does not contain poly-(A) sequences (Verkleij *et al.*, 1982). The complete nucleotide (nt) sequence of the genome of TSWV isolate BR-01 has been elucidated (De Haan *et al.*, 1989; De Haan *et al.*, 1990; De Haan *et al.*, 1991; Kormelink *et al.*, 1992c), and revealed that TSWV has a unique genome organization among plant viruses (Fig. 1). The S and M RNA display an ambisense gene arrangement, each containing two open reading frames (ORFs). The S RNA (2916 nt) encodes a non-structural protein of 52.2 kDa (NSs) in the viral sense and the N protein in the viral complementary sense (vc) (De Haan *et al.*, 1990). The function of the NSs protein has remained unknown, antiserum raised against this protein specifically labeled elongated flexible filaments or paracristalline arrays in immunogold decoration studies (Kormelink *et al.*, 1991; Kitajima *et al.*, 1992). The M RNA (4821 nt) encodes a non-structural protein

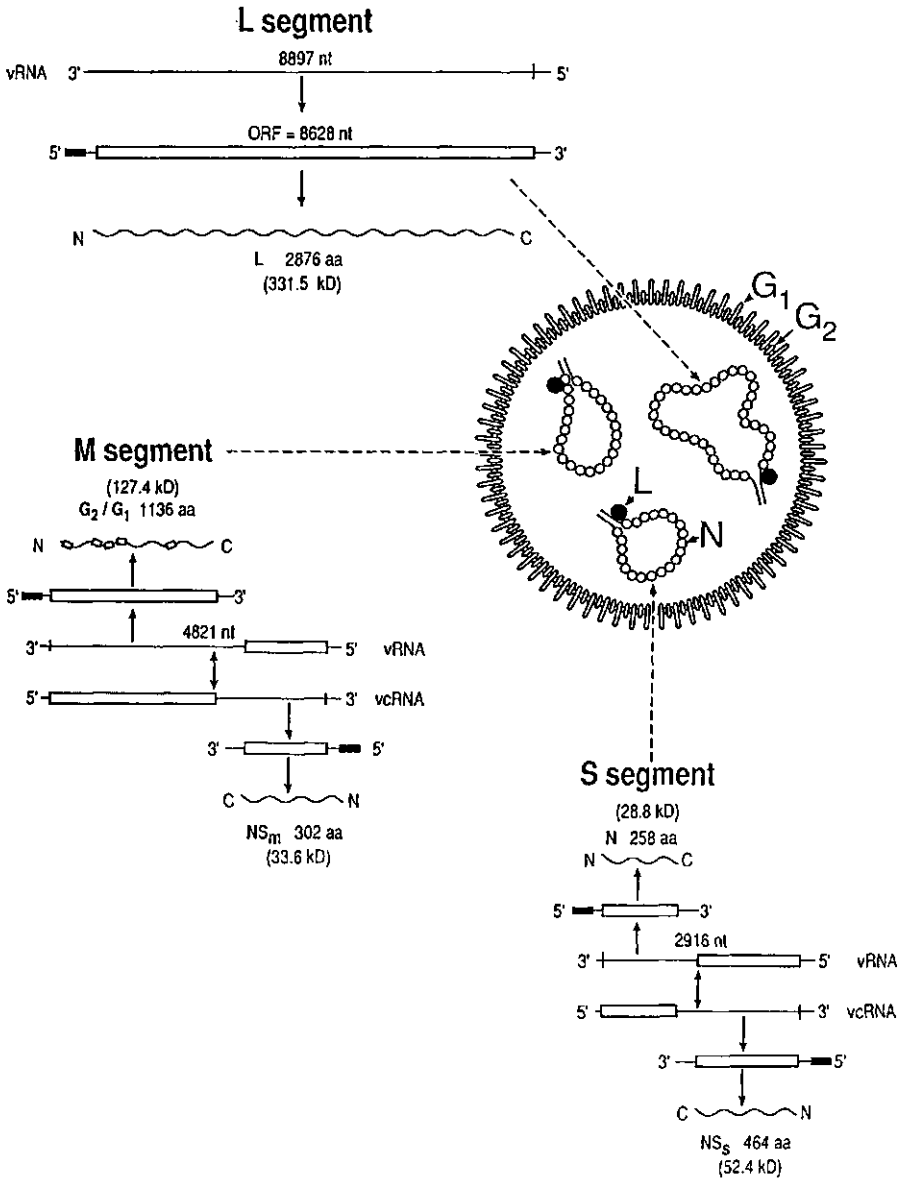

Figure 1. Structure and gene expression of tomato spotted wilt virus. The black boxes at the 5' ends of the mRNAs represent heterogeneous sequences.

of 33.6 kDa in the viral sense, which represents the movement protein (Kormelink *et al.*, 1994; Storms *et al.*, 1995), and a common precursor to the glycoproteins (127.4 kDa) in the viral complementary sense. The glycoprotein precursor contains a RGD motif which is characteristic for cellular attachment domains (Kormelink *et al.*, 1992c). Both the S and the M RNA are translationally expressed via subgenomic mRNAs, transcribed from either the viral or viral complementary strands (Kormelink *et al.*, 1992a). These mRNAs probably terminate in the intercistronic region, at a long stable A-U hairpin (De Haan *et al.*, 1990; Kormelink *et al.*, 1992b). The L RNA (8897 nt) is of complete negative polarity and contains a single ORF in the vc sense corresponding with a theoretical translation product of 331.5 kDa (De Haan *et al.*, 1991). Comparison with the polymerase proteins of other negative-strand viruses indicates that this protein most likely represents the viral RNA dependent RNA polymerase. Expression of the L RNA occurs via the synthesis of a full-length mRNA (Kormelink *et al.*, 1992a). All segments contain complementary 3' and 5' termini which can be folded in a stable panhandle structure, and are probably responsible for the formation of the pseudo-circular nucleocapsids (De Haan *et al.*, 1989; De Haan *et al.*, 1991; Kormelink *et al.*, 1992c).

Although the role of the individual viral proteins in the transcription/replication process has not been elucidated for TSWV yet, a comparison to the negative strand RNA viruses might shed some light on this.

Transcription/replication strategies of negative-strand RNA viruses

Negative-strand RNA viruses are defined by having a genome that by itself is neither translatable nor infectious. In order to be infectious the RNA must be tightly associated with the nucleocapsid protein and an RNA-dependent RNA polymerase must be provided. Upon entry of a host cell this viral RNA dependent RNA polymerase starts transcribing the genomic RNA to give rise to primary transcripts. This basic feature is the starting point for a range of transcription and replication strategies followed by negative-strand RNA viruses as will be discussed in the following paragraphs with emphasis on the genetic manipulation of negative-strand RNA genomes. A major distinction is made between viruses whose genome consists of a single RNA molecule (Order Mononegavirales, comprising the families *Rhabdoviridae*, *Paramyxoviridae* and *Filoviridae*) and those possessing multipartite (segmented) genomes (the families *Orthomyxoviridae*, *Arenaviridae* and *Bunyaviridae*). Elements essential for replication and gene expression have been retained throughout the negative-strand RNA viruses indicating that they have originated from a common ancestor

(Tordo *et al.*, 1992).

Non-segmented negative-strand RNA viruses

The genomic organization of rhabdoviruses as well as of paramyxoviruses share striking similarities which point to a common strategy in their gene expression and replication. The gene order in the genome of rhabdoviruses, e.g. vesicular stomatitis virus (VSV), is 3' l-(leader)-N-NS-M-G-L 5' whereas paramyxoviruses have a similar genetic organization but possess more genes. The genes are separated by gene junction sequences which consist of a polyadenylation signal, a few non-transcribed nucleotides and a restart signal.

After infection of a cell by a rhabdo- or paramyxovirus, the ribonucleoprotein complex (RNP) serves as a template for primary transcription. The viral RNA dependent RNA polymerase, consisting of a catalytic subunit (L) and a non-catalytic cofactor (P), enters at the 3' end of the RNA and sequentially synthesizes the short leader RNA, followed by the mRNAs for the N, NS, M, G and L genes by terminating and restarting at each gene junction region. As a result of this transcription strategy, the molar abundance of each mRNA is regulated by the location of its gene, with the N mRNA being the most abundant and the L mRNA the least abundant of the transcripts. The mRNAs are capped at their 5' end and are polyadenylated at their 3' end. Polyadenylation itself appears to be the result of polymerase "slippage" at a short polyuridylate tract. Both capping and polyadenylation are intimately coupled with the transcription process; preformed RNAs added to the transcription complex or to the polymerase do not undergo these modifications (for detailed reviews see Banerjee, 1987; Banerjee and Barik, 1992; Galinski, 1991). The P genes of paramyxoviruses express multiple proteins through a mechanism known as RNA editing (Ohmigoto *et al.*, 1990; Thomas *et al.*, 1988; Vidal *et al.*, 1990). This most likely occurs by a stuttering mechanism of the viral polymerase (Vidal *et al.*, 1990).

At a later stage in infection the transcriptase switches to a replicative mode for synthesis of full-length RNA. In contrast to transcription, the replication product is not free RNA, but an RNP with an encapsidated full-length RNA. Since constant protein synthesis is a prerequisite for replication of all negative-strand RNA viruses, it is assumed that RNA polymerization and encapsidation of the growing RNA chain into nucleoprotein are linked. Concurrent encapsidation involves the participation of preformed N-P/NP-L and P-L complexes (Horikami *et al.*, 1992). The origin of encapsidation is located close to the 3' end of the genome (Moyer *et al.*, 1991). The switch from transcription to replication is most likely affected by the amount of intracellular, unassembled nucleocapsid RNA (Vidal and Kolakofsky, 1989).

A major drawback in obtaining a manipulatable system for negative-strand viruses has been the fact that the viral polymerase, unlike that of positive strand viruses, cannot use

nucleic acid directly as a template but only after encapsidation with the nucleoprotein. Luytjes *et al.* (1989) were the first who described a system for a negative-strand RNA virus that allowed successful generation of biologically active RNPs containing artificial RNA (see next section). For non-segmented viruses this *in vitro* encapsidation is ineffective, which is probably due to the tighter RNP structure (Baudin *et al.*, 1994), although a few nucleotides corresponding to the genome ends of VSV could be associated *in vitro* with nucleoprotein (Moyer *et al.* 1991; Smallwood and Moyer, 1993). Park *et al.* (1991) demonstrated that a transfected short, artificial RNA construct could be rescued upon infection of the transfected cells by Sendai virus. This model genome contained the CAT reporter gene sequence which was bordered by the viral 3'- terminal sequence including the putative promoter for the polymerase and the signal(s) involved in leader RNA transcription/release and initiation of mRNA transcription. The 5' end contained the transcription stop/polyadenylation signal derived from the 5'-terminal cistron (L) and encoded the antigenomic promoter for replication. The results obtained with this artificial RNA construct confirmed that all *cis*-acting sequences required for encapsidation, initiation of replication and transcription of this paramyxovirus reside in the terminal sequences of the genome. Successful rescue of transfected RNAs by infectious helper virus has been described for respiratory syncytial virus (Collins *et al.*, 1991), parainfluenza virus type 3 (Dimock and Collins, 1993; De and Banerjee, 1993) and measles virus (Sidhu *et al.*, 1995). No extra nucleotides are tolerated at 3' end of the transcript as replication cannot proceed from an internal site (Collins *et al.*, 1991; De and Banerjee, 1993), in contrast to the 5' end which appeared to tolerate additional nucleotides (De and Banerjee, 1993). Promoter sequences can be studied by using the reporter gene assay (Harty and Palese, 1995) as well as internal transcription signals by using bicistronic model genomes containing two different reporter genes (Kuo *et al.*, 1994).

Naturally occurring defective interfering particles (DIs) have extensively been used to elucidate the *trans*-acting factors required for their propagation, especially those of the non-transcribing copy-back type. These DIs possess the parental 5' terminus and a complementary 3' end, therefor only allowing replication. Proteins expressed from transfected plasmids carrying a T7 promoter in the presence of a recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3; Fuerst *et al.*, 1986) were shown to support replication of a VSV DI (Pattnaik and Wertz, 1990) and a Sendai virus DI (Curran *et al.*, 1991). The minimal set of proteins required for replication consisted in both cases of the N, P and L proteins. Moreover, assembly and budding of infectious particles was observed in cells co-infected with the VSV DI and recombinant vTF7-3 and expressing all viral proteins from transfected plasmids (Pattnaik and Wertz, 1991). This approach not only provided a powerful tool for studying the *trans*-acting factors but also obviated the need for helper virus.

The use of the vaccinia virus/bacteriophage T7 RNA polymerase expression system

allowed the expression of viral proteins from individual plasmids and the intracellular generation of genome analogues from transfected plasmids. A crucial point in this approach was the generation of correct termini of transcripts which was achieved by the development of plasmid vectors designed to yield genome-like 3' termini by the autolytic activity of ribozyme sequences (Ball, 1992; Pattnaik *et al.*, 1992). Expression of the DI-T RNA from the transfected plasmid (which contains the DI-T sequence flanked by ribozyme sequences) in vTF7-3-infected cells, which also expressed the VSV N, P and L genes from simultaneously transfected plasmids, resulted in efficient encapsidation and replication of the RNA (Pattnaik *et al.*, 1992). These studies confirmed that a precise 3' end was more crucial for VSV replication than a correct 5' end of the transcript. Although transcripts with short extensions or deletions were encapsidated no replication was observed. Remarkably, extra, non-viral G residues at the 5' end were removed during replication (Pattnaik *et al.*, 1992). Constructs corresponding to transcribing or non-transcribing model genomes of several non-segmented negative-strand RNA viruses have been expressed in the vaccinia virus/bacteriophage T7 expression system (for a recent review see Conzelmann, 1996). These studies were aimed at the elucidation of *cis*- and *trans*-acting factors and involved promoter mutagenesis as well as the analysis of particular properties of the viral polymerase and the viral assembly process (Conzelmann, 1996).

For the recovery of infectious viruses from cloned cDNA the use of antigenome rather than genome transcripts was crucial for success (Schnell *et al.*, 1994). A major difference with the above described experiments is the presence of N, P and L coding sequences in full-length transcripts of the genome-sense which may lead to hybridization of large amounts of mRNAs encoding N, P and L proteins. Recombinant rabies viruses (Schnell *et al.*, 1994) and recombinant VSV viruses (Lawson *et al.*, 1995; Whelan *et al.*, 1995) were recovered by using transcripts from the full-length antigenome. No recovery was possible using the plasmids yielding genome-sense transcripts.

Segmented negative-strand RNA viruses

Orthomyxoviridae

Members of the *Orthomyxoviridae* family contain six (Thogoto virus), seven (Dhori virus and members of the genus *Influenza C*) or eight (members of the genus *Influenza A, B*) RNA segments. This section will focus on influenza A virus. The coding sequences (in negative polarity) are flanked in each influenza virus RNA segment by short non-coding regions. The first 12 and 13 nucleotides of the 3' and 5' ends of each RNA segment, respectively, are highly conserved among different RNA segments of the same virus and also among different influenza A virus strains. These ends are partially complementary and are responsible for the panhandle structure of viral RNAs, as observed in both virions and infected cells (Hsu *et al.*,

1987).

Upon infection of a cell, when released in the cytoplasm, influenza virus nucleocapsids directly migrate to the nucleus where transcription and replication takes place. For expression of the genome four viral proteins are essential: the nucleoprotein (NP) and the three subunits of the polymerase (PB1, PB2 and PA, reviewed by Krug *et al.*, 1989). Influenza virus uses a remarkable mechanism for the synthesis of viral mRNAs. In contrast to the polymerases of the Mononegavirales, those of the segmented negative-strand viruses do not possess capping activity. Therefore, viral mRNA synthesis requires initiation by host cell primers, specifically capped (m^7GpppNm-containing) RNA fragments derived from host cell RNA polymerase II transcripts (reviewed by Krug, 1981). Host cell primers are generated by a viral cap-dependent endonuclease that cleaves the capped cellular RNAs 10-18 nucleotides from their 5' end preferentially at a purine residue (Krug, 1981), a process commonly referred to as "cap-snatching". The PB2 subunit recognizes and binds to the 5' cap-structure of the host cell (Blaas *et al.*, 1982; Ulmanen *et al.*, 1981; Braam *et al.*, 1983), and cleaves host-cell mRNAs (Shi *et al.*, 1995). This subunit functions only as the trimeric enzyme (Shi *et al.*, 1995) and is not required for replication (Nakagawa *et al.*, 1995). Interestingly, recombinant influenza virus polymerase requires both 5' and 3' viral ends for endonuclease activity (Hagen *et al.*, 1994). Transcription is initiated by addition of a G residue to the primer, complementary to the penultimate nucleotide at the 3' end of the viral RNA strand (Krug *et al.*, 1989). Chain elongation is performed by the PB1 subunit which proceeds to a stretch of U residues 17 to 22 nucleotides away from the 5' end of the viral RNAs, where transcription terminates and which serves as a polyadenylation signal (Robertson *et al.*, 1981). Two of the viral mRNAs (coding for the M1 protein and for the NS1 protein) are spliced to form smaller mRNAs (reviewed by Krug, 1989). The switch from viral mRNA synthesis to the synthesis of full-length RNAs is regulated by the amount of free nucleoprotein in the nucleus (Beaton and Krug, 1986). Later during infection NP accumulates in the nucleus which results in transcription antitermination and subsequent formation of viral complementary RNAs. These template RNAs are encapsidated, indicating that the encapsidation signal is most likely located at the 5' ends of the RNA molecules (Krug *et al.*, 1989). Transcripts initiated with a capped primer fragment cannot be antiterminated in the presence of NP (Beaton and Krug, 1986) suggesting that the capped primer sequence blocks the binding of the NP. The nucleus is also the site of vRNA synthesis, the complementary RNA strands remain here, whereas the nascent encapsidated viral RNA molecules are transported out of the nucleus as nucleocapsid structures to yield progeny virions (Shapiro *et al.*, 1987).

Amplification, transcription, and rescue of synthetic RNA molecules derived from a negative-strand RNA virus was achieved for the first time by Luytjes *et al.* (1989). A

biologically active influenza virus RNP complex was reconstituted using synthetic RNA and purified viral proteins, and amplification and expression of the reporter gene was driven by an influenza helper virus (Luytjes *et al.*, 1989). The results obtained demonstrated that the signals required for replication, transcription, and packaging of the viral RNA are located in the 26 3' terminal and the 22 5' terminal nucleotides (Luytjes *et al.*, 1989). Different methods for *in vitro* RNP reconstitution have been reported, differing in both the source of the NP protein and the helper functions (for a review see Garcia-Sastre and Palese, 1993). In all these systems, replication and transcription of the input RNP is driven either by superinfection with a helper virus or by the proteins contained in the purified RNP cores, which makes it difficult to examine the functions of the individual proteins during replication. Using recombinant vaccinia viruses expressing influenza virus proteins the minimum subset of influenza virus proteins needed for specific replication and expression of a synthetic NS-like gene was found to be the three polymerase proteins (PB1, PB2 and PA) and the nucleoprotein (Huang *et al.*, 1990). Similar systems have been used to study the *trans*-acting domains of the polymerase proteins involved in replication (reviewed by Garcia-Sastre and Palese, 1993). Promoter sequences recognized by the viral polymerase in both vRNA and cRNA have been studied by a reverse genetics approach (Li and Palese, 1992; Parvin *et al.*, 1989; Piccone *et al.*, 1993; Seong and Brownlee, 1992a and b; Yamanaka *et al.*, 1991). Promoter sequences have been defined within the first 12-14 nucleotides at the 3' end of the vRNAs (Parvin *et al.*, 1989; Piccone *et al.*, 1993; Seong and Brownlee, 1992a and b; Yamanaka *et al.*, 1991), and within the first 12-14 nucleotides at the 3' end of cRNAs (Li and Palese, 1992; Seong and Brownlee, 1992a; Yamanaka *et al.*, 1991), although there are some discrepancies resulting from the polymerase preparation and template used. As mentioned before these nucleotide positions are highly conserved in influenza virus RNA segments. Luo *et al.* (1990) showed that a stretch of 5-6 uridine residues close to the 5' end of the vRNA and juxtaposed to the RNA panhandle structure is required for efficient polyadenylation of the mRNA. Mutations affecting either uridine stretch or the panhandle structure reduced mRNA formation. These data support a stuttering mechanism for poly-(A) addition of viral mRNAs for which 5 to 7 uridine residues have been shown to be the optimal length of the U stretch (Li and Palese, 1994). The upstream sequence at the 5' end is not involved in polyadenylation and the optimal distance between the 5' end and the U stretch is 16 nucleotides (Li and Palese, 1994). The 5' terminus of the vRNA plays an important role in the regulation of transcription as well, as has been shown by Hagen *et al.* (1994) who demonstrated that the endonuclease activity of recombinant polymerase was strictly dependent on the presence of a template RNA containing both 3' and 5' viral sequences. Fodor *et al.* (1994 and 1995), proposed an RNA-fork model involved in the initiation of transcription to account for the involvement of both 3' and 5' termini.

Arenaviridae

Members of the *Arenaviridae* are characterized by a bipartite genome. Both single-stranded RNA segments, denoted L (large) and S (small) contain two ORFs in an ambisense gene arrangement (reviewed by Bishop, 1990). The genes are separated by a stable secondary structure in the form of a hairpin. Viral S mRNA transcription has been shown to terminate in the hairpin region in Tacaribe (TAC) virus (Iapalucci *et al.*, 1991) and lymphocytic choriomeningitis (LCM) virus (Meyer and Southern, 1993). The mRNAs contain one to five, heterogeneous, extra nontemplated bases at their 5' ends, which are capped. Remarkably, the 5' ends of genomes and antigenomes also contain a single nontemplated G residue. The precise mechanism of transcription and replication in the family *Arenaviridae* is still unclear.

A model of arenavirus transcription and replication has been proposed based on three methods of analysis: primer extensions with TAC and Pichinde (PIC) virus mRNA and with genomic and antigenomic templates (Raju *et al.*, 1990), sequence analysis of cDNA clones representing the 5' ends of TAC virus NP mRNA and antigenomes (Garcin and Kolakofsky, 1990) and the 5' and 3' termini of LCM virus mRNAs for the NP and GPC proteins (Meyer and Southern, 1993), and *in vitro* RNA synthesis of LCM and TAC virus RNAs (Fuller-Pace and Southern, 1989; Garcin and Kolakofsky, 1992). Initiation of transcription may be fundamentally different from that of influenza virus (Krug, 1989) and bunyaviruses (Kolakofsky and Hacker, 1991), as the 5'-terminal sequences of arenaviral mRNAs are considerably shorter (Polyak *et al.*, 1995). A polymerase slippage model of virus RNA replication has been proposed to account for the nontemplated 5'(p)ppG at the ends of genomes and antigenomes (Garcin and Kolakofsky, 1992).

Bunyaviridae

The genome of members of the *Bunyaviridae* typically consists of three single stranded RNA segments, denoted L, M and S, which are tightly wrapped by the nucleocapsid protein. Members of the *Phlebovirus* and *Tospovirus* genera contain one or two RNA segments, respectively, that display an ambisense gene arrangement (Elliott, 1990; Murphy *et al.*, 1995). Transcriptase activity has been detected in detergent-disrupted virions of Lumbo virus (Bouloy and Hannoun, 1976), Germiston (Gerbaud *et al.*, 1987), La Crosse (Patterson *et al.*, 1984), Uukuniemi (Ranki and Petterson, 1975), Hantaan (Schmaljohn and Dalrymple, 1983) and tomato spotted wilt virus (Adkins *et al.*, 1995). An endonuclease activity was detected which cleaved methylated capped mRNAs *in vitro* (Patterson *et al.*, 1984; Vialat and Bouloy, 1992). Analysis of viral mRNAs revealed the presence of 10-18 non-viral sequences at their 5' ends, showing that bunyaviruses, like influenza and arenaviruses, use cap-snatching to prime transcription. The bunyaviral mRNAs are not polyadenylated. There seems to be no universal signal for transcription termination although it has been suggested that purine-rich

sequences, which seem to occur around the termination sites, might be involved (Raju and Kolakofsky, 1986). The mRNAs are about 60 to 100 nucleotides shorter than full-length transcripts. For both La Crosse (Bellocq *et al.*, 1987) and Germiston (Vialat and Bouloy, 1992) virus a translational requirement for transcription has been reported, which, at least for La Crosse virus, has been shown to be cell-type dependent (Raju *et al.*, 1989). A model to explain these findings has been proposed, which suggests that interactions between the nascent mRNA chain and its template causes premature termination. In the presence of concomitant protein synthesis ribosomes moving along the nascent mRNA behind the polymerase prevent the mRNA from hybridizing to its template and the polymerase reads through to the major termination site (Bellocq and Kolakofsky, 1987).

Jin and Elliott (1991) described a reverse genetics approach for Bunyamwera virus. Recombinant vaccinia viruses containing the complete L gene under control of the vaccinia P7.5 promoter or the bacteriophage T7 promoter were obtained. For functional expression of the L protein from the latter recombinant vaccinia virus coinfection with a second recombinant vaccinia virus which synthesizes T7 RNA polymerase (vTF7-3, Fuerst *et al.*, 1986) was required. Both systems express a functional protein as was shown in a nucleocapsid transfection assay: recombinant vaccinia virus-infected cells were tranfected with purified Bunyamwera virus nucleocapsids, and subsequently, total cellular RNA was analyzed by Northern blotting. No Bunyamwera virus RNA was detected in control transfections but both positive- and negative-sense Bunyamwera virus S segment was detected in cells previously infected with recombinant vaccinia viruses expressing the L protein (Jin and Elliott, 1991). This assay was used to study the effect of site-specific amino acid substitutions in the L protein. It was shown that residues strictly conserved between the L proteins of different viruses in the family *Bunyaviridae* were obligatory required for activity, whereas non-conserved residues could be substituted without abolishing RNA synthesis capability (Jin and Elliott, 1992). Furthermore, using the transfection assay it was shown that the L protein contains the endonuclease activity which generates the primers required for transcription initiation. The results obtained indicate that the recombinant L protein has both transcriptase and replicase activities (Jin and Elliott, 1993).

Studying both *cis*- and *trans*-acting signals has become feasible with the development of a system for analyzing bunyavirus transcription using a recombinant (BUNSCAT) RNA template derived from a cDNA construct containing the exact 5' and 3' untranslated regions of the Bunyamwera virus S RNA segment flanking the (negative-sense) reporter gene chloramphenicol acetyltransferase (CAT) (Dunn *et al.*, 1995). The system permits investigation of both the protein and RNA sequence requirements for transcription. Only the bunyavirus L and N proteins were needed for transcription of the BUNSCAT RNA.

Scope of the investigation

The aim of the research described in this thesis was to gain more insight into the transcription/replication process of TSWV.

At the onset of this research there was only limited information available with respect to the synthesis of TSWV RNAs and the viral proteins involved. Preliminary sequence analysis of the L RNA revealed a number of motifs, characteristic for RNA-dependent RNA polymerases, which indicated that the L RNA most likely encodes the putative viral RNA dependent RNA polymerase. The size of the ORF (corresponding with a translation product of 331.5 kDa) predicted by this sequence, however, was in conflict with reported sizes (110-220 kDa) of a large protein co-purifying with TSWV particles (Mohamed *et al.*, 1973; Peters *et al.*, 1991; Tas *et al.*, 1977). Sequence analysis of the L RNA of impatiens necrotic spot virus (INSV) belonging to a different serogroup within the *Tospovirus* genus, revealed that the L RNA is of comparable size to that of TSWV BR-01, and that its coding product (L protein) is the most conserved protein (Chapter 2). To allow unequivocal detection and to study the expression of the L protein, antisera were raised against bacterial expression products. Using these antisera it was shown that the (331.5 kDa) tospoviral L protein is present in an unprocessed form in both purified virus and nucleocapsid preparations (Chapter 3).

Partial purification and sequence analysis revealed that the 5' ends of viral mRNAs contained non-viral heterogenous sequences, probably derived from (capped) host mRNAs via a process referred to as "cap-snatching". Sequence analysis showed that there was no strict base preference at the cleavage site as has been reported for some other members of the *Bunyaviridae* (Chapter 4). Stimulated by the finding of Adkins *et al.* (1995), showing that measurable amounts of *in vitro* polymerase activity can be obtained from purified virus particles, studies were initiated to investigate whether this *in vitro* system would lent itself for studying the role of the viral proteins and template requirements in more detail. Thus it was shown that the *in vitro* RNA synthesizing reaction was completely dependent on manganese and that mainly S RNA-specific products were formed (Chapter 5). Although the *in vitro* transcription assay will be useful for defining the basic characters of the transcription/replication process it appears to be of limited value for the unravelling of all *cis*- and *trans*-acting factors involved in this process. For that purpose a manipulatable system would be required, in which at the one hand the template requirements can be tested by adding specific and modified viral genome sequences, and at the other hand the involvement of all (viral) proteins can be scrutinously determined. As it was known that TSWV also replicates in its insect vector (Wijkamp *et al.*, 1993) it was therefore decided to set up an *in*

vivo reconstitution system based on the expression of TSWV replication genes in *Spodoptera frugiperda* insect cells driven by a baculovirus/bacteriophage T7 hybrid vector system (Chapter 6). This system was shown to support the transient expression of heterologous genes supplied in T7 promoter-containing plasmids. The attempts to exploit this system for *in vivo* reconstitution of the TSWV trancription/replication machinery failed however, at least within the time limits of this Ph.D. research, due to the apparent impossibility to obtain a translatable full-length cDNA copy of the TSWV L RNA (Chapter 3). In Chapter 7 suggestions for successful exploitation of the baculovirus/bacteriophage T7 expression system will be discussed, including the possibility to center the approach around INSV in stead of TSWV, when cloning of a full-length copy of its L RNA, based on the sequence information presented in Chapter 2, may turn out less cumbersome.

CHAPTER 2

Completion of the impatiens necrotic spot virus genome sequence and genetic comparison of the L proteins within the family *Bunyaviridae*.

Summary

The nucleotide sequence of the large (L) genome segment of impatiens necrotic spot virus (INSV) has been determined, and herewith the complete nucleotide sequence of the entire, tripartite genome of this important tospovirus has been elucidated. The L RNA is 8776 nucleotides long and of negative polarity, containing one large open reading frame (ORF) on the viral complementary strand. Comparison of the deduced amino acid sequence of the INSV L RNA primary translation product (330.3 kDa) with those of the L RNAs of other members of the *Bunyaviridae* reveals that this protein represents the putative viral RNA-dependent RNA polymerase. A cluster dendrogram of the (putative) RNA polymerases indicates that the genus *Tospovirus* and the genus *Tenuivirus*, though both encompassing ambisense, plant-infecting viruses, have different affinities to the animal-infecting *Bunyaviridae*, tospoviruses being most closely related to the genus *Bunyavirus*, and tenuiviruses to the genus *Phlebovirus*, respectively.

Parts of this chapter have been submitted as:
Van Poelwijk, F., Prins, M. and Goldbach, R. (1996). Completion of the impatiens necrotic spot tospovirus genome sequence and genetic comparison of the L proteins within the family *Bunyaviridae*.

Introduction

Most members of the *Bunyaviridae*, a large family of enveloped, arthropod-born RNA viruses (Elliott, 1990; Murphy *et al.*, 1995) infect animals but some are able to infect plants. These latter bunyaviruses are classified into a separate genus, the genus *Tospovirus*, named after the type species tomato spotted wilt virus (TSWV). Tospoviruses are exclusively transmitted by thrips in a propagative manner (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993). Due to the recent worldwide spread of one of the most efficient vectors, the Western flower thrips (*Frankliniella occidentalis* Pergande), not only TSWV (Goldbach and Peters, 1994) but also a second tospovirus, impatience necrotic spot virus (INSV) has recently emerged and is gaining in economic impact, having become a serious threat for the cultivation of ornamental plants both in Northern America and in Europe (Law and Moyer, 1990; DeAngelis *et al.*, 1994; Vaira *et al.*, 1993). Thusfar molecular studies have been mainly focussed on TSWV whereas information on INSV is limited. Both the small (S) and the middle (M) segment of the tripartite RNA genome of INSV have been sequenced (De Haan *et al.*, 1992; Law *et al.*, 1992) but molecular data on the largest (L) genomic segment were lacking. Here we report on the determination of the nucleotide sequence of the INSV L RNA, thereby providing the sequence of the complete genome of this tospovirus.

Materials and Methods

Virus and plants

INSV isolate NL-07 (De Avila *et al.*, 1992) was maintained on *Nicotiana benthamiana* plants by mechanical inoculation and nucleocapsids were purified as described by De Avila *et al.* (1990). RNA was extracted as previously described (De Haan *et al.*, 1989).

Primers and RT-PCR

Reverse transcription was carried out at 37 °C with viral or viral complementary specific primers and Moloney murine leukemia virus reverse transcriptase (M-MLV, Gibco BRL). Primer pairs were added to the first strand reaction for both v and vc sense followed by amplication using Taq polymerase (Supertaq, SphaeroQ). Primers used to amplify PCR fragment P1 (1: 5'-TTTTTTTCTAGAGCAATC-3' and 2: 5'-GACAGCATGCTGTATCT CC-3'), P2 (3 :5'-AATGGACCCCCAAC-3' and 4: 5'-ATTTGCATCATGTCC-3'), P3 (3 and 5: 5'-TGTTCTCATCAGCCC-3'), P4 (6: 5'-AAAAAGTGTCTGAAG-3' and 7: 5'-TTC

AATTTCACATAC-3'), P5 (8: 5'-TGGAGCTATTATACC-3' and 9: 5'-ACACAGCAGTGT CC-3'), P6 (10: 5'-GGACAGAAGAACAAG-3' and 12: 5'-AGAGCAATCAGGCA-3') and P7 (11: 5'-CAAAGAACTTGATC-3' and 12). PCR amplified fragments were cloned into a T-vector (Promega) and sequenced on an ABI 373A automatic DNA sequencing system.

Results

Cloning and sequencing of the INSV L RNA

Sequence information for the primers used in PCR was initially derived from a number of cDNA clones that were obtained by random priming of nucleocapsid RNA. The identity of these clones was confirmed by Northern analysis. These clones are indicated with L to distinguish them from PCR-derived clones, which are marked P. The cloning strategy is depicted in Figure 1. Primer pairs 1-2, 3-4, 3-5, 6-7, 8-9, 10-12 and 11-12 were used to amplify seven fragments of 421, 593, 1609, 564, 1702, 2071 and 200 nucleotides, respectively (Fig. 1).

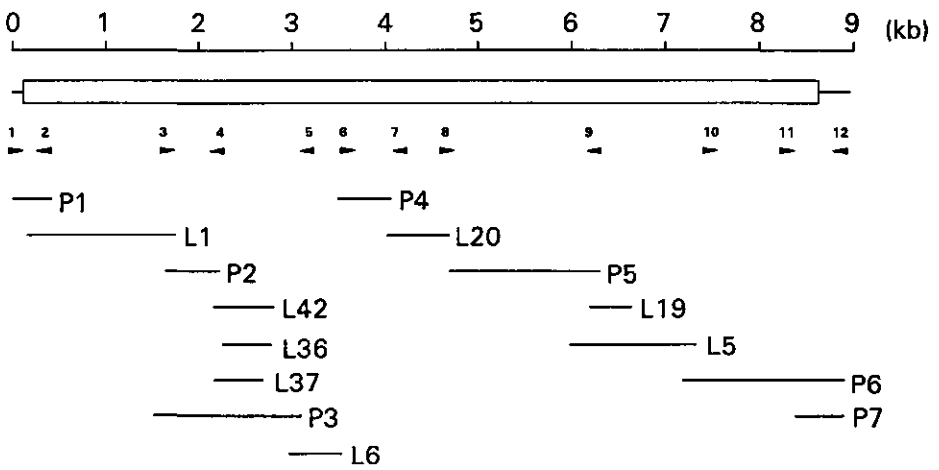

Figure 1. Cloning strategy of the INSV L RNA. The viral complementary strand (vRNA) is shown in 5' to 3' polarity, the box corresponds with the large ORF. Primers shown (arrowheads) are used for RT-PCR. cDNA clones are indicated with L and PCR-derived cloned fragments are indicated with a P.

All amplified fragments were cloned into a pGEM-T vector. The first nine terminal nucleotides of all tospoviruses sequenced to date (mainly based on S RNA sequences, except for TSWV and INSV for which also the M RNA sequence is known) are conserved (vRNA: 3'-UCUCGUUAG-5'). This feature allowed to design a primer that could be used to amplify fragments encompassing both the 5' and 3' ends. This is possible as the termini are complementary and both viral and viral complementary strands are present in RNA purified from nucleocapsid preparations. The cloned PCR fragments were selected for their size, subjected to dideoxy nucleotide sequencing and run on an automatic DNA sequencing system.

The complete nucleotide sequence of the L RNA was determined from either cDNA- or PCR derived clones. Most of the sequence was determined by sequencing both strands, less than 5% was determined from one strand only. The entire sequence is shown in Figure 2 and is available from the EMBL, Genbank and DDBJ nucleotide databases under the accession number X93218.

```
1     AGAGCAATCAGGCAACAACAATTTATTCAGAATGAACAATTACAAAGCAAGATTATTAATAGAAAATTCTGTTACTCTACTATCGTCAATTGATGATTGT
                                  M  N  N  Y  K  A  R  L  L  I  E  N  S  V  T  L  L  S  S  I  D  D  C

101   ATCAAAAGCAATTTAGAGTTAAGCAGAGACCTCCATAAAAAGAACCCAGATGAAATAAGTGAAGATATTATTATAAATAACAATGCTAAAAACTATGAAG
      I  K  S  N  L  E  L  S  R  D  L  H  K  K  N  P  D  E  I  S  E  D  I  I  I  N  N  N  A  K  N  Y  E  A

201   CTTTAAGAACTCTAATAGCAAGAATTACTAGGGATGGTGAAGGGATTGAAACAGGATTGGCTACAGTTGACATGAAAAAGATATCTGAAGACATGACTCT
        L  R  T  L  I  A  R  I  T  R  D  G  E  G  I  E  T  G  L  A  T  V  D  M  K  K  I  S  E  D  M  T  L

301   CCTTGAGCAGAAATATCTTGAAACTGAATTAGCTAGACATGATATGTTCGGTGAGCTGGTTTCAAGACATTTGCATTTAAAACCTAAAAAAAGACATGAT
      L  E  Q  K  Y  L  E  T  E  L  A  R  H  D  M  F  G  E  L  V  S  R  H  L  H  L  K  P  K  K  R  H  D

401   GTGGAGATAGAGCATGCTGTCAGAGAGTATTTTGAAGAGCTAAGCAAGAAAGAATGTTCTAACAGACTGTCTGAAGAAGATTTCAAAAAGGTCAGTAAAG
      V  E  I  E  H  A  V  R  E  Y  F  E  E  L  S  K  K  E  C  S  N  R  L  S  E  E  D  F  K  K  V  S  K  E

501   AATATGTTGCTACCAACGCTACTCCTGATAACTTTGTGATATACAAAGAATCCAAGAGTGGTCCTCTTTGCATGATGATCTATGATTGGAAAATTTCTGT
        Y  V  A  T  N  A  T  P  D  N  F  V  I  Y  K  E  S  K  S  G  P  L  C  M  M  I  Y  D  W  K  I  S  V

601   TGATGCTAAAACAGAAACTAAAACAACAGAGAAATATTACAAAAACATATGGAAATCATTGAAAGATGTGAAAGTCAAGGGTAAATCTTTCTTGGAAGAA
        D  A  K  T  E  T  K  T  T  E  K  Y  Y  K  N  I  W  K  S  L  K  D  V  K  V  K  G  K  S  F  L  E  E

701   CATCCCATATTCATCTCTATTGTTATTCTTAAACCTATTGGATCTATGCCTATAGTGGTCACGACTAGCAGAGTCTTAGAGAAATTTGAAGATTCAGAAT
      H  P  I  F  I  S  I  V  I  L  K  P  I  G  S  M  P  I  V  V  T  T  S  R  V  L  E  K  F  E  D  S  E  S

801   CTGCATTGCATGCCAACAGGTTGAGACACGCAAGCCAATCAAAGCTTGTCGGGGTGTCTAACATAGGCCGGATAATTGGCACAACTCCTACTGTTGTTAG
        A  L  H  A  N  R  L  R  H  A  S  Q  S  K  L  V  G  V  S  N  I  G  R  I  I  G  T  T  P  T  V  V  R

901   AGAATTTTATGCAGACACCCAAAAGTTAAAAATTGAATTTAGGAGCATACTGGGTGAAGAGTTCGGCTCTAAAGACATATTTTTTAGTCATTGGACAAAT
      E  F  Y  A  D  T  Q  K  L  K  I  E  F  R  S  I  L  G  E  E  F  G  S  K  D  I  F  F  S  H  W  T  N

1001  AAATATAAAGACAGAGATCCAACACAGATTGCTCATTCAGAGGATTTGGAGAAGATAATTGAATCAATGGTAACAGATGACATAAGTAGGGAAGAAATTG
      K  Y  K  D  R  D  P  T  Q  I  A  H  S  E  D  L  E  K  I  I  E  S  M  V  T  D  D  I  S  R  E  E  I  V

1101  TCCATTTTATGTTTGGGAATTTTTGCTTGCATATAGAGACAATGAATGATCAACATATTGCTGATAGATTTAATGGGTATAGATCCTCTTGTGTGAATCT
        H  F  M  F  G  N  F  C  L  H  I  E  T  M  N  D  Q  H  I  A  D  R  F  N  G  Y  R  S  S  C  V  N  L

1201  GAATGTAGAACCTAAGAAAGACATTTCAGAATTAAAGGATCATCTGTTATCTACTAAGGGTTTGTGGGAATCTTTATATGATCATCACTTGATTAAGGTT
      N  V  E  P  K  K  D  I  S  E  L  K  D  H  L  L  S  T  K  G  L  W  E  S  L  Y  D  H  H  L  I  K  V

1301  ATGGATAGGATAAAAACGAAAAAACAAAAAGAAAAGATTATACCTGACATAATGACTGCATTCAACTTGAATGCTGAAGAGTACGAAAAGAAATACCCAA
      M  D  R  I  K  T  K  K  Q  K  E  K  I  I  P  D  I  M  T  A  F  N  L  N  A  E  E  Y  E  K  K  Y  P  N

1401  ATTGTTTCACAAGTGATCTCTCTGAAACAAAGACAAATTTTTCTGTCACTTGGTCACCTTGCACTGATATGGTGGAACTAGGAAACCAAGATTATAACAA
        C  F  T  S  D  L  S  E  T  K  T  N  F  S  V  T  W  S  P  C  T  D  M  V  E  L  G  N  Q  D  Y  N  N

1501  TGCAGTTATTGATCGGTTCAGGAAAGCTTTCCTTAGCAATCCTAGGATCAGATATAATTCAGCTTATAGCAAAGAGATGAATGTGACCAGCAAAGCAAAG
      A  V  I  D  R  F  R  K  A  F  L  S  N  P  R  I  R  Y  N  S  A  Y  S  K  E  M  N  V  T  S  K  A  K

1601  GATGTGACTGAACTTGTCAGGTCCTGTCTAACAACTCTAAGCTGTGATACAAGTGGAATGGACCCCCAACAATTAGAAGACGAAATTGACATAAGCACAG
      D  V  T  E  L  V  R  S  C  L  T  T  L  S  C  D  T  S  G  M  D  P  Q  Q  L  E  D  E  I  D  I  S  T  G

1701  GTGGTATAAAAGTGGAAAGAACTAGCAAATCACAAGAATGGATTAAGAAAAATGATTGTTTGACTAGAAATAGAAATGAGTTTAACATGAGGGAAACCAG
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      G I K V E R T S K S Q E W I K K N D C L T R N R N E F N M R E T S

1801  TAAAGATAATAAAGTGATCTATTTCAAAGGTTTGAGTGTTATGAACGTGAGCATGAGTAAAAAGAAAAGGATAATAAAGCATGAGGAACTAAAAGGGAAA
      K D N K V I Y F K G L S V M N V S M S K K K R I I K H E E L K G K

1901  ATCACAAAAAGGTCTAGAATATGATACATCAGAGAGACAGTATGACCCTAATGATGATTATGTAAGTTTGGATTTATCTTCATTCACCCACGCAAAGAAGC
      I T K G L E Y D T S E R Q Y D P N D D Y V S L D L S S F T H A K K L

2001  TTATCAGACATGATAATGAAGAGAGCTTAGAATGGTGCTCACAGATCCAAGATGGATTATTTGTTCTCCATAACTCGGACATCAGAGAAAATTGCAAAGT
      I R H D N E E S L E W C S Q I Q D G L F V L H N S D I R E N C K V

2101  GGCTACTGTTTACAACAATTATACAAAAAATCCTGAAAATCTTTTCACTCAATCAACTATCATAAAAACAGAAATGGAAACTTGTAAAAAGATAAACAAA
      A T V Y N N Y T K N P E N L F T Q S T I I K T E M E T C K K I N K

2201  TTATGCAATGATCTGGCTATATATAATTATGCTGAGGACATGATGCAAATATCTAAAGGTTTGATGGTTGCTGACAGGTACATGACTAAAGAAAGTTTCA
      L C N D L A I Y N Y A E D M M Q I S K G L M V A D R Y M T K E S F K

2301  AGATATTGACAACATTCAATACTAGCATGCTAGTTTTGGCTTTTAAAGGAGATGGGCTAAACACAGGAGGGTCAGGAGTCCCTTACATACTAGTCCACAT
      I L T T F N T S M L V L A F K G D G L N T G G S G V P Y I L V H M

2401  GGTGGAAGAGACTCTATCTGAGCAGTTTAGTGTGTGTTACACAAAAGAAATATATAGTCATTTCTCATTTGGTAGTCATGTTGTATATATTATGAGGCCG
      V E E T L S E Q F S V C Y T K E I Y S H F S F G S H V V Y I M R P

2501  CAAAGGCTGAACCAAGTTAGACTTCTTAGCCTTTTCAAATCTCCCAGCAAAGTTCCAGTTTGTTTTGCTCAGTTTTCCAAAAAAGCTCATGAATTAGAAG
      Q R L N Q V R L L S L F K S P S K V F V C F A Q F S K K A H E L E G

2601  GGTGGTTGAAAATAAAAGATATGCAAGAAGTGCAAACACTAAGCATGAGCTCTAATGTAAGAAGAATAATGAGAAATATTGTCTTTTCTTCTGTCATGAT
      W L K I K D M Q E V Q T L S M S S N V R R I M R N I V F S S V M I

2701  AGGAACAGTCACAAAGCTTAGCAGGATGGGAATCTTTGACTTCATGAGATATGCAGGATTCCTACCTTTATCAGACTATTCAAATATAAAAGAATACATA
      G T V T K L S R M G I P D P M R Y A G F L P L S D Y S N I K R Y I

2801  CAAGATAAATTTGATCCAGACATAACAAATGTTGCTGACATGTTCTTTGTTGAAGGAATAAAAAAGCTTCTTCTCAAAATGGAGAACTTAAATTTAAGTA
      Q D K F D P D I T N V A D M F F V E G I K K L L L K M E N L N L S T

2901  CAAGTGCAAAACCTGTGGTTATTGATCATGAGAATGATGTGATAGGGGGAATCACAAATTTGAACATAAAATGCCCAATCACAGGTGCTACTCTTAAGAC
      S A K P V V I D H E N D V I G G I T N L N I K C P I T G A T L K T

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      L E D L Y N N V Y L A I Y M M P K S L H N H F H N L T S L L N V P

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      N G V L N L K S L S D Y Y A S T V E N V G L M R T E I E N K E D F

3301  CTTGAGTCCTTGTTACAAGATTTCCACTTGAAATCTTCAAAAAAGTGTTCTCAATCTAATATAATTTGTTCAGATGATATAATTAACTGTCTTCAAGAA
      L S P C Y K I S T L K S S K K C S Q S N I I C S D D I I N C L Q E

3401  GCAAATGTTAGATCACTGGAAGATTTGAGTCCAAAAAATTTGGCAATCTTGAAAGGTTTGCTTAGAACTCTTCATGAAGATAAAAACAGGTTGTATGAAT
      A N V R S L E D L S P K N L A I L K G L L R T L H E D K N R L Y E F

3501  TTTTTGAAGACCACTCTGAAAACCCTTATTACCTTATGGAAAAAATGAAGACAATCAAATCTTCTGAAAAAATAACAACAGGTAAATCAAAAACATCTAA
      F E D H S E N P Y Y L M E K M K T I K S S E K I T T G K S K T S K

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      E E L Y D L I K Q Y H N I M D I D M E S I M N L G K G L E G K K H T

3801  CTTTCCTGCAAATGTTAGAATTCGTCATGTCTAAAGCTAAAAATGCTTCTGATGCAATAGATTTTTTAGTTTCTGTATTTGAAAAGATGCAAAGAACTAA
      F L Q N L E F V M S K A K N A S D A I D F L V S V F E K M Q R T K

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      T D R E I Y L M S M K V K M M L Y F I E H T F K H V A Q S D P S E

4001  GCCATATCCATTAGTGGTGACAATAAAATAAGAGCATTATCTATGTTGTCGATGGACACAATAACATCATACAATGATATATTGAAAAACAGCAAGAACA
      A I S I S G D N K I R A L S M L S M D T I T S Y N D I L K N S K N K

4101  AATCTAAGTTAGCATTTTTATCAGCTGATCAATCTAAGTGGTCAGCTTCTGACCTAACATACAAGTATATATTGGCCATTATAATGAATCCTATATTGAC
      S K L A F L S A D Q S K W S A S D L T Y K Y I L A I I M N P I L T

4201  ATCAGGTGAGTGCACATTAATGGTTGATTGTCTGATGATGTATGTGAAATTGAAAAAGGTGTGCATTCCAACAGACATTTTCTTAGGTTTAAGGAATTCA
      S G E C T L M V D C L M M Y V K L K K V C I P T D I F L Q L R N S

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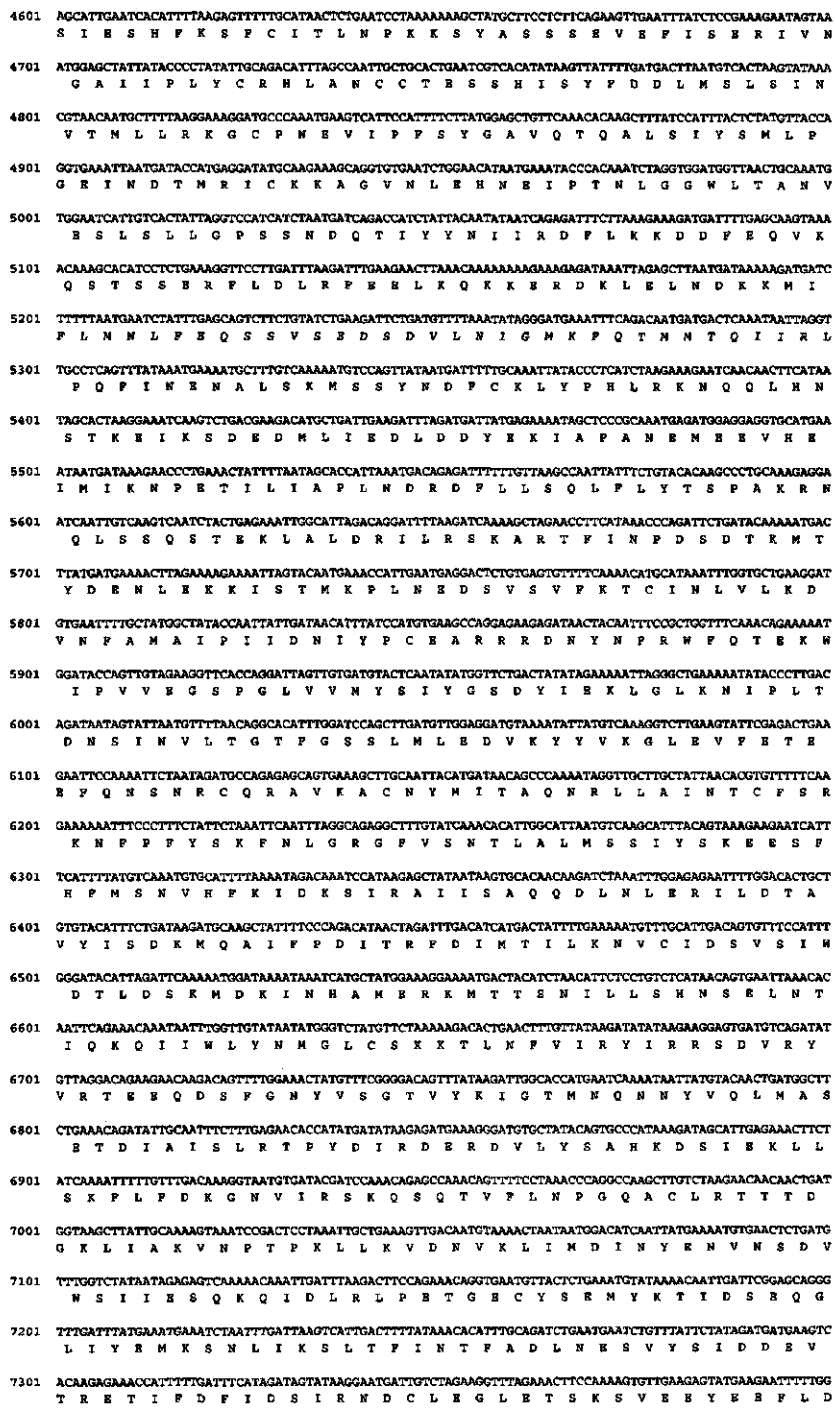

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S I E S H F K S F C I T L N P K K S Y A S S S E V E F I S E R I V N

4701 ATGGAGCTATTATACCCCTATATTGCAGACATTTAGCCAATTGCTGCACTGAATCGTCACATATAAGTTATTTTGATGACTTAATGTCACTAAGTATAAA
G A I I P L Y C R H L A N C C T E S S H I S Y F D D L M S L S I N

4801 CGTAACAATGCTTTTAAGGAAAGGATGCCCAAATGAAGTCATTCCATTTTCTTATGGAGCTGTTCAAACACAAGCTTTATCCATTTACTCTATGTTACCA
V T M L L R K G C P M E V I P F S Y G A V Q T Q A L S I Y S M L P

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G E I N D T M R I C K K A G V N L E H N E I P T N L G G W L T A N V

5001 TGGAATCATTGTCACTATTAGGTCCATCATCTAATGATCAGACCATCTATTACAATATAATCAGAGATTTCTTAAAGAAAGATGATTTTGAGCAAGTAAA
E S L S L L G P S S N D Q T I Y Y N I I R D F L K K D D F E Q V K

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Q S T S S E R F L D L R F E E L K Q K K E R D K L E L N D K K M I

5201 TTTTTAATGAATCTATTTGAGCAGTCTTCTGTATCTGAAGATTCTGATGTTTTAAATATAGGGATGAAATTTCAGACAATGATGACTCAAATAATTAGGT
F L N N L F E Q S S V S E D S D V L N I G M K F Q T N N T Q I I R L

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P Q F I N E N A L S K M S S Y N D F C K L Y P H L R K N Q Q L H N

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S T K E I K S D E D M L I E D L D D Y E K I A P A N E M E E V H E

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Q L S S Q S T E K L A L D R I L R S K A R T F I N P D S D T K M T

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Y D E N L E K K I S T M K P L N E D S V S V F K T C I N L V L K D

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I P V V E G S P G L V V M Y S I Y G S D Y I E K L G L K N I P L T

6001 AGATAATAGTATTAATGTTTTAACAGGCACATTTGGATCCAGCTTGATGTTGGAGGATGTAAAATATTATGTCAAAGGTCTTGAAGTATTCGAGACTGAA
D N S I N V L T G T F G S S L M L E D V K Y Y V K G L E V F E T E

6101 GAATTCCAAAATTCTAATAGATGCCAGAGAGCAGTGAAAGCTTGCAATTACATGATAACAGCCCAAAATAGGTTGCTTGCTATTAACACGTGTTTTTCAA
E F Q N S N R C Q R A V K A C N Y M I T A Q N R L L A I N T C F S R

6201 GAAAAAATTTCCCTTTCTATTCTAAATTCAATTTAGGCAGAGGCTTTGTATCAAACACATTGGCATTAATGTCAAGCATTTACAGTAAAGAAGAATCATT
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6301 TCATTTTATGTCAAATGTGCATTTTAAAATAGACAAATCCATAAGAGCTATAATAAGTGCACAACAAGATCTAAATTTGGAGAGAATTTTGGACACTGCT
H F M S N V H F K I D K S I R A I I S A Q Q D L N L E R I L D T A

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6501 GGGATACATTAGATTCAAAAATGGATAAAATAAATCATGCTATGGAAAGGAAAATGACTACATCTAACATTCTCCTGTCTCATAACAGTGAATTAAACAC
D T L D S K M D K I N H A M E R K M T T S N I L L S H N S E L N T

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6901 ATCAAAATTTTTGTTTGACAAAGGTAATGTGATACGATCCAAACAGAGCCAAACAGTTTTCCTAAACCCAGGCCAAGCTTGTCTAAGAACAACAACTGAT
S K F L F D K G N V I R S K Q S Q T V F L N P G Q A C L R T T T D

7001 GGTAAGCTTATTGCAAAAGTAAATCCGACTCCTAAAATTGCTGAAAGTTGACAATGTAAAACTAATAATGGACATCAATTATGAAAATGTGAACTCTGATG
G K L I A K V N P T P K L L K V D N V K L I M D I N Y E N V N S D V

7101 TTTGGTCTATAATAGAGAGTCAAAAACAAATTGATTTAAGACTTCCAGAAACAGGTGAATGTTACTCTGAAATGTATAAAACAATTGATTCGGAGCAGGG
W S I I E S Q K Q I D L R L P E T G E C Y S E M Y K T I D S E Q G

7201 TTTGATTTATGAAATGAAATCTAATTTGATTAAGTCATTGACTTTTATAAACACATTTGCAGATCTGAATGAATCTGTTTATTCTATAGATGATGAAGTC
L I Y E M K S N L I K S L T F I N T F A D L N E S V Y S I D D E V

7301 ACAAGAGAAACCATTTTTGATTTCATAGATAGTATAAGGAATGATTGTCTAGAAGGTTTAGAAACTTCCAAAAGTGTTGAAGAGTATGAAGAATTTTTGG
T R E T I F D F I D S I R N D C L E G L E T S K S V E E Y E E F L D

7401 ACACCCACGGGTTTAGGGAAACTGTTAGCCTTTTAAAAACATGATAGAGAGTTTAGAGTCATTGGATGCTGAATATAGCCCTATATTCTTGAATATAAC
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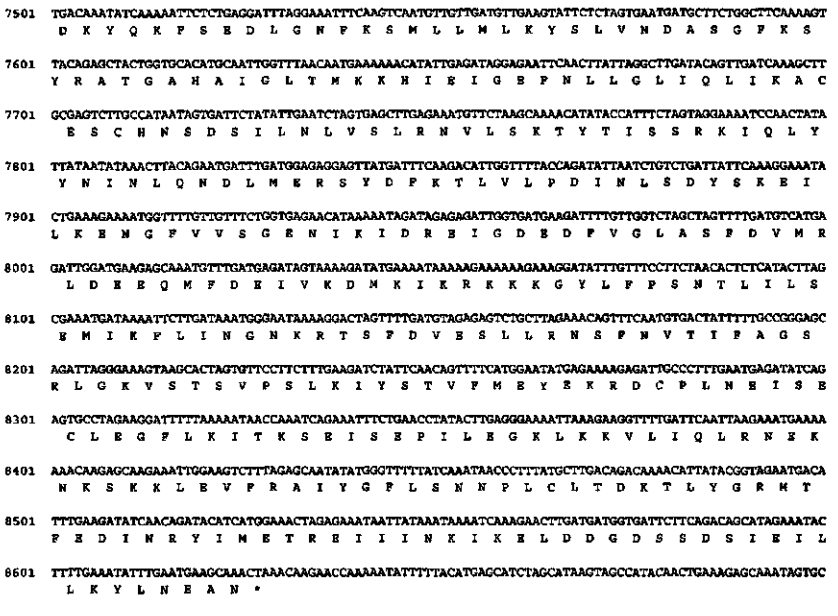


Figure 2. The complete nucleotide sequence of INSV L RNA (numbered from the 5' end of the viral complementary strand) and its predicted gene product. The deduced amino acid sequence of the protein encoded by the viral complementary RNA is written below the RNA sequence. The asterisk (*) indicates the UAA termination codon.

Characteristics of the INSV L RNA sequence

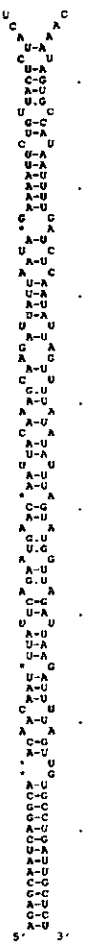
The INSV L segment is 8776 nucleotides long (Fig. 2) and contains 37% A, 30% U, 14% C and 19% G (or 67% AU and 33% GC). The 5' and 3' ends of INSV L RNA are complementary and can be folded into a hairpin structure, a typical feature of all segmented negative strand viruses, which is thought to play a role in transcription/replication (Fig. 3).

Predicted gene product of INSV L RNA

Analysis of the six different reading frames of the viral (v) and viral complementary (vc) RNA revealed the presence of one large open reading frame (ORF) in the viral complementary strand (Fig. 4). The ORF starts with an AUG codon at position 8744 (numbered from the 5' end of the viral RNA) and extends to an UAA stop codon at position 139, resulting in a primary translation product of 2865 amino acids with a predicted molecular weight of 330.3 kDa. Analysis of the predicted protein reveals several short hydrophobic regions (Kyte and Doolittle, 1982).

Comparison of INSV L RNA and TSWV L RNA

Comparison of the INSV L segment with that of TSWV revealed 68.9% identity in nucleotide sequence whereas at the amino acid level 69.5% identity and 83.6% similarity was found, using the GAP function of the GCG package from University of Wisconsin. The alignment of TSWV and INSV L proteins revealed a frameshift in the L ORF of TSWV. Upon resequencing of the original clone 806 (De Haan *et al.*, 1991), an insertion of an U residue at position 4206 was found and an U residue at position 4129 was deleted. After restoring these errors the reading frame showed 100% match in this region.



Interestingly, the L RNA sequence of INSV is 124 nucleotides shorter than that of TSWV (8897 nt), resulting in an ORF which is 10 amino acids shorter, lacking the acidic tail (Fig. 5). Furthermore, the 3' nontranslated region of INSV L RNA (vc sense) is 140 nucleotides long whereas for TSWV this region comprises 242 nts (a duplication of the AUUU sequence at position 55 was found in the TSWV L sequence). The INSV L sequence was verified by cloning and sequencing of several PCR fragments in this region derived from independent amplification experiments. Primer extension analysis of nucleocapsid RNA confirmed that the L RNA of INSV is indeed shorter (data not shown). Sequence analysis of US-01, an American isolate of INSV (Law and Moyer, 1990) revealed the same sequence in this region, including the shortened ORF (Fig. 5) and 3' end (data not shown).

Analysis of all three RNA segments of INSV and TSWV in a dot plot revealed that the L RNA is the most conserved RNA segment, whereas the S RNA is least conserved (Fig. 6). A genetic comparison of individual gene products of INSV and TSWV shows that the L protein is the most conserved gene product (Table 1).

Comparison of the INSV L protein with those of other Bunyaviruses

Comparison of the L protein of INSV with that of *Bunyaviridae* belonging to the other genera (Elliott, 1989; Schmaljohn, 1990; Antic *et al.*, 1991; Stohwasser *et al.*, 1991; Müller *et al.*, 1991; Elliott *et al.*, 1992; Accardi *et al.*, 1993; Roberts *et al.*, 1995) revealed that significant homology was found with Bunyamwera virus L protein and La Crosse

Figure 3. The complementary sequences at the termini of the INSV L RNA. The numbers represent the position to the 5' end of the vRNA.

L protein. Homology with the other members of the *Bunyaviridae* was restricted to a shorter internal stretch of approximately 200-250 residues. This stretch contains five types of short consensus sequences which are characteristic for RNA polymerases that display RNA template specificity, the polymerase motifs (Fig. 7)(Poch *et al.*, 1989; Tordo *et al.*, 1992). Of these conserved motifs the SDD motif has been shown to be of functional importance in both Bunyamwera L protein (Jin and Elliott, 1992) and influenza A polymerase (PB1 subunit, (Biswas *et al.*, 1994)). RNA dependent RNA polymerase activity has been demonstrated to be associated with purified virus preparations of TSWV (Adkins *et al.*, 1995) and with purified nucleocapsid preparations of both TSWV and INSV (see Chapter 5) although direct evidence linking RNA polymerase activity to the L protein is lacking.

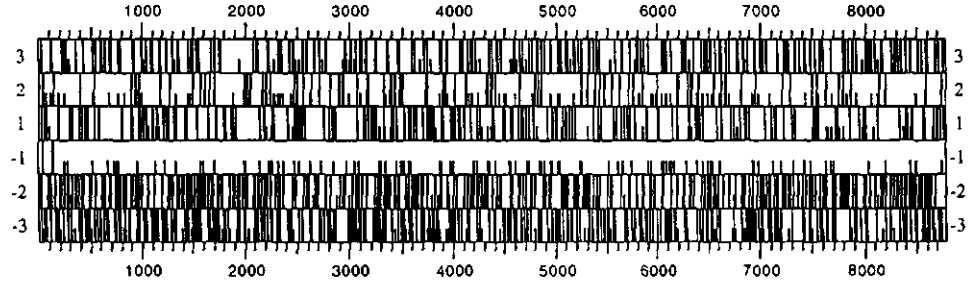


Figure 4. Distribution of translation initiation (short vertical bars) and termination (long vertical bars) codons in the three possible reading frames of the viral (1, 2, and 3) and viral complementary (-1, -2, and -3) L RNA strands.

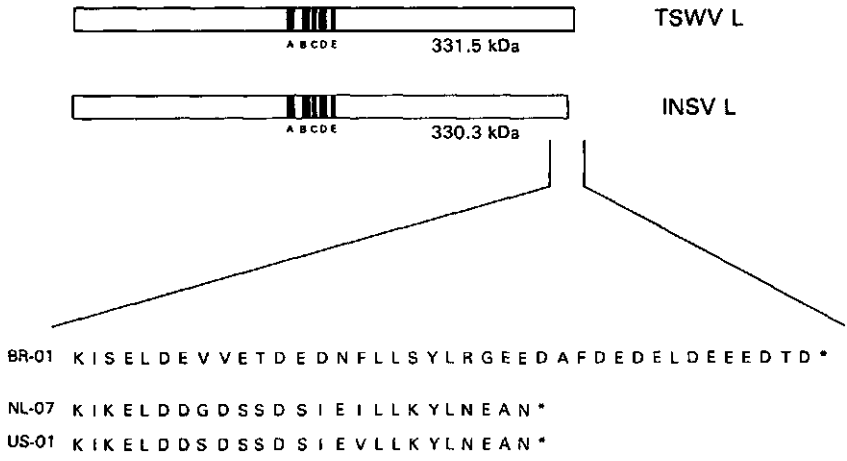


Figure 5. Comparison of the L ORFs and the C-terminal sequences of TSWV (BR-01), INSV (NL-07) and INSV (US-01). Polymerase motifs shown in Fig. 7 are depicted as bars.

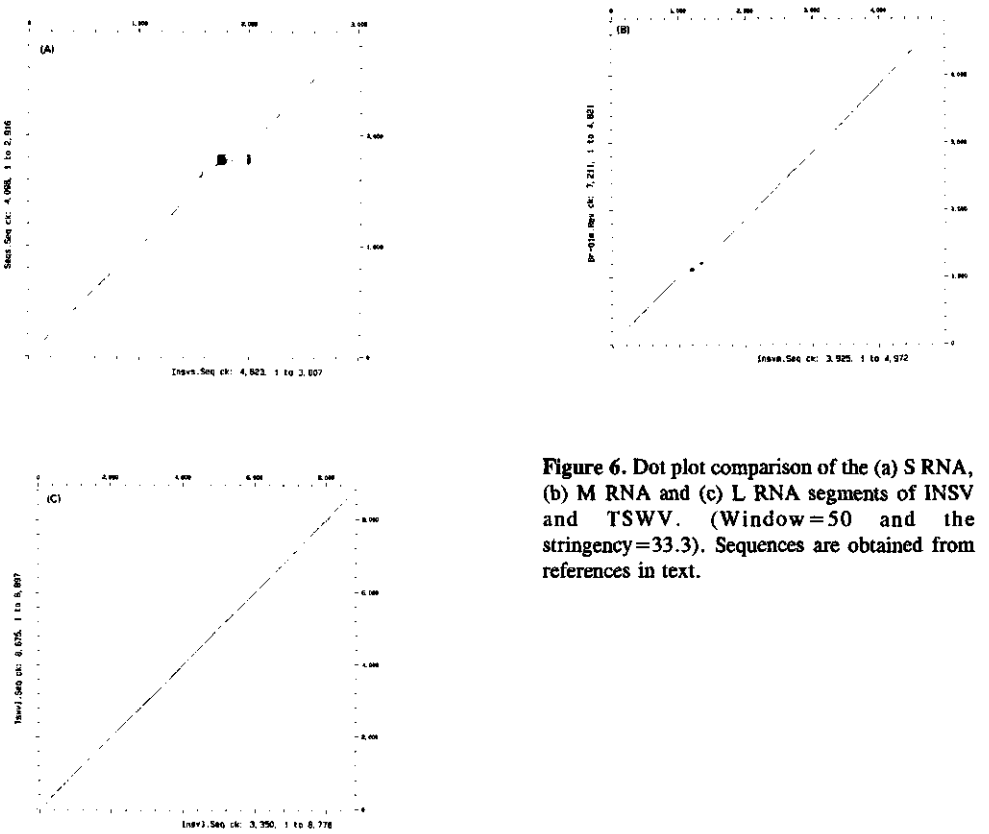


Figure 6. Dot plot comparison of the (a) S RNA, (b) M RNA and (c) L RNA segments of INSV and TSWV. (Window=50 and the stringency=33.3). Sequences are obtained from references in text.

Discussion

With the elucidation of the L RNA sequence, INSV is the second tospovirus of which the complete genomic sequence has been determined. The INSV L RNA is 8776 nucleotides long which is comparable to the size of the L RNA of TSWV. Both tospoviral L RNAs (and their corresponding gene products) are significantly longer than those of their animal-infecting counterparts. Like the S and M RNA, the L RNA contains complementary 5' and 3' termini of about 80 nucleotides that can form a panhandle structure which may be involved in the appearance of circular nucleocapsids in virus particles (Peters *et al.*, 1991).

INSV L RNA contains a single ORF in the viral complementary strand corresponding to a protein with a predicted molecular mass of 330.3 kDa, the putative RNA dependent RNA polymerase. Comparison of the INSV L protein with that of TSWV (De Haan *et al.*, 1991) reveals 69.5% identity and 83.6% similarity thus being the most conserved protein.

Strikingly, the L protein of INSV lacks the typical acidic tail at the C-terminus (Fig. 5.). The significance of this extremely acidic C-terminus (5 glutamic acid and 5 aspartic acid residues out of 15, see Fig. 5) in the TSWV L protein, and its absence in that of INSV is not clear yet, but it is very likely that the exposure and folding of the two tospoviral polymerases will be rather different in this region. The acidic tail might be involved in interactions with the (basic) nucleocapsid protein, although the lacking acidic residues of the INSV L protein do not seem to have an effect on *in vitro* transcription/replication activity (see Chapter 5).

	MOTIF A	MOTIF B		
INSV	**K**SKLAFLSADQ**G**KW**S**	STNSYPVSMN**W**LQ**G**NLNYLS**S**VY**H**		
TSWV	**K**SRLAFLSADQ**S**KW**S**	TTNTYPVSMN**W**LQ**G**NLNYLS**S**VY**H**		
La Crosse	**K**GLKMEINADM**S**KW**S**	AQ.DVFYKYN**W**LQ**G**NFNYT**S**SY**H**		
Bunyamwera	**K**ALKLEINADM**S**KW**S**	AQ.DVFYKYN**W**LQ**G**NFNYI**S**SY**H**		
RVFV	PVWTCATSDDAR**K**W**N**	.QGHFVTK**F**GMMQGILHYT**S**SLL**H**		
Toscana	SVWTCATSDDAR**K**W**N**	.QGHYVTK**F**GMMQGILHFT**S**SLL**H**		
Uukuniemi	HHETVATSDDAA**K**W**N**	.QCHHVTK**F**GMMQGILHYT**S**SLL**H**		
Hantaan	**K**RKLMYV**S**ADAT**K**W**S**	P.*GDNSAKFNWLQGNLNKCSSLFG*		
Seoul 80-39	**K**RKLMYV**S**ADAT**K**W**S**	P.GDNSA**K**FN**W**LQ**G**NLNKC**S**SLFG		
Puumala	**K**RKLMYV**S**ADAT**K**W**S**	P.GDNSA**K**FN**W**LQ**G**NLNKC**S**SLFG		
	MOTIF C	MOTIF D	MOTIF E	
INSV	IVH**S**DDNATSLI	SHFK**S**FCITLNPK**K**SYA**S**	**S**SEV.**E**F**I**S	
TSWV	IVH**S**DDNATSLI	AHFK**S**FCITLNPK**K**SYA**S**	**S**SEV.**E**F**I**S	
La Crosse	LVH**S**DDNQTSIT	LTFGC.QA..NM**K**KTYVT	NCIK.**E**F**V**S	
Bunyamwera	MVH**S**DDNQTSLA	LTFGC.QA..NM**K**KTYIT	HTCK.**E**F**V**S	
RVFV	MQG**S**DD**S**SMLIS	KELGVYLAIYPSEK**S**TA**N**	TDFVME**Y**N**S**	
Toscana	MQG**S**DD**S**SMIIS	K**S**LGTYIGIYPSEK**S**TPN	TDFVME**Y**N**S**	
Uukuniemi	LQ**S**SDD**S**GMMIS	KVIGKYLGIY**S**SV**K**ST**N**N	TLHLLE**F**N**S**	
Hantaan	AHH**S**DDALFIYG	LLLG**S**IKI..SPK**K**TTVS	PTNA.**E**F**L**S	
Seoul 80-39	AHH**S**DDALFIYG	LLLG**S**IKI..SPK**K**TTL**S**	PTNA.**E**F**L**S	
Puumala	AHH**S**DDALFIYG	LLMG**S**IKI..SPK**K**TTV**S**	PTNA.**E**F**L**S	

Figure 7. Amino acid homology between *Bunyviridae* L proteins. Conserved amino acids were identified using the GAP and PILEUP options of the GCG package from the University of Wisconsin. Conserved amino acids are in bold. The position of the motifs are illustrated in Fig. 5 (see text for references).

Classification of tospoviruses has been based on serological differences of the (S RNA-derived) nucleocapsid protein. The L sequence reported here is determined from a Dutch isolate, NL-07, obtained from infected impatiens plants, i.e. the same isolate for which also the S RNA has been described by De Haan *et al.* (1992), whereas the M RNA sequence is determined from an American isolate, US-01 (Law *et al.*, 1992). Parts of the M RNA of NL-07 have been amplified by PCR, cloned and sequenced (results not shown). Out of the more than 700 nucleotides sequenced, only few nucleotide changes with the US-01 M RNA sequence were found and these changes did not lead to differences on amino acid level. This result indicates that the isolates NL-07 and US-01 are almost completely identical.

Comparison of the L proteins of tospoviruses INSV and TSWV with those of animal-infecting members of the *Bunyaviridae* indicates that tospoviruses are most closely related

to the genus *Bunyavirus* (Fig. 8). This is an interesting observation as tospoviruses have an ambisense S RNA, a feature they only share with members of the genus *Phlebovirus*. This may suggest that the generation of an ambisense gene arrangement is a relatively late event during bunyaviral evolution. The observation that tospoviruses have been adapted to plant hosts by inclusion of the NSm gene, in an ambisense arrangement within the M RNA segment, supports this hypothesis (Kormelink *et al.*, 1994; Storms *et al.*, 1995).

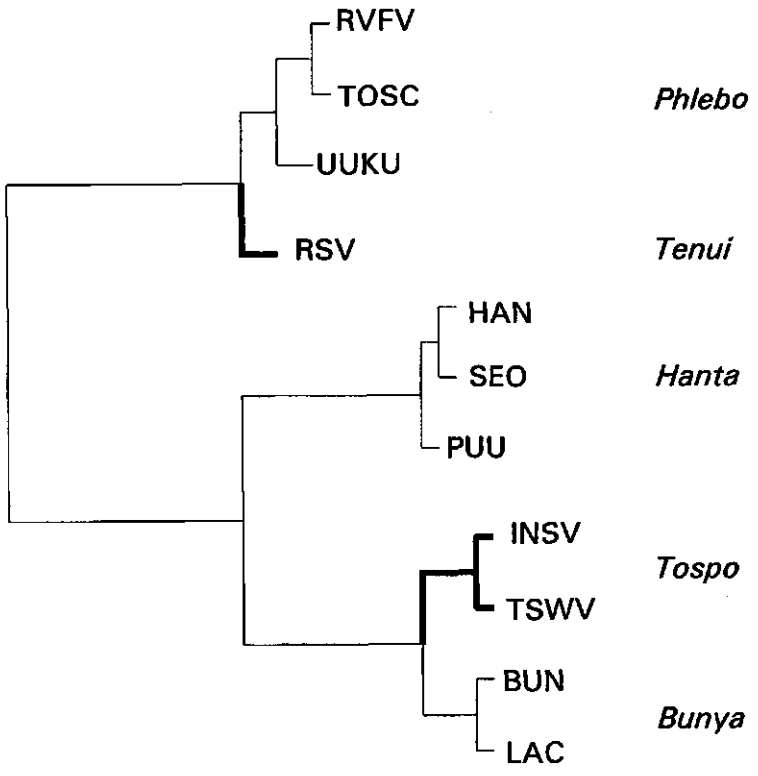


Figure 8. Genetic comparison of bunyaviral L proteins. The pile-up and growtree options of the GCG package from the University of Wisconsin were used to construct the phylogenetic tree. Rift Valley fever (RVFV), Toscana (TOS) and Uukuniemi (UUKU) virus belong to the genus *Phlebovirus*, Hantaan (HAN), Seoul (SEO) and Puumala (PUU) virus to the genus *Hantavirus*, Bunyamwera (BUN) and La Crosse (LAC) virus to the genus *Bunyavirus*, impatiens necrotic spot (INSV) and tomato spotted wilt virus (TSWV) to the genus *Tospovirus* and rice stripe virus (RSV) to the (floating) genus *Tenuivirus*.

Comparison of the bunyaviral L proteins with that of rice stripe virus (Toriyama *et al.*, 1994), belonging to the floating genus *Tenuivirus* (Murphy *et al.*, 1995), indicates that tenuiviruses have most close genetic relationship to the genus *Phlebovirus* (Fig. 8) and are only distantly related to the tospoviruses. Hence, it seems that these two genera of ambisense, plant-infecting viruses have been descended from the animal-infecting *Bunyaviridae* by two independent evolutionary pathways. This observation, together with the fact that tenuiviruses have 4 and sometimes even 5 genomic segments, indicates that tenui- and tospoviruses, though both representing ambisense, plant-infecting RNA viruses, are not easily to be harboured in a single virus family.

Acknowledgements

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CHAPTER 3

Detection of the L protein of tomato spotted wilt virus and cloning of its cistron

Summary

The 5'-terminal and 3'-terminal parts of the single open reading frame (ORF) in the L RNA of tomato spotted wilt virus (TSWV) were expressed using a prokaryotic expression system. Using antibodies raised against the obtained translational products, a 330 kDa protein could be specifically detected in preparations of purified virions and in nucleocapsid preparations from TSWV-infected leaf tissue. The results obtained indicate that the L protein of TSWV, though much larger than that of the animal-infecting bunyaviruses, is present in virus particles in an unprocessed, intact form.

A full length cDNA copy of the L RNA was constructed and cloned into an AcNPV transfervector. Recombinant baculoviruses were obtained that expressed a 67 kDa protein which reacted with a L-specific antiserum. Sequence analysis of the transfervector demonstrated that a deletion of 80 basepairs, causing the introduction of two premature stop codons, was present in the cloned, viral cDNA, resulting in a truncated L protein.

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Introduction

Based on both particle morphology and genetic organization of its tripartite RNA genome the plant virus TSWV has been placed into the family *Bunyaviridae*, which comprises a large group of arthropod-born, enveloped viruses. TSWV, together with a small number of closely related but less characterized viruses, forms a separate genus, denoted *Tospovirus*, within this family (Francki *et al.*, 1991; Peters *et al.*, 1991). Particles of TSWV consist of a lipid envelope, containing two glycoprotein species (G1 and G2), in which the genomic RNA segments are complexed with nucleocapsid (N) protein to form pseudo-circular structures. The S RNA of TSWV (2916 nucleotides (nts)) encodes, like that of phleboviruses, the N protein (28.8 kDa) and a non-structural protein (NSs, 52.4 kDa) in an ambisense arrangement (De Haan *et al.*, 1990; Giorgi *et al.*, 1991; Ihara *et al.*, 1984; Kormelink *et al.*, 1992a; Marriot *et al.*, 1989; Simons *et al.*, 1990). The M RNA is also ambisense encoding the precursor to both glycoproteins (127.4 kDa) and another non-structural protein (NSm, 33.6 kDa) (Kormelink *et al.*, 1992).

The L RNA is of complete negative polarity and contains one open reading frame (ORF) in the viral complementary (vc) sense, which corresponds with a primary translation product of 331.5 kDa which by occlusion most likely represents the viral polymerase (De Haan *et al.*, 1991). The predicted protein is homologous to the L proteins of the animal-infecting *Bunyaviridae* and contains several amino acid motifs that are conserved among RNA polymerases of negative-strand viruses (De Haan *et al.*, 1991; Poch *et al.*, 1989).

While the proteins coded for by the M and the S RNA have all been detected in either virus particles or infected plant tissues, the L protein, theoretically encoded by the L RNA, has not yet been identified. Several reports have claimed the occurrence of a "large" protein in purified TSWV preparations with an estimated size varying between 110 kDa and 220 kDa (Mohamed *et al.*, 1973, Peters *et al.*, 1991; Tas *et al.*, 1977).

For some of the animal-infecting bunyaviruses the predicted molecular weight of the L protein (deduced from the open reading frame in the L RNA) matches the experimentally determined sizes of this protein (as determined by SDS-PAGE), which is about 250 kDa (Elliot *et al.*, 1984; Elliott, 1989; Schmaljohn, 1990). The first aim of this research was to detect the L protein both in purified TSWV virions and in TSWV-infected plant tissues, and to investigate the apparent discrepancy between the predicted (331.5 kDa) and the reported sizes (110-220 kDa) for this protein.

The second aim was to construct a recombinant baculovirus which contains a full-length cDNA copy of the ORF encoding the TSWV L protein. Until recently, it has not been possible to introduce genetic changes into cloned copies of negative stranded RNA virus

genomes and to express infectious transcripts from these clones. A major obstacle to the development of such techniques is the fact that deproteinized genomic RNA of negative stranded RNA viruses is not infectious. In order to be infectious, the RNA must be encapsidated with a nucleocapsid protein to form an active template and provided with the RNA dependent RNA polymerase.

Luytjes *et al.* (1989) were the first who described a system in which an RNA derived by transcription from a cDNA clone, containing a foreign gene flanked by regulatory sequences of an influenza virus genome segment, could be introduced into cells and amplified with a helper virus. A different reverse genetics approach was introduced for Bunyamwera virus, belonging to the genus *Bunyavirus* (Jin and Elliott, 1991). In this system a recombinant vaccinia virus expressing the L protein of Bunyamwera virus was used to transcribe and replicate *in vivo* transfected nucleocapsids which were not infectious by itself. This system proved to be very useful to delineate functional domains within the Bunyamwera virus L protein (Jin and Elliott, 1992, 1993). Recently, a similar system has been described (Lopez *et al.*, 1995) for Rift Valley fever virus (a phlebovirus) where the L protein can rescue viral RNP's and transcribe synthetic genome-like RNA molecules. As a first step towards a reverse genetics system for TSWV the construction and expression of a full length cDNA copy of the L RNA is described.

Materials and Methods

Virus purification

The TSWV isolate BR-01 was maintained on *Nicotiana rustica* by mechanical inoculation. Virus particles and nucleocapsids were purified as described by De Avila *et al.*, (1990). Virus was concentrated after sucrose gradient centrifugation by mixing with an equal volume of resuspension buffer and subsequent centrifugation for 1.5 hrs at 32000 rpm using a Beckman TL100 ultracentrifuge (TL55 rotor).

Primers and PCR

PCR fragment L-n (900 bp) was constructed using Taq polymerase (SpheroQ) and primers J062 (5'-CCCCCATGGAAATCCAGAAAATACAAAAA-3') and J029 (5'-TGTGGGTGTG GTTCCAAC-3'). The PCR fragment L-c (2 kbp) was made using primers ZUP40 (5'-CCC ATGGTTGATAAAGTG-3')andJ025(5'-CCCGGATCCTGCAGAGCAATCAGGTACAAC TAAAACATATAACCTCTCCAC-3'). P1V is a PCR derived clone which was amplified

with Vent DNA polymerase, and primers J035 (5'-TAGCATGATGCTATTAGC-3') and J037 (5'-GGTATACAAACCTTC-3'). P2 was amplified using primers J038 (5'-AATGTAACAG TTTTAAAG-3') and J033 (5'-CCACTTGCTGTTGAATTG-3') PCR fragment P3 (906 bp) was constructed using primers M11 (5'-CCCGGATCCTGCAGAGCAATCCAG-3') and JO29 (5'-TGTGGGTGTGGTTCCAAC-3'). P5 was amplified using PDH3 (5'-AGGGAACATTTC TTGTC-3') and J063 (5'-CCCGGATTCCTGCAGAGCAATCAGGTACAA-3').

Plasmid constructions

All PCR fragments were cloned into a T-vector (Marchuk *et al.*, 1990) and L-n and L-c were subcloned as a *Nco*I-*Bam*HI (N-B) fragment into a pET11t expression vector and transformed into *Escherichia coli* DH5αF'. The inserts were checked by partial sequencing. DNA-isolation, digestion with restriction enzymes, and agarose gel electrophoresis were carried out using standard procedures (Sambrook *et al.*, 1989).

Expression of 5'- and 3'- terminal parts of the L ORF in *Escherichia coli* BL21 cells

Overnight cultures containing pET11t/L-n, pET11t/L-c and pET11t were diluted 1:100 in fresh LB-medium containing ampicillin (50 μg/ml). Cells were grown for 4 hrs at 37 °C and subsequently induced by adding isopropylthiogalactoside (IPTG) to a final concentration of 0.4 mM. Cells were harvested 2 hrs post induction, centrifuged and resuspended in lysis buffer (50 mM Tris-HCl, pH=8.0; 5% SDS and 10mM ß-mercaptoethanol). Protein expression was analyzed by Coomassie Brilliant Blue (CBB) or silver staining according to Morrisey, (1981).

Antisera production

Both expression products L-n and L-c were purified from polyacrylamide gels as described (Kormelink *et al.*, 1991). Portions of 50 to 100 μl of purified protein were emulsified in Freund's incomplete adjuvants (Difco Laboratories) and injected into rabbits at days 1, 14, and 28. From day 28 on the rabbits were bled several times at two week intervals and gamma-globulin fractions isolated as reported (Clark and Adams, 1977).

Construction and isolation of recombinant baculoviruses

A full length cDNA copy of the L RNA was cloned as a *Bam*HI fragment into the unique *Bam*HI site of pAcDZ1 (Zuidema *et al.*, 1990), resulting in the transfervector pAc/L. *Spodoptera frugiperda* cells (Sf-21) were grown in Hink's medium supplemented with 10% fetal calf serum (FCS, Vaughn *et al.*, 1977). Sf-21 cells were cotransfected by lipofectin with the recombinant transfervector pAc/L and 1 μg of *Bsu*I cut viral DNA (AcPAK6) per 10^6 cells according to Kitts *et al.* (1993). The virus collected from the cotransfection supernatant

5 days post transfection was subjected to three rounds of plaque purification according to Zuidema *et al.* (1990). Two recombinants, denoted AcNPV/L1 and AcNPV/L2, were selected, amplified and the titer determined by an end point dilution assay (O'Reilly *et al.*, 1992). These recombinants were further analyzed for production of L protein after infection of either Sf-21 or T. ni 5B1-4 cells by resolving the proteins in a 5-15% polyacrylamide gel and immunostaining using the L-n and L-c antisera.

Results

Detection of the L protein of TSWV

To allow unequivocal identification of the L protein and to study possible post-translational cleavages, antibodies were raised against both the N- and C-terminus (Fig. 1A). The N- and C-terminal sequences were separately expressed using a prokaryotic expression system (pET system; Studier *et al.*, 1986). For this purpose two PCR-fragments were amplified, a 900 base pair (bp) construct encompassing the N-terminal (L-n) region of the L ORF, and a 1.8 kbp construct, corresponding with the C-terminal part of the L ORF (L-c). Primers J062 and ZUP40 contained a *Nco*I site (CCATGG) to place the ATG in the proximity of the Shine and Dalgarno sequence of the pET11t vector. As a consequence, in construct L-n the second codon in the L ORF was changed from AAC (Asn) to GAA (Glu), and in construct L-c the second codon was changed from TTT (Phe) to GTT (Val). Expression of these constructs resulted in polypeptides of expected size, i.e. 33 kDa for construct L-n and 67 kDa for construct L-c, respectively (Fig. 1B, lanes 1 and 2). Both products were purified from polyacrylamide gels as described by Kormelink *et al.* (1991). Antisera were prepared as described in Materials and Methods. The titer and specificity of antisera obtained were tested with protein blots containing the *E. coli* expressed L RNA-specific polypeptides (Fig. 1B, lanes 5 and 6). The detection limit of the antiserum raised against the N-terminal domain of the L protein (L-n) was approximately 2 ng and for the antiserum against the C-terminal domain (L-c) 4 ng (data not shown).

Both of the antisera reacted specifically with a large protein in both TSWV virion (Fig. 2, lanes 2 and 5) and TSWV nucleocapsid (Fig. 2, lanes 4 and 8) preparations. This protein comigrated with the 330 kDa protein of the high molecular weight markers. No reaction was obtained with healthy plant material (Fig. 2, lanes 3 and 7), indicating that the protein detected indeed represents the L protein of TSWV. Especially in nucleocapsid fractions some smaller immunoreactive proteins of unknown origin were detected (Fig. 2, lanes 2 and 5). These proteins probably do not represent proteolytic processing products of the L protein since they reacted both with the L-n and L-c antisera. So far no L protein could be detected

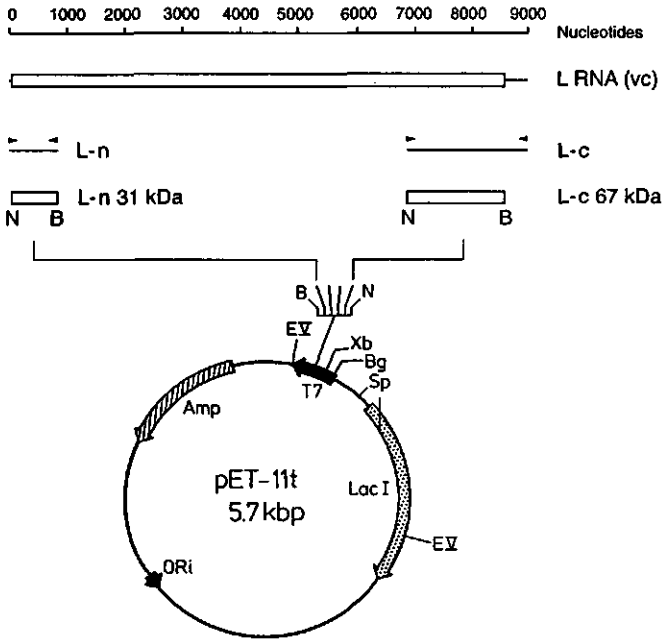

Figure 1A. Genetic organization of the TSWV L RNA (vc strand) and localization of PCR fragments used for expression in *E. coli*. Open reading frames are depicted as open bars.

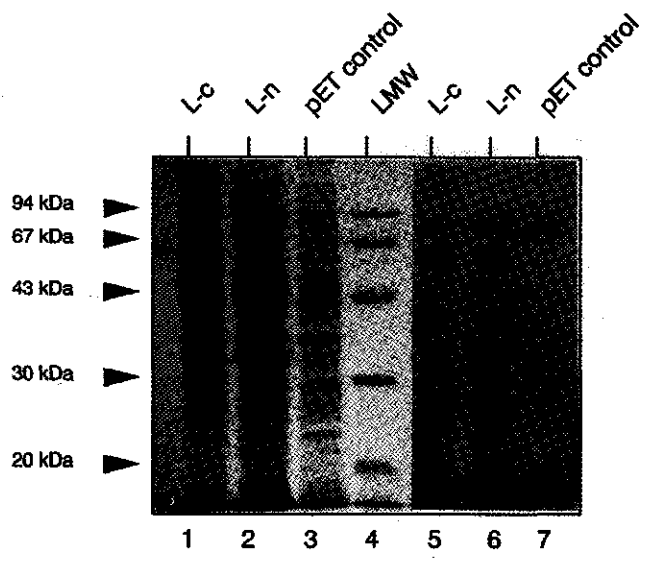

Figure 1B. Expression of constructs L-n and L-c in *E. coli* (BL21) cells. Proteins were resolved on a 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue (lanes 1-4) or blotted on Immobilon (lanes 5-7). The protein blot was analyzed using 1 μg/ml L-n or L-c IgG. LMW size markers, indicated on the left, included phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (20.1 kDa).

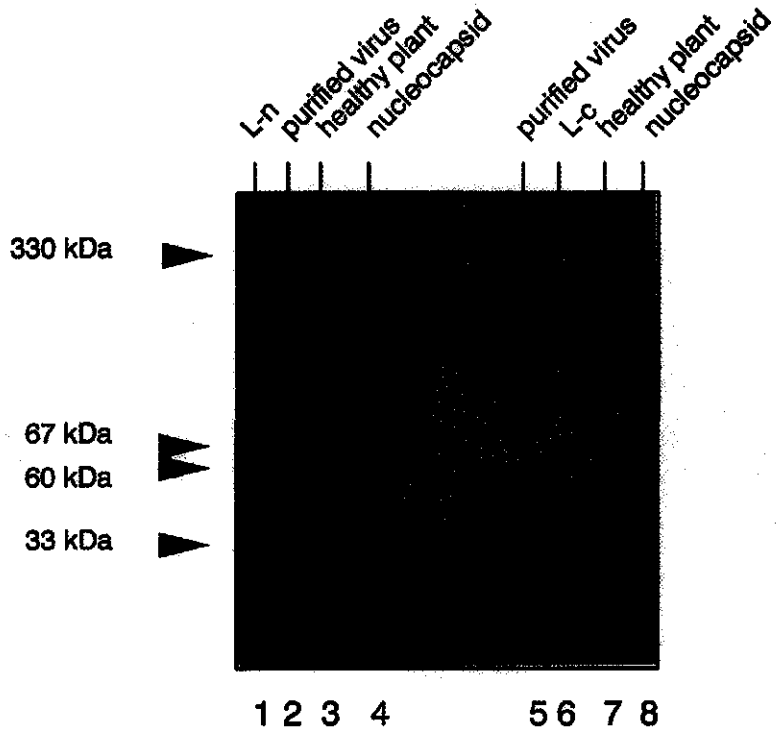

Figure 2. Detection of the L protein in purified virus and nucleocapsid preparations from TSWV-infected *Nicotiana rustica* leaves. After electrophoresis on a linear 5-15% gradient polyacrylamide gel the proteins were analyzed by silverstaining (Morrisey, 1981) or blotted and immunostained, using either anti-L-n (lanes 1-4) or anti-L-c (lanes 5-8) immunoglobulins. HMW size markers are indicated on the left.

in extracts of infected leaf tissue by using either western blotting, ELISA or immuno-gold decoration, probably due to the low concentrations in which this protein may occur in extracts.

Construction of a full-length cDNA clone of the L RNA

The cloning strategy followed to obtain a full-length cDNA clone of the L RNA is depicted in Fig. 3. The cDNA clones used in this work are described by De Haan *et al.* (1991); clones indicated with P are PCR derived. PCR fragment P3 (Fig. 3) was amplified to obtain the complete 3' terminal sequence (viral sense) including a unique *Bam*HI site to facilitate cloning into the baculoviral transfervector pAcDZ1. This PCR fragment was cloned into a T-vector and subsequently subcloned as a *Bam*HI-*Spe*I fragment into pKS$^+$ (clone P3). Clone P3 was extended by the *Spe*I-*Hin*dIII fragment of clone 662 to yield T1 (Fig. 3). Clone

T2 was obtained after addition of the *Eco*RI-*Hin*dIII fragment of clone 803 to an *Eco*RI-*Hin*dIII linearized clone T1. P1V is a Vent PCR derived clone, comprising nucleotides 2316-4262.

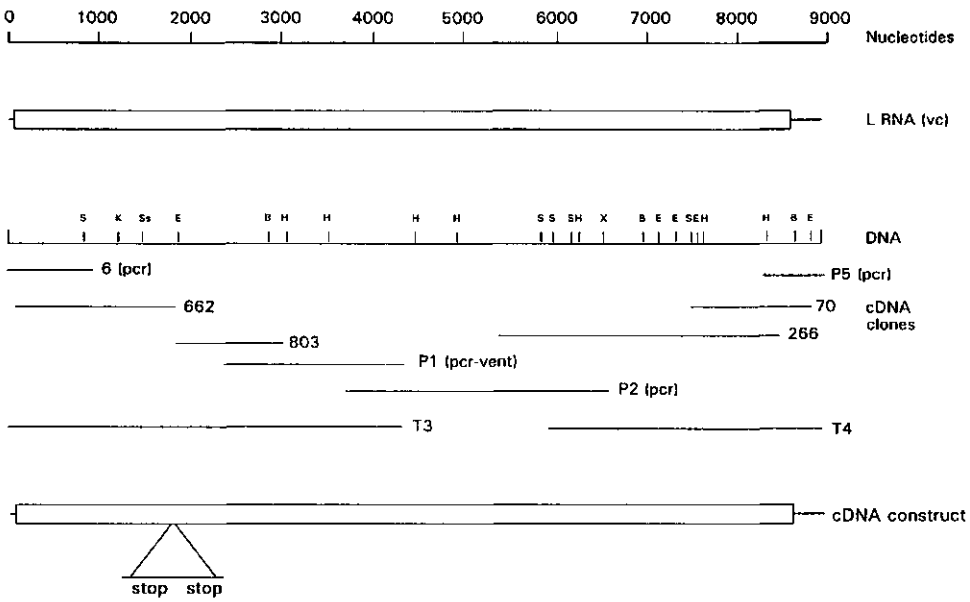

Figure 3. Cloning strategy used for the construction of a full-length cDNA copy of the L RNA of TSWV. cDNA clones are described by De Haan *et al.* (1991), PCR derived clones are denoted as P.

The *Bgl*II-*Xho*I fragment of P1V was subcloned into clone T2 to give rise to clone T3 which starts at the 3' end of the viral RNA and encompasses the unique *Nhe*I site.
PCR fragment P5 was constructed to obtain the 5' end (viral sense) including a *Bam*HI site (Fig. 3). The amplified fragment was cloned into a T-tailed *Eco*RV site of pKS$^+$ (clone P5). The *Spe*I-*Nru*I fragment of clone 266 was added to clone P5, giving rise to clone T4 (Fig. 3). The *Spe*I fragment of P2 was added to T4 yielding T5 which consists of the region corresponding to the 3' terminus of the vc L RNA and encompasses the *Nhe*I site. The full-length construct was obtained by cloning the *Nhe*I-*Xho*I fragment of T3 into a *Nhe*I-*Xho*I linearized T5 vector.

Construction and expression of a recombinant baculovirus containing the L ORF

The full-length cDNA copy of the L RNA was cloned as a *Bam*HI fragment into the unique *Bam*HI site of pAcDZ1. The resulting transfer vector, pAc/L, was analyzed by

restriction enzyme analysis and nucleotide sequence determination (results not shown). The L cDNA clone was transferred to AcNPV by cotransfection of *Spodoptera frugiperda* cells with a mixture of linearized AcNPV PAK6 DNA and the transfervector. DNA was isolated from nonoccluded virus and analyzed for the presence of a full-length L gene by *Bam*HI restriction enzyme digestion and subsequent Southern blot analysis (data not shown). Production of L protein was analyzed by comparing the protein patterns of AcNPV/L infected *Spodoptera frugiperda* cells with that of wild type AcNPV-infected Sf-21 cells. An expression product of 67 kDa, present in large amounts, was observed upon infection of Hi5 cells with AcNPV/L (Fig. 4). However, no product of the expected size (330 kDa) was detected. This result prompted us to resequence the transfer vector in the region where a stop codon was expected. Analysis of the sequence showed that there was a 80 bp deletion from nucleotide position 1715 to 1795, giving rise to two adjacent stopcodons. To reassure whether this deletion was the result of a cloning artefact, the original cDNA clone used to construct the full-length clone, was resequenced. The same deletion was indeed present in the original cDNA clone (clone 662). In order to restore the 80 bp deletion a PCR fragment was constructed comprising the 5' terminal 1.8 kb of the vc L RNA. This fragment was cloned into pET/11t and the expression product obtained was of the expected size and specifically reacted with the L-n antiserum, indicating that this clone contained the correct open reading frame. This PCR fragment started at position 34 (same primer used as for L-n), thus lacking the leader sequence. To analyze both the influence of the substituted second codon and the absence of the leader sequence on expression levels a recombinant AcNPV containing this PCR derived fragment was constructed. The expression product obtained after expression in either Sf-21 or T. ni 5B1-4 cells could only be detected using L-n antiserum (Fig. 4, left panel). This indicates that, although the PCR derived construct is expressed in *E. coli* and the ATG is in a rather optimal context according to the Kozak rules, for expression of the L gene in insect cells, a construct including the original leader is favored. However, a negative effect of the substituted second codon on the expression level cannot be ruled out.

Discussion

In previous studies on the protein composition of TSWV conflicting data were reported with respect to the molecular weight of the largest structural protein. Moreover, there is a considerable discrepancy between the size of the L protein as estimated from protein gels and

the predicted size based upon the nucleotide sequence as determined by De Haan *et al.* (1991). To allow unequivocal identification of the L protein, antisera were raised against prokaryotic expression products corresponding to the N- and C-terminus.

From protein blot analysis (data not shown) it was deduced that about 2 μg of virus is

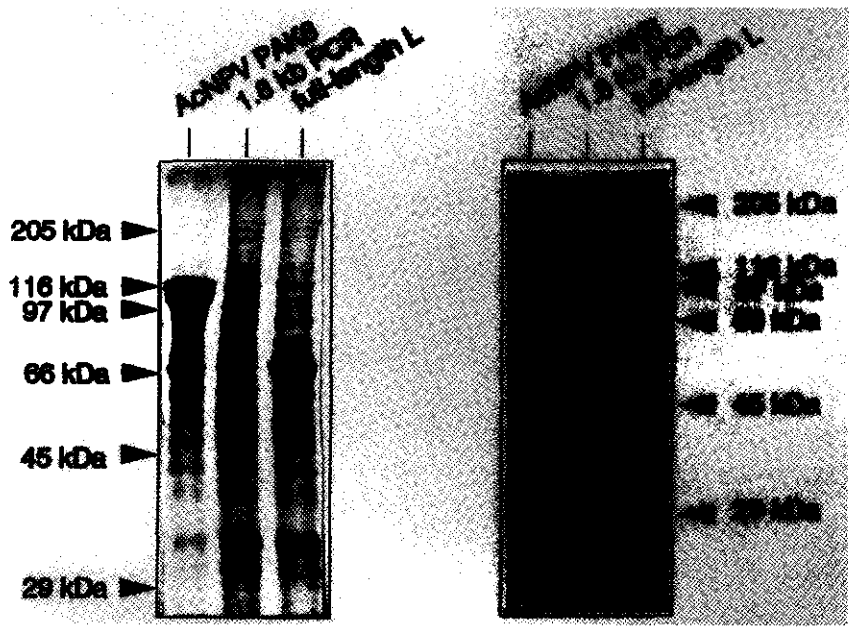

Figure 4. Analysis of expression products obtained after infection of Sf-21 cells with AcNPV/PCR or AcNPV/L. Proteins were resolved on a 5-15% PAGE and analyzed by CBB staining (left panel) or blotted and immunostained using anti-L-n IgG (right panel).

needed to detect the L protein. The detection limit of the antiserum is 4 ng for the L-c polypeptide which corresponds to 19.8 ng of L protein. This means that approximately 1% of the TSWV particle would consist of L protein. Based on this percentage, the diameter and density of the virus particle and estimates from silverstained polyacrylamide gels the number of L protein copies was calculated to range between 10-20 per TSWV particle. This number is comparable to the estimates that Jin and Elliott (1992) made for the Bunyamwera virus L protein (25 copies per virus particle).

Although the existence of functional, processed forms of the L protein *in situ* cannot be ruled out, our results demonstrate that TSWV virions as well as infectious nucleocapsid cores purified from infected cells contain a non-processed, viral L protein of 330 kDa. It is tempting to assume that this relatively large size of TSWV L protein reflects an adaptation

of this bunyavirus for being able to replicate in plant cells.

Analysis of the function of the L protein in the transcription/replication process of TSWV has been hampered by the lack of a manipulative genome. To unravel the function(s) of the L protein in the infection process of TSWV and shed some light on the existence of additional domains present on the L protein, a reversed genetics system is required. Several approaches have been described for both unsegmented and segmented negative strand RNA viruses. Within the family *Bunyaviridae* two reverse genetics systems have been reported (Jin and Elliott, 1991; Lopez *et al.*, 1995). For both Bunyamwera virus and Rift Valley fever virus the L protein is supplied by a recombinant vaccinia virus containing a full-length cDNA copy of the L RNA. The functionality of this protein was tested by an *in vivo* assay in which cells are infected with the recombinant vaccinia virus and subsequently transfected with natural ribonucleoproteins or artificial genome-like templates. In this chapter the construction of a full-length CDNA clone of the L RNA of TSWV is described. This clone is composed of both cDNA clones described by De Haan *et al.* (1991) and of PCR fragments. Upon expression of the L gene in insect cells using recombinant AcNPV containing the full-length construct an expressed product of only 67 kDa was detected. This product, which specifically reacted with the L-n antiserum, was of similar size as the TSWV-specific polypeptide expressed by a recombinant AcNPV containing the 1.8 kb N-terminal PCR fragment. Re-sequencing of this region in the full-length clone revealed a 80 bp deletion, giving rise to two adjacent stop codons, which later appeared to be present in the original CDNA clone 662 also. Comparing the expression levels of the full-length and the 1.8 kb PCR fragment revealed that the latter had a much lower expression level. The large difference in expression levels of the full-length construct and the 1.8 kb N-terminal PCR-derived construct could not be explained, but, in view of similar transcription levels, has to be on the level of translation.

It has been suggested that extra leader sequences in the chimaeric mRNA might impede expression in the baculovirus/insect cell system but this does not seem to be the case here. The presence or absence of a leader sequence does not unequivocally account for different expression levels of several baculovirus recombinants expressing different genes of TSWV (Kormelink, 1994). The low level of expression can also not be explained by an unfavorable AUG context (Kozak, 1981, 1986; optimal consensus A/GCCAUGG), as it is rather optimal for the recombinant containing the PCR fragment (G at position +4). The N-end rule (Bachmair *et al.*, 1989; Tobia *et al.*, 1991), which predicts the turnover speed of proteins due to the presence of rather unfavorable N-end amino acid residues downstream the methionine codon probably does also not account for the lower expression level as the amino acid substitution should decrease this turnover speed. Therefore, the full-length construct has to be repaired to overcome the 80 bp deletion and a part of the 1.8 kb PCR fragment can be used for this purpose.

Acknowledgements

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CHAPTER 4

Sequence analysis of the 5' ends of tomato spotted wilt virus N mRNAs.

Summary

Messenger RNAs transcribed from the tomato spotted wilt virus (TSWV) RNA genome have characteristic extra, non-templated heterogenous sequences at their 5' ends which may be the result of a cap-snatching event involving cellular mRNAs. In order to investigate the genetic origin of these extra sequences and to gain more insight in the process of cap-snatching as performed by TSWV, nucleocapsid protein (N) mRNAs derived from the TSWV S RNA were cloned and sequenced. Twenty clones were obtained which contained 5'-proximal sequences of non-viral origin, ranging in length from 12 to 21 nucleotides. None of the sequences analyzed were identical and no strict base preference at the endonucleolytic site was observed.

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Van Poelwijk, F., Kolkman, J. and Goldbach, R. (1996). Sequence analysis of the 5' ends of tomato spotted wilt virus N mRNAs. *Archives of Virology* **141**, 177-184.

Introduction

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus*, a genus belonging to the large family of arthropod-born *Bunyaviridae* (Francki *et al.*, 1991). The tospoviruses differ from the other members of this family by infecting plants instead of animals.

Typical for all bunyaviruses, the enveloped TSWV virion contains three single stranded RNA segments, denoted L, M and S, which are tightly associated with the nucleocapsid (N) protein. In addition 10-20 copies of the L protein are present per virion (Van Poelwijk *et al.*, 1993). The L segment is of negative polarity whereas the M and S segments have an ambisense gene arrangement. The M segment encodes the glycoproteins and a non-structural protein (NSm, the putative movement protein (Kormelink *et al.*, 1992c)), and the S segment encodes the N protein and another non-structural protein (NSs) (De Haan *et al.*, 1990; Kormelink *et al.*, 1991). The L segment encodes the L protein (331.5 kDa) which is thought to be the viral RNA-dependent RNA polymerase, although direct proof is lacking (De Haan *et al.*, 1991; Van Poelwijk *et al.*, 1993).

All segmented negative strand viruses studied so far contain non-viral sequences at the 5' ends of their mRNAs, indicating that the viral transcriptase utilizes RNA primers to prime transcription (Bishop *et al.*, 1983; Bouloy and Hannoun, 1978; Bouloy *et al.*, 1990; Collet, 1986; Eshita *et al.*, 1985; Garcin and Kolakofsky, 1990; Gerbaud *et al.*, 1987; Huiet *et al.*, 1993; Ihara *et al.*, 1985; Kormelink *et al.*, 1992b; Patterson and Kolakofsky, 1984; Raju *et al.*, 1990; Simons and Petterson, 1991). These primers are presumably derived from cellular mRNAs by a process referred to as cap-snatching, i.e. the 5' end of a cellular mRNA is cleaved off by an endonuclease and subsequently used to prime viral transcription. This process was first described for influenza virus (for review see Krug, 1981) for which it has been proposed that mRNA synthesis is initiated at the penultimate nucleotide of the template RNA, and that basepairing between the 3' end of the primer and the template is not required (Krug, 1981). Transcriptase activity has been detected in detergent disrupted preparations of a number of bunyaviruses (Bouloy and Hannoun, 1976; Gerbaud *et al.*,1987; Patterson and Kolakofsky, 1984). The low activity, compared to other negative strand viruses such as vesicular stomatitis virus, has hampered more detailed analysis of the transcription process. Patterson and Kolakofsky (1984) demonstrated that La Crosse virions contained a polymerase which was stimulated by dinucleotides (e.g. ApG), cap analogs (e.g. mGpppAm), and natural mRNAs (e.g. alfalfa mosaic virus RNA 4). In addition a methylated cap-dependent endonuclease activity was detected. Jin and Elliott (1991 and 1993a) were able to transcribe and replicate Bunyamwera S RNA *in vivo* by using the L protein that was expressed from

recombinant vaccinia virus. These experiments suggested that the L protein has the endonuclease activity which generates the primers needed for initiation of transcription.

For influenza virus it has been suggested that a specific sub-set of host cell mRNAs are used to prime influenza virus mRNA synthesis, given the preference for G-C-A terminated primer fragments (the terminal sequence of positive sense RNA is 5'- AGCA, (Shaw and Lamb, 1984)). Jin & Elliott (1993b) suggested a controlled polymerase slippage model for Bunyamwera virus to account for an apparent preference for the 3' end of a primer which resembles the 5' end of the viral RNA. This preference at the 3' end of the primer has also been demonstrated for snowshoe hare (Bishop *et al.*, 1983), Germiston (Vialat and Bouloy, 1992) and Dugbe virus (Jin and Elliott, 1993b) but not for Uukuniemi virus (Simons and Petterson, 1991). To gain more insight in the transcription process with TSWV, especially in the initiation process, viral mRNAs extracted from infected plants were analyzed. Sequence analysis of the 5' ends of TSWV mRNAs should reveal the origin of the extra non-templated sequences (viral or non-viral) and whether there is a base preference at the 3' end of the primer resembling the 5' end of viral RNA. For this purpose the 5' proximal parts of N mRNAs were cloned and analyzed.

Materials and Methods

Plants, virus and cDNA clones

The Brazilian isolate BR-01 of TSWV was maintained in *Nicotiana rustica* by mechanical inoculation. Complementary DNA clones representing the different RNAs of TSWV have been described previously (De Haan *et al.*, 1989, 1990 and 1991).

Total RNA extraction

Young seedlings of *Nicotiana rustica* were mechanically inoculated with extracts of TSWV BR-01 infected leaves. After inoculation, systemically infected leaf samples were taken at 8 days post infection (p.i.). Total RNA was extracted from TSWV-infected *Nicotiana rustica* according to De Vries *et al.* (1982).

Sucrose gradient centrifugation

Total RNA extracted from TSWV-infected *Nicotiana rustica* was resolved by centrifugation through 15-22.5% sucrose gradients in 50 mM Tris-HCl, pH 8.0, 1mM EDTA and 0.5% SDS. Prior to loading, the RNA was denatured with methyl mercuric hydroxide at a final concentration of 25 mM. Centrifugation was for 17 hrs at 24,000 rpm at 20°C in

a SW41 rotor. RNA was recovered from individual fractions of the gradient and subsequently resolved in 1% agarose gels (Baily and Davidson, 1976). The RNA was blotted onto Hybond (Amersham), and hybridized to ^{32}P-labelled strand specific probes corresponding to the 3' or 5' terminal region of the S RNA. Relevant fractions were pooled and the RNA was ethanol precipitated.

Cloning 5' ends of mRNAs

Specific cloning of the 5' ends was essentially done as described by Dumas *et al.* (1991), using the 5'-ampliFINDER™ RACE kit (Clontech). The procedure is schematically represented in Fig. 1A. The N mRNA was reversed transcribed using primer S1 identical to nucleotides 2404-2421 of TSWV S vRNA (5'-CTTAGATTTGATAGTATT-3'), based on the sequence as described by De Haan *et al.* (1990). After removal of the template by alkaline hydrolysis the (single stranded) cDNA was purified with GENOBIND glassmilk and ligated to the 3' blocked anchor primer 1 (3'NH_3-GGAGACTTCCAAGGTCTTAGCTATCA CTTAAGCAC-P 5') using T4 RNA ligase. The cDNA was amplified by PCR; 30 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min and extension at 72 °C for 1 min using oligonucleotides S2, identical to nts 2473-2493 of vRNA (5'-ATCAAGCCTTC TGAAGGTCAT-3'), and anchor primer 2 (5'-CTGGTTCGGCCCACCTCTGAAGGTTCCA GAATCGATAG-3'), which is partially complementary to anchor primer 1.

Results

Enrichment for subgenomic TSWV N mRNAs

In previous studies it has been shown that low amounts of S-specific mRNAs and viral complementary (vc) S RNA are present relative to the full-length viral (v) S RNA (Kormelink *et al.*, 1992a and b). Due to this low abundance partial purification of the mRNAs was required in order to obtain relatively enriched fractions. To this end total RNA was isolated from infected *N. rustica* and resolved on 15-22.5% sucrose gradients. Collected fractions were analyzed for their absorbance at 254 nm and after ethanol precipitation their RNA content was analyzed on a 1% agarose gel (Bailey and Davidson, 1976), transferred to Hybond membrane and hybridized to strand-specific probes corresponding to the N coding region in TSWV S RNA (Fig. 1). The enriched fractions were pooled.

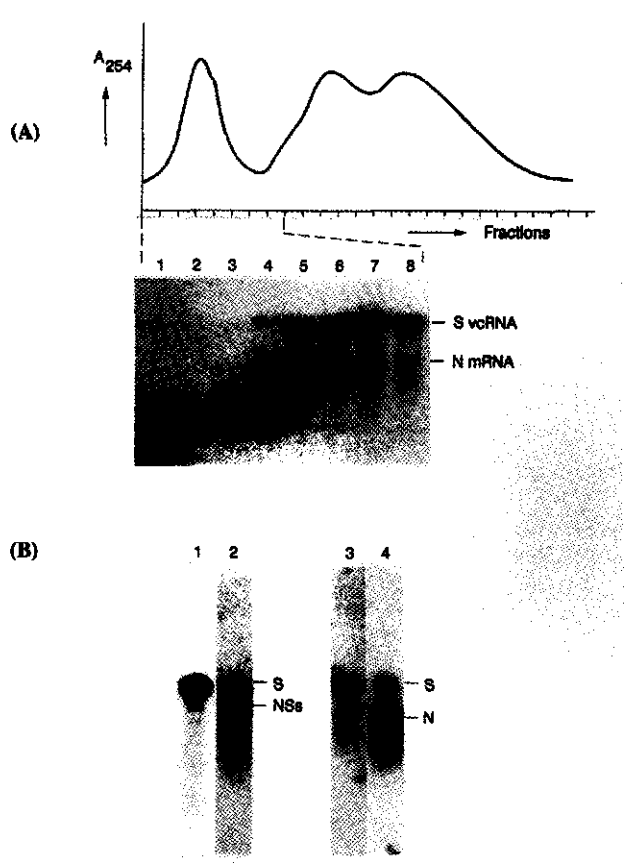

Figure 1. Sedimentation analysis and separation of RNA species from TSWV-infected *Nicotiana rustica* plants, 8 days post inoculation. Five hundred μg of total RNA from infected tissues was layered on a 15-22.5% sucrose gradient. (a) Absorbance profile of RNA fractions collected from the gradient. Sedimentation was from the left to the right. Two μg RNA from each fraction was resolved on a 1% agarose gel, transferred to Genescreen membrane and hybridized to riboprobe S2-v, specific for the N gene. (b) RNA pellets obtained after centrifugation through a CsCl cushion, enriched for the N mRNA (lane 2) and for the S vcRNA (lane 1), analyzed on a Northern blot using riboprobes S2-v. Riboprobes were prepared as described by Kormelink *et al.* (1992a).

Cloning of 5' ends of N mRNAs

The pooled, enriched fractions were either used directly for cloning or purified on a CsCl cushion according to Kormelink *et al.* (1992b). In Fig. 2a the strategy for cloning the heterogeneous 5' ends of TSWV N mRNAs is depicted. Briefly, first strand synthesis is performed using primer S1. Subsequently the RNA is hydrolyzed and anchor primer 1 is ligated to the cDNA using T4 RNA ligase. This fragment is then amplified by PCR using an upstream S2 primer, which enhances the specificity of the reaction, and anchor primer 2 which is partially complementary to anchor primer 1. The amplified product had the expected size of approximately 450 bp (Fig. 2b) and was cloned into a T-vector (Promega). Plasmids containing a cloned insert of the correct size were analyzed by dideoxynucleotide-chain termination sequencing using alkali-denatured dsDNA as a template (Sambrook *et al.*, 1989) and primer S3 (5'-CCCGCAGTCGTTTCTTAG-3') identical to nts 2832-2849 of S vRNA. The sequence data obtained are listed in Fig. 3. The viral sequence is given in capital letters

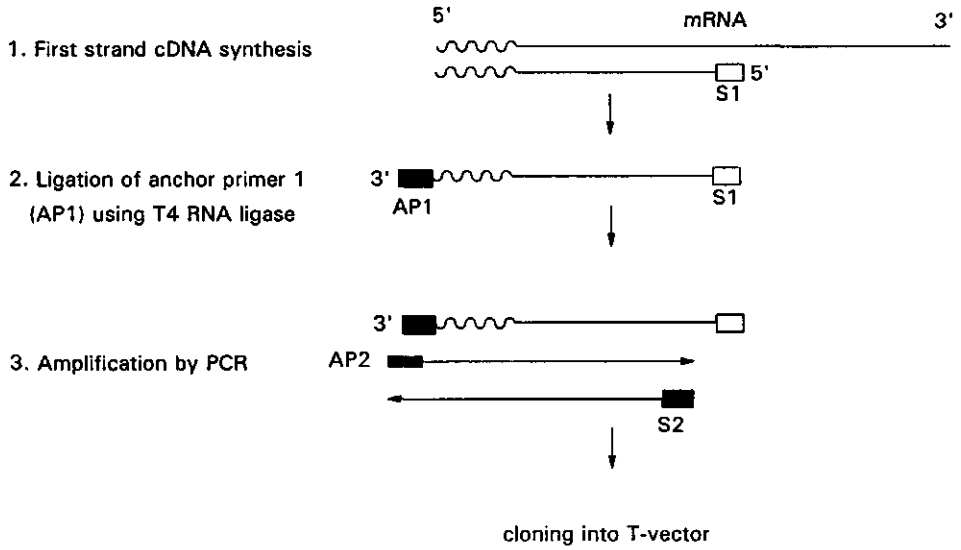

Figure 2. (a) Strategy for cloning the heterogeneous 5' ends of TSWV N mRNAs. Partially enriched mRNA fractions were used. Extra, non-viral leader sequences in the mRNA are indicated by a waved line and the location of primers used for amplification by boxes.

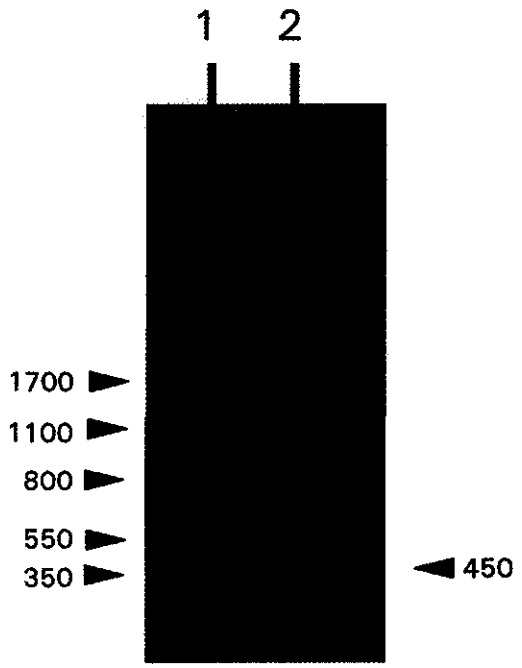

Figure 2. (b) Analysis of the PCR-amplified DNA fragments on a 1% agarose gel. A ± 450 bp product of expected size was obtained (lane 2). In lane 1 lambda DNA digested with endonuclease *PstI* is used as a molecular weight marker.

whereas the extra, non-templated sequence is given in lowercase letters. The 3' terminal sequence of the S RNA as described by De Haan *et al.* (1989), was confirmed with one exception. All clones analyzed revealed a C to U base substitution at position 2908 which is within the first nine terminal nucleotides that areconserved within all tospoviruses sequenced so far. The substitution occurred in the same position in three independent cloning experiments (including independent PCR amplifications) indicating that it is probably not due to a misincorporation by Taq DNA polymerase.

Of 36 cloned cDNA sequences, 20 revealed additional sequences between the 5' end of the genomic TSWV S RNA sequence and the anchor primer (Fig. 3). The 16 clones that did not contain additional sequences were probably derived from vcRNA, the replicative intermediate. The inserts contained the complete 5' end of TSWV S vcRNA (including the C to U substitution, data not shown). The mRNA leader sequences obtained were heterogeneous, ranging in length from 12 to 21 nucleotides and contained an overall base composition of 33% A, 30.4 % U, 19.6% C and 17% G residues (or 63.4% A+U, 36.6% G+C). The 3' proximate nucleotide of the primer consists in 9 out of 20 clones of a U residue while 4 clones contained an A residue, 4 a C residue and 3 a G residue, suggesting a slight preference for a U residue at this position.

clone	-10	+1
1	gguugauaggaaaau	GAGCAAUUGUGU
2	ggacgucguacuugu	GAGCAAUUGUGU
3	caauucgaauuucg	AGAGCAAUUGUGU
4	aaucugauucaguguggcgau	GAGCAAUUGUGU
5	gaugauaaauacacccuuuc	AGAGCAAUUGUGU
6	caauacuaucaaaucua	AGAGCAAUUGUGU
7	gguauuuaguuauuuu	AGAGCAAUUGUGU
8	gugugugugaauau	AGAGCAAUUGUGU
9	aagcaaaacaaucu	AGAGCAAUUGUGU
10	aauaauauacuaguacu	AGAGCAAUUGUGU
11	gcucuuugcucua	AGAGCAAUUGUGU
12	gauaggcaccgcauucc	AGAGCAAUUGUGU
13	gaucaauagcugg	AGAGCAAUUGUGU
14	uucuugucgcauc	GAGCAAUUGUGU
15	acacgcuaaacaa	AGAGCAAUUGUGU
16	auaacuucacaaacaa	AGAGCAAUUGUGU
17	uacaacccucuu	AGAGCAAUUGUGU
18	aaucgaccagaaguuau	AGAGCAAUUGUGU
19	ggacagcucucucuuc	AGAGCAAUUGUGU
20	ggauauaaauuaaaaag	AGAGCAAUUGUGU

Figure 3. Host-derived sequences at the 5' ends of TSWV N mRNAs. The viral complementary RNA sequence is shown in capital letters whereas the non-viral sequences are given in lowercase letters. The ultimate 5' nucleotide of genomic TSWV S vcRNA (A, which is complementary to nucleotide 2916 of the vRNA) is at the +1 position.

Table 1. Presence of host-derived sequences in mRNAs of segmented negative strand RNA viruses. See text for references.

Family	Genus	Species	mRNA	non-viral primer	3'-terminal base of primer
Bunyaviridae	Tospo	TSWV	N, NSs	12-20 nts	preference for U
	Phlebo	Rift Valley fever	G	12-14	
		Punta Toro	N, NSs	11-18	
		Uukuniemi	N, NSs	7-25	no consensus
		Toscana	N, NSs	9-15	
	Bunya	Bunyamwera	N	12-17	U
		Germiston	N, G	12-18	U,C
		Snowshoe hare	N, NSs, G	12-17	A
		LaCrosse	N, NSs	10-14	
	Nairo	Dugbe	N	5-16	C
Arenaviridae		Tacaribe	N	1-4	no consensus
Orthomyxoviridae	Orthomyxo	Influenza A	M (RNA7) NS (RNA8) H (RNA4)	10-18	A,G
	Tenuiviruses	Maize stripe	NCP(RNA4)	10-15	no consensus

Discussion

In this chapter the presence of non-viral heterogeneous sequences at the 5' ends of TSWV N and NSs mRNAs has been demonstrated. Primer extension analysis of enriched NSm mRNA fractions revealed the presence of 12-18 extra, non-templated nucleotides (results not shown). Such extra sequences have previously been found for other members of the *Bunyaviridae* indicating that it can be listed as a family characteristic. In order to gain more insight in the process of cap-snatching as performed by TSWV, nucleocapsid protein (N) mRNAs derived from the TSWV S RNA were cloned and sequenced. Twenty clones were obtained which contained 5'-proximal non-templated sequences, ranging in length from 12-21 nucleotides, and corresponding to the previously described primer extension products (Kormelink *et al.*, 1992b). These leaders must be host mRNA-derived as no homology is found with TSWV genomic sequences and therefore most likely result from a cap-snatching process. A search in the EMBL databank did not reveal any homology to known plant gene sequences.

For some viruses which utilize cap-snatching a marked preference concerning the identity of the 3'-proximate nucleotide (-1 position) in the host mRNAs derived leader sequence is observed while for other viruses there is no or hardly any consensus for this position (see Table 1). Comparison of the primer sequences of the TSWV mRNAs seems to indicate a slight preference for a U residue at the -1 position, as 9 out of 20 clones contain this residue at this position (Fig. 3). Remarkably, in 4 cases the A at position +1 was missing (Fig. 3). Similar results have been reported for mRNAs of influenza virus (Beaton and Krug, 1981), snowshoe hare (Bishop *et al.*, 1983), Germiston (Bouloy *et al.*, 1990), Bunyamwera (Jin and Elliott, 1993a) and maize stripe virus (Huiet *et al.*, 1993). This suggests that basepairing of the terminal nucleotide may not be absolutely required for an RNA to act as a primer in transcription initiation.

For the genera *Bunyavirus, Phlebovirus, Nairovirus* and *Tospovirus* the process of viral mRNA synthesis has been investigated and revealed a common mechanism for initiation of mRNA transcription. It is interesting that, on the one hand some bunyaviruses (e.g. Bunyamwera virus and Dugbe virus) show a strict sequence specificity for the 3' terminal nucleotide of the primer, while for Uukuniemi virus there seems to be no consensus at this position. Our results indicate that TSWV seems to take an intermediate position in this respect, with some preference (9 out of 20 mRNAs) for a U residue. This indicates that, although cap-snatching is a commonly used mechanism to prime transcription, there are differences in this process, not only between distinct families but even within a given family.

Acknowledgements

The authors wish to thank Drs. Richard Kormelink and Dick Peters for helpful discussions. This work was supported by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for Scientific Research (N.W.O.).

CHAPTER 5

Optimizing *in vitro* polymerase activity associated with purified nucleocapsid and virus preparations of tomato spotted wilt virus

Summary

An RNA-dependent RNA polymerase activity was found associated with both purified virions and nucleocapsids of tomato spotted wilt virus (TSWV). Trichloroacetic acid-precipitable products were obtained after incubation of detergent-disrupted TSWV virions in a transcriptase reaction mixture containing radiolabeled [α-^{32}P] CTP. The conditions for optimal incorporation were investigated. The reaction was manganese dependent and reaction products were RNAse sensitive whereas no DNA template was required. Reaction products hybridized with all three genomic RNAs of TSWV and with cDNA clones of all five viral genes, whereas no hybridization was detected to RNA extracted from healthy plant material. No specific inhibition of the *in vitro* polymerase reaction was observed upon incubation with available antisera against TSWV encoded proteins.

Introduction

Tomato spotted wilt virus (TSWV) is the type species of the *Tospovirus* genus within the large family of the arthropod-born *Bunyaviridae* (Murphy *et al.*, 1995). The virus is exclusively transmitted by thrips in a circulative/propagative manner (Wijkamp *et al.*, 1993). Virions consist of spherical, enveloped particles ranging in diameter from 80-110 nm. The three genomic RNAs, denoted L (large), M (medium), and S (small), are tightly associated with the nucleocapsid protein (N) to form ribonucleoprotein complexes (nucleocapsids). These nucleocapsids appear as pseudocircular structures in electron microscopy studies due to the complementary termini of the viral RNAs resulting in a panhandle formation. In addition, the virions contain 10-20 copies of the L protein, the putative viral RNA-dependent RNA polymerase (RdRp) (see Chapter 3).

Transcriptase activity has been detected in detergent-disrupted preparations of Lumbo (Bouloy and Hannoun, 1976), Germiston (Gerbaud *et al.*, 1987), La Crosse (Patterson *et al.*, 1984), Uukuniemi (Ranki and Petterson, 1975) and Hantaan virus (Schmaljohn and Dalrymple, 1983) representing three of the five *Bunyaviridae* genera. Moreover, transcriptase activity has directly been assigned to the L protein of Bunyamwera virus (Jin and Elliott, 1991). The transcriptase activity reported was weak compared to those of other viral taxa (e.g. Rhabdoviridae and Orthomyxoviridae), which has hampered a more detailed understanding of this process. Patterson *et al.* (1984a) demonstrated that bunyaviral polymerase activity can be stimulated by addition of oligonucleotides such as ApG, cap analogues (e.g. m^7GpppAm) and natural mRNAs (e.g. alfalfa mosaic virus RNA 4) and evidence was obtained that these acted as primers for mRNA synthesis. Furthermore, an endonuclease activity was detected which cleaved methylated caps *in vitro* (for review see Elliott, 1990). These results correlated with analysis of mRNAs from infected cells, which indicated the presence of heterogeneous non-viral sequences at their 5' ends (for review see Jin and Elliott, 1993). Both the presence of an unprocessed L protein in purified virus- and nucleocapsid preparations of TSWV (Chapter 3) and non-viral sequences at the 5' ends of viral mRNAs (Chapter 4) have previously been demonstrated. Recently, Adkins *et al.* (1995) were able to demonstrate *in vitro* transcriptase activity associated with virions of TSWV. Product analysis revealed that predominantly double-stranded RNA products were formed. Activity was manganese dependent and independent of a DNA template. Using (α-^{32}P)CTP incorporation of up to $300*10^3$ cpm was obtained in assays containing 13 μl of a concentrated virion preparation. Since it was not known whether this level of incorporation is sufficient to allow any further detailed studies with respect to product analysis, involvement of individual proteins or inhibition studies, we have tried to optimize the *in vitro* transcriptase

assay. Furthermore, transcriptase activity from two different tospoviruses, TSWV and INSV, were isolated from different source plants, as to determine possible host or virus-dependent differences. This chapter summarizes the efforts to increase the *in vitro* transcriptase activity and analysis of products formed using both purified virions and nucleocapsids.

Materials and Methods

Plants, virus, RNA extractions and cDNA clones

The Brazilian isolate BR-01 of TSWV was maintained in *Nicotiana rustica* and *Datura stramonium*. The Dutch isolate NL-07 of impatiens necrotic spot virus (INSV) was maintained in *Nicotiana benthamiana*. Complementary DNA clones representing the different RNAs of TSWV have been described previously (De Haan *et al.*, 1989, 1990, 1991; Kormelink *et al.*, 1994). RNA extractions were done as described by Kormelink *et al.* (1992b). Alfalfa mosaic virus RNA 4 was kindly donated by Dr. John Bol.

Virus and nucleocapsids purification

Young seedlings of *N. rustica* or *D. stramonium* were mechanically inoculated with extracts of TSWV BR-01-infected leaves whereas *N. benthamiana* was mechanically inoculated with extracts of INSV-infected leaves. TSWV and INSV were purified essentially as described by Mohamed *et al.* (1973) from systemically infected leaves harvested at 10-12 days post infection (p.i.). Leaf material was homogenized in a blender using 3 ml extraction buffer (0.1 M phosphate buffer pH 7.0, containing 0.01 M Na_2SO_3) per gram leaf material. The solution was strained through cheesecloth and centrifuged for 10 min at 10,000 rpm in a GSA rotor. The pellets were resuspended by stirring gently for 30 min at 4 °C in 1 ml 0.01 M Na_2SO_3 per gram of leaf material. After centrifugation for 15 min at 10,000 rpm in a SS34 rotor, the supernatant was centrifuged for 30 min in a Ti45 rotor at 29,000 rpm. The pellet was resuspended in 2.5 ml 0.01 M Na_2SO_3 for 30 min and subsequently centrifuged for 45 min on a 10-40% sucrose gradient at 23,000 rpm in a SW28 rotor. The virus band was collected, diluted 1:1 with 0.01 M Na_2SO_3 and centrifuged for 45 min at 24,000 rpm in a SW41 rotor. The pellet was resuspended in 200 μl 0.01 M Na_2SO_3.

Nucleocapsids were purified as described by De Avila *et al.* (1990) with modified buffers. The extraction buffer consisted of 0.1 M Tris-HCl, 0.01 M Na_2SO_3, pH 8.0 whereas the resuspension buffer contained 0.01 M Tris-HCl, 0.01 M Na_2SO_3, 0.5% Nonidet P-40, pH 8.0.

In vitro polymerase assay

The reaction was carried out in transcription reaction mixture consisting of 5.0 mM $MnCl_2$, 2.5 mM $MgCl_2$, 25 mM Tris-HCl (pH 8.0), 5.0 mM dithiothreitol (DTT), 0.5% (v/v) Triton X-100, 1 mM each of ATP, GTP and UTP, 10 μCi (α-^{32}P)CTP (3000 Ci/mmol, Amersham) and 10 μg virus. The reaction mixtures were incubated for 1 hr at 30 °C. Assays including reticulocyte lysate were carried out in a 50 μl volume, containing 40% (v/v) rabbit reticulocyte lysate, 50 μM tRNA and 20 μM amino acid mixture (Promega). After incubation for 1 hr at 30 °C the reaction mixture was phenol/chloroform extracted twice and the RNA subsequently ethanol precipitated. The pellet was resuspended in 10 μl RNase free H_2O and 5 μl was analyzed on gel.

TCA precipitations

Trichloroacetic acid (TCA) precipitations were essentially done as described by Sahal and Fujita-Yamaguchi (1987). In short, the 25 μl reaction mixtures were spotted onto Whatman 3MM or phosphocellulose Whatman paper (P-81) and the filters washed with cold 10% TCA for 20 min on a rotating shaker. Washing was repeated at room temperature with 5% TCA until the washing medium showed insignificant radioactivity (generally twice for 20 min). The papers were washed with ethanol, air-dried, squares cut out of the matrix and each was counted in a scintillation vial (Cerenkov counting, Beckman scintillation counter).

Results

Virus purification

Intact, enveloped particles of TSWV were purified from both *N. rustica* or *D. stramonium* as described in Material and Methods. The entire isolation procedure was carried out within 5-6 hrs and at 4 °C as to preserve optimal polymerase activity. The purity of the virion preparation was verified by electron microscopy (Fig. 1) and by analyzing the visible virus

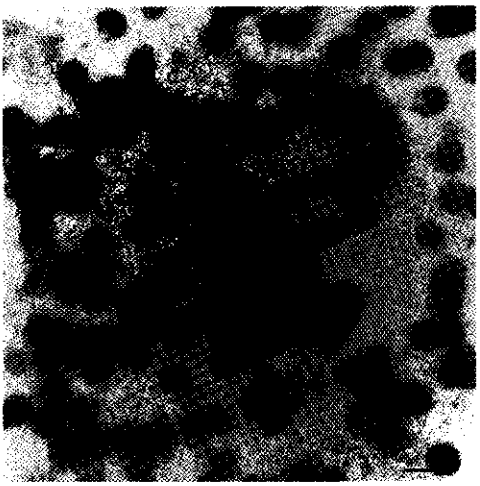

Figure 1. Electron micrograph of TSWV particles, purified according to the rapid procedure described in Materials and Methods. The bar represents 200 nm.

band of the 10-40% sucrose gradient for its protein content using SDS-PAGE (results not shown). A typical purification from 120 g of TSWV-infected leaf material yielded approximately 400 μg of virus.

In vitro polymerase activity

Purified virus was assayed for *in vitro* transcriptase activity, in principle following the assay conditions described by Adkins *et al.* (1995). Activity associated with purified virions could only be detected upon disruption by a non-ionic detergent. Incorporation of [α-^{32}P] CTP into TCA-precipitable products increased both in time (Fig. 2) and with increasing virus concentration (results not shown). Taken these results into account an incubation time of one

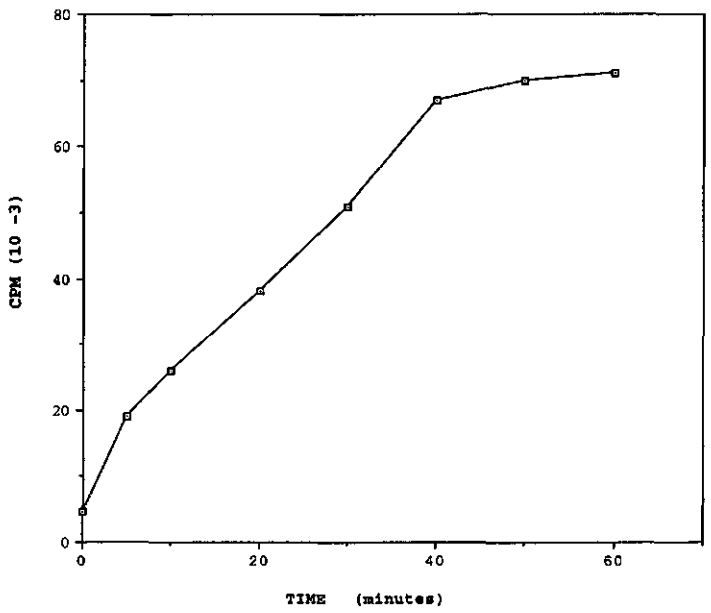

Figure 2. Time course analysis of the *in vitro* transcription assay using purified TSWV. For description of the assay components see Materials and Methods.

hours was chosen. The effects of omitting one or several of the standard transcriptase reaction mixture ingredients (see Materials and Methods) on the incorporation level were analyzed (Fig. 3). Assays were performed in triplicate as individual TCA precipitations can exhibit large variation leading to misinterpretation of the results. Using these assay conditions incorporation of up to $45*10^3$ cpm/μg of virion preparation could be reached, values (though somewhat higher) that confirm those reported by Adkins *et al.* (1995). It should be noted here that activity varied with the virus batch, the incorporation was on the average $30*10^3$ cpm/μg of virion preparation. Omitting virus from the reaction leads to incorporation levels that are near background (Fig. 3). Further proof that the transcriptase

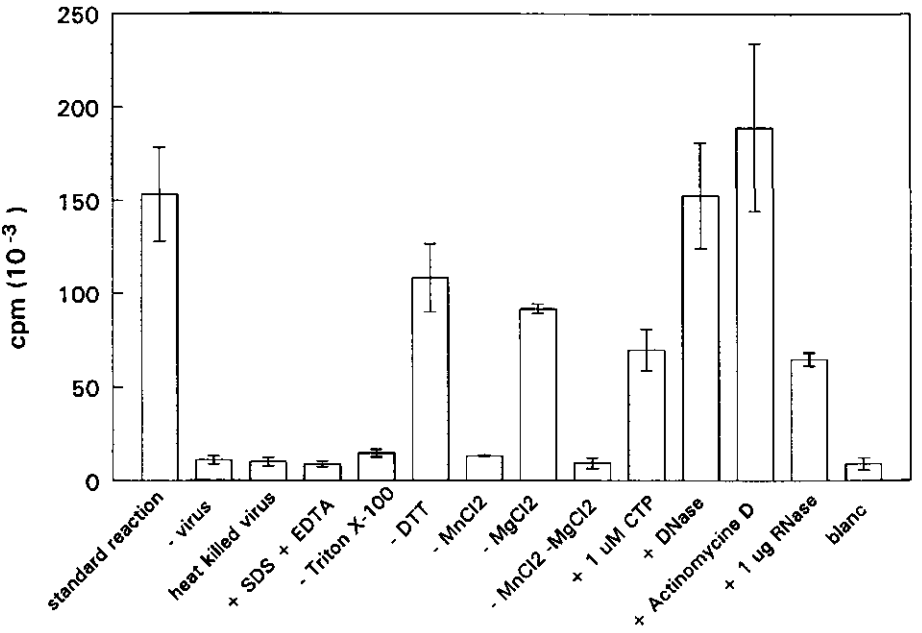

Figure 3. Analysis and optimization of the *in vitro* transcription reaction. The effect of omission of individual components of the standard *in vitro* transcription reaction on [^{32}P]CMP incorporation levels were measured by Cerenkov counting in a liquid scintillation counter. 1= standard reaction (see Materials and Methods), 2= -virus, 3= heat killed virus (20 min pre-incubation at 60°C), 4= + 1% SDS and 100 mM EDTA (final concentrations), 5= - Triton-X100, 6= -DTT, 7= -$MnCl_2$, 8= -$MgCl_2$, 9= -$MnCl_2$ and $MgCl_2$, 10= +1 μM CTP, 11= +RNase-free DNase, 12= +Actinomycine D, 13= +RNase (1μg), 14= blanc, (Whatmann 3MM paper only).

activity was unique for TSWV came from simultaneously treated fractions of healthy *N. rustica* which did not support any incorporation. The virus was effectively heat killed by (pre-) incubation for 20 min at 60 °C (Fig. 3). Adding SDS to a final concentration of 1% in combination with 100 mM EDTA also effectively abolished all polymerase activity (Fig. 3). Actinomycine D (a DNA-dependent RNA polymerase inhibitor) did not inhibit the reaction at a concentration of 100 μg/ml (Fig. 3), suggesting that the activity was not dependent on a DNA template.

Attempts to optimize the TSWV transcription assay.

To further optimize the *in vitro* CMP-incorporating activity of purified TSWV particles the following controls and treatments were carried out. Firstly, it was verified whether all virions lysed after the Triton-X100 treatment. Analysis of purified virus preparations before and after treatment with 0.5% Triton X-100 by electron microscopy revealed that after

treatment with this non-ionic detergent no virus particles were observed demonstrating complete lysis of the virions (results not shown).

Secondly, optimal concentrations of the various assay components were tested. Omission of DTT reduced incorporation to approximately 65% of the standard reaction (Fig. 3). The optimum manganese concentration in the reaction mixture was found to be 5 mM. The reaction was completely manganese-dependent as omission of this divalent metal cation results in background levels of incorporation (Fig. 3). In contrast, the effect of magnesium chloride was less prominent as omission of this component reduced the incorporation approximately 40%. The incorporation increased with $MgCl_2$ concentrations and remains at a constant level for all magnesium concentrations above 2.5 mM, confirming the results obtained by Adkins *et al.* (1995). If both manganese and magnesium chloride were omitted no specific incorporation could be detected. The addition of 1 μM (and higher concentrations) unlabeled CTP caused a reduction of 50% of the incorporation (Fig. 3).

Table 1. Effect of the source plant and tospovirus species on the *in vitro* transcription reaction. *Nicotiana rustica* is not a systemic host for INSV, therefore no *in vitro* transcriptase activity was tested for this combination. Reaction conditions are described in the Materials and Methods section.

VIRUS	HOST	ACTIVITY (10^3 CPM/μg)
TSWV	Datura stramonium	21 ± 2
TSWV	Datura stramonium	17 ± 1.5
TSWV	Datura stramonium	30 ± 3
TSWV	D. stramonium + N. rust.	10 ± 1.2
TSWV	Nicotiana rustica	10 ± 0.5
TSWV	Nicotiana rustica	32 ± 6
TSWV	Nicotiana rustica	44 ± 5
NUCLEOCAPSID	**HOST**	**ACTIVITY (10^3 CPM/μg)**
TSWV	Nicotiana rustica	10 ± 1
INSV	Nicotiana benthamiana	15 ± 2

Effect of host plant and virus species

Attempts to optimize the *in vitro* transcription assay in terms of higher incorporation levels also involved analysis of different tospoviruses versus preparations obtained from different

plant species e.g. *N. benthamiana*. Polymerase activity found associated with purified nucleocapsids of INSV was comparable to that of TSWV nucleocapsids isolated from *N. rustica* or *D. stramonium* (Table 1). Virus purification of TSWV from infected *D. stramonium* yielded about twice as much virus compared to purification from infected *N. rustica*. As a consequence more leaf material was used for virus purification from infected *N. rustica*. The observed variation in activity between individual virus preparations may be due to several factors, e.g. time of harvesting or synchronicity of virus infection (yield/gram leaf material). The variation in transcriptase activity using preparations of a given host is as large as the variations between the preparations from two different hosts, indicating that there is no host effect (Table 1). The manganese-dependency was less prominent in the experiments with nucleocapsid preparations which was probably due to impurities in these preparations. *In vitro* transcription activity was not observed in nucleocapsid preparations that were further purified on a $CsSO_4$ gradient. This is probably due to the dissociation of the L protein from the nucleocapsids. No L protein could be detected in these preparations using specific antisera (data not shown). The loss of *in vitro* transcription activity corresponds to the loss of infectivity as tested by a local lesion assay on *Petunia* leaves.

Qualitative analysis of the products formed in the *in vitro* transcription assay.

In their analysis of the *in vitro* transcription assay of TSWV, Adkins *et al.* (1995) describe *de novo* formed RNA products with an average length of 250 nucleotides in the standard reaction. These products hybridized to transcripts generated from cDNA clones of parts of the TSWV genomic RNAs. A discrete product of approximately 3 kb appeared upon the addition of unlabelled CTP in the assay. The addition of wheat germ extract yielded the same product profile. Whether this was due to the presence of CTP in the extract or reflects a requirement of TSWV *in vitro* RNA synthesis for ribosome binding to the nascent strand remained unclear.

In the following experiments attempts were made to elucidate the nature and specificity of the products obtained by the *in vitro* transcriptase reaction.

RNase-free DNase (Fig. 3) did not effect the incorporation indicating that the products formed are not DNA. Addition of RNase A (1 μg), however, drastically reduced the incorporation, which was even lower when 5 μg RNase A was added (approximately 10% of the standard, results not shown). These results confirm those of Adkins *et al.* (1995) and demonstrate that the templates, products, or both were RNA.

Genomic RNA of TSWV, total RNA from healthy *N. rustica* and alfalfa mosaic virus RNA 4 (as a control) was spotted onto a Hybond-N filter and hybridized to a 50 μl *in vitro* reaction mixture. Specific hybridization was obtained with TSWV genomic RNA whereas no hybridization was obtained with alfalfa mosaic virus RNA 4 or total RNA from healthy *N.*

rustica (Fig. 4a). The reaction products hybridized to all three genomic RNAs (Fig. 4b). The intensity of the hybridization signals was strongest for the S RNA and decreased in the order of S > M > L RNA. To investigate whether this hybridization pattern reflects the ratio of the genomic RNAs in the virus preparations used in the *in vitro* reaction or reflects a selective preference of the polymerase for the individual RNA segments, the RNA was purified and analyzed by northern blotting and probed with segment-specific probes (Fig. 4c). The hybridization patterns obtained reflect the molar ratio of the segments as observed in ethidium bromide stained RNA patterns of purified, enveloped virions. To further dissect the signals observed with all three genomic RNA segments, five cDNA clones containing all five genes of TSWV, were spotted onto Hybond-N and probed with the *in vitro* reaction products (Fig. 4d). All five genes revealed a specific hybridization pattern whereas no hybridization was obtained with control plasmid (Fig. 4d). Spotting equal amounts of DNA led to hybridization signals of different intensity, which decreased in the order of N > GP, L >

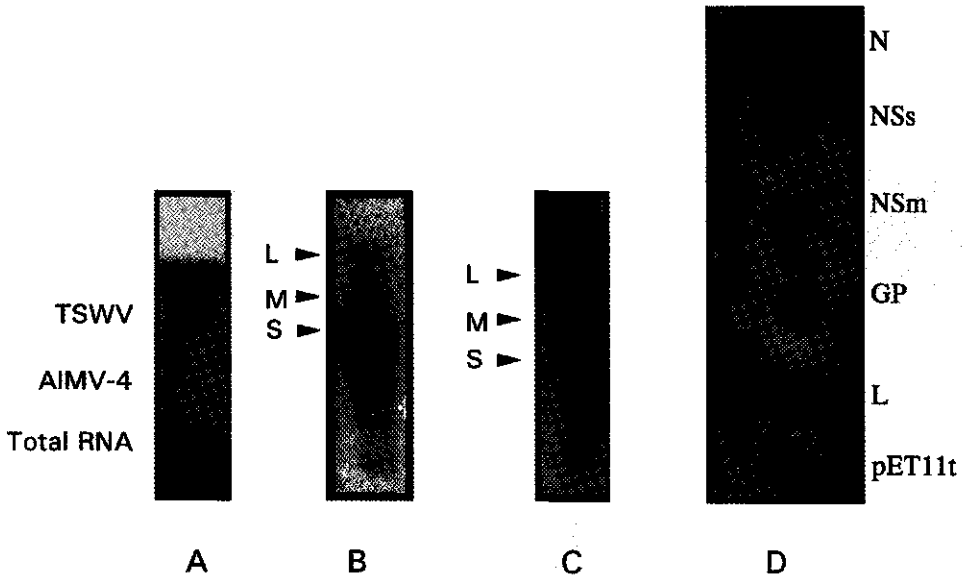

Figure 4. Analysis of products of the *in vitro* transcription reaction. (a) Genomic RNA (0.5 μg) of TSWV (purified from nucleocapsids), alfalfa mosaic virus RNA4 (1 μg) and total RNA (8μg) from healthy *N. rustica* plants was spotted onto Hybond-N and hybridized to the [^{32}P]-labelled products of an *in vitro* transcription reaction. (b) RNA was extracted from purified virus as described in Materials and Methods, subjected to electrophoresis and blotted onto Hybond-N. The [^{32}P]-labeled products of an *in vitro* transcription reaction were used as a probe. (c) As (b) but three segment specific (L, M, S) probes were used. (d) equal amounts (0.5 μg) of cDNA of all five genes of TSWV were spotted onto Hybond-N and hybridized to the *in vitro* transcription reaction.

NSs> NSm (Fig. 4d). The lower hybridization signal obtained for the NSs and NSm genes corresponds with the lower abundancy of viral complementary RNA in the virion (Kormelink *et al.*, 1992b). These results suggest that the genomic RNA hybridization pattern as observed in Figure 4b reflects the molar ratio of the segments.

Analysis of the products of the *in vitro* reaction on a 1% agarose gel revealed that no major, discrete products were synthesized but heterogenous products ranging in size from 400 nucleotides and smaller. Analysis of these reaction products on a 2.8% sequencing gel confirmed the previous observation, revealing several discrete products of 450 nucleotides and smaller. This observation is in contrast to the results with *in vitro* transcription reactions with Rift Valley fever virus (RVFV) where only full-length N mRNAs are formed (Dr. M. Bouloy, personal communication). The *in vitro* transcription reaction of RVFV exhibits a translational dependency whereas for TSWV this has remained unclear (Adkins *et al.*, 1995).

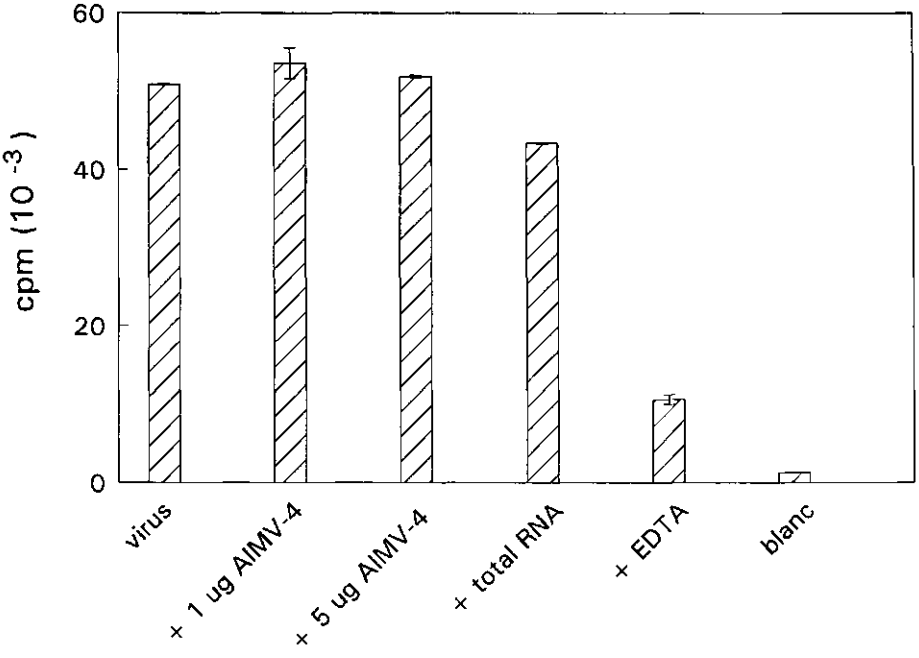

Figure 5. Effect of alfalfa mosaic virus RNA 4 on the *in vitro* transcription reaction. Alfalfa mosaic virus RNA 4 (1 or 5 μg) or total RNA (8 μg) from *N. rustica* was added to the *in vitro* transcription reaction and analyzed for stimulation of transcription.

Effect of alfalfa mosaic virus RNA 4

Transcriptase activity of La Crosse virus was stimulated by addition of cap analogues and natural mRNAs such as alfalfa mosaic virus RNA 4 (Patterson *et al.*, 1984a). In order to

study a possible stimulatory effect of natural mRNAs on the *in vitro* transcription reaction of TSWV, 1.0 or 5.0 μg alfalfa mosaic virus RNA 4 were added to the reaction mixture (Fig. 5). However, no significant increase in incorporation was observed. The addition of 10 μg total RNA isolated from healthy *N. rustica* (including capped mRNAs) did not stimulate the incorporation either (Fig. 5). Due to the lack of stimulation by capped primers it is not possible to discriminate between transcription or replication in these experiments.

Effect of TSWV specific antisera on the *in vitro* transcriptase reaction

Although the *in vitro* CMP-incorporating activity could not be further improved than $30*10^3$ cpm/μg of virus, preliminary experiments with various IgG preparations were performed as to investigate the requirement of individual viral proteins in this process. Various antisera directed against individual TSWV proteins were tested for their ability to inhibit incorporation of the *in vitro* transcriptase assay. In the initial experiments all reactions that contained IgGs (1μg) showed a slightly decreased incorporation (results not shown). Addition of RNase inhibitor (RNasin, Promega) did not have any effect, indicating that the decrease was not due to the introduction of RNase activity in the IgG preparations. Upon

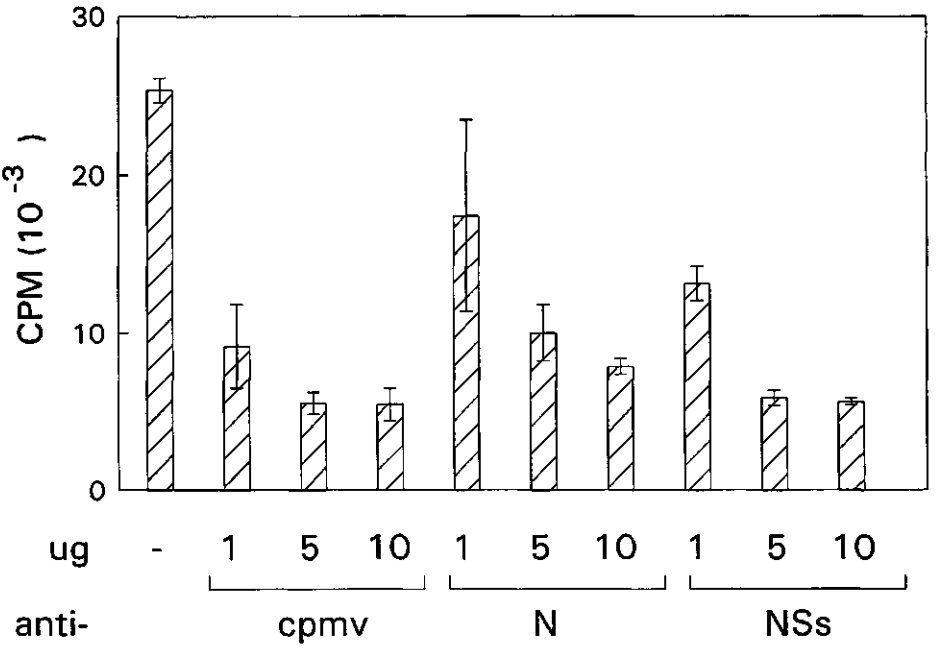

Figure 6. Effect of addition of IgGs (directed against cowpea mosaic virus, TSWV- N or NSs) to the *in vitro* transcription reaction. Virions were pre-incubated with 1, 5 or 10 μg IgG at 30 °C for 15 minutes in the presence of Triton X-100.

further investigation there was no specific inhibition in incorporation by using up to 10 μg of IgGs directed against complete virus, the N protein, the L protein (both anti L-n and L-c, Chapter 3), NSs (Kormelink *et al.*, 1991), or NSm (Kormelink *et al.*, 1994) compared to unrelated antisera (directed against cowpea mosaic virus). Preincubation of the virus for 15 min at 30 °C in the presence of 0.5% Triton X-100 and antiserum (directed against N, NSs and cowpea mosaic virus) resulted in a decreased incorporation, in a non-specific, IgG concentration dependent manner (Fig. 6). The data obtained in these experiments, using the currently available antisera, did not allow the assignment of a function of a particular protein in the transcription/replication process.

Discussion

Recently, polymerase activity associated with purified virus preparations was demonstrated using a rapid virus purification procedure (Adkins *et al.*, 1995). In this chapter these results have been reproduced and the *in vitro* transcription activity was further analyzed. The reaction requires manganese as has previously been described for Lumbo virus (Bouloy and Hannoun, 1976), Uukuniemi virus (Ranki and Petterson, 1975), and Hantaan virus (Schmaljohn and Dalrymple, 1983) but is not required for Germiston virus (Gerbaud *et al.*, 1987) or La Crosse virus (Patterson *et al.*, 1984a). For these latter viruses a translational dependency of the transcription process has been reported (Bellocq and Kolakofsky, 1987; Bellocq *et al.*, 1987; Vialat and Bouloy, 1992). A mechanism was proposed where ongoing translation prevents annealing of the newly synthesized strand to the template RNA thus causing premature termination of the transcription process. Adkins and coworkers (1995) found a 3 kb reaction product when wheat germ extract was included into the TSWV *in vitro* reaction mixture. The same product was also found when only the CTP concentration was increased. These results may indicate that one (or more) component(s) in the reaction mixture was limiting. Although in the experiments described in this chapter no 3 kb product was obtained the addition of rabbit reticulocyte lysate did not lead to an increased product size. Taken these data and those of Adkins *et al.* (1995) into account, it appears that for TSWV transcription is not depending on translation.

No further improvement in CMP incorporation levels was obtained. Moreover, the addition of alfalfa mosaic virus RNA 4 or total RNA of *N. rustica* did not stimulate the incorporation in contrast to what has been found for La Crosse virus (Patterson *et al.*, 1984a). However, the presence of (co-purified) cap-structures in purified virus preparations of TSWV cannot be ruled out. Therefore it should be concluded that in the current *in vitro*

system it is not yet possible to discriminate between transcription and replication. The products of the *in vitro* reaction hybridized to cDNA of all five genes, indicating that, at least for the S and M RNA, both viral and viral complementary RNA are present and transcribed. The products formed seem to reflect the molar ratio of the RNA segments present in the virion.

Since neither the addition of the antibodies directed against the N- or C-terminal part of the L protein nor the addition of antibodies directed against any of the other viral proteins did show any significant effect, the developed *in vitro* transcription assay may be of limited value for unravelling the role of the individual viral proteins.

CHAPTER 6

Development of a hybrid baculovirus/bacteriophage T7 transient expression system

Summary

A hybrid recombinant baculovirus-bacteriophage T7 expression system was developed for transient expression in insect cells of plasmids with foreign genes provided with a T7 promoter. The coding sequence for T7 RNA polymerase, with or without a nuclear localization signal, was inserted into the genome of *Autographa californica* nuclear polyhedrosis virus. Recombinant viruses stably expressed T7 RNA polymerase in insect cells. Upon transfection of infected insect cells with plasmids containing the genes for chloramphenicol acetyltransferase (CAT), the hepatitis B virus precore-, core- or e- antigens under control of the T7 promoter, transient expression of these genes was detected by ELISA. The results obtained indicate that this baculovirus/T7 system provides a simple and widely applicable tool for transient gene expression studies.

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Introduction

Heterologous and controlled expression of cloned genes has become an important and widely used technique in molecular biology. There are various expression systems available today, derived from both prokaryotic and eukaryotic cells. Prokaryotic expression systems are efficient and easy to apply but they impose a number of limitations for synthesis of eukaryotic proteins. Protein folding, proteolytic processing, glycosylation, phosphorylation, secretion and subunit assembly may not occur properly, if at all. Therefore eukaryotic expression systems, employing virus vectors such as vaccinia virus and baculovirus, are often preferred for functional expression of eukaryotic genes (Moss, 1992; Luckow and Summers, 1988).

The baculovirus-insect cell expression system is widely used and perhaps the most efficient for the high-level production of eukaryotic proteins. The expression system relies on the construction of recombinant baculoviruses through homologous recombination of wild type virus and special transfer vectors (Luckow and Summers, 1988). Some applications however, e.g. the construction of a large number of recombinant baculoviruses, or expression of toxic products, are either time consuming, or can only be obtained with great difficulty. Transient-expression of foreign genes in a hybrid vector system that would utilize the highly active and specific T7 RNA polymerase in a eukaryotic environment might therefore offer an attractive alternative for rapid screening and analysis.

Several attempts have been made to exploit bacteriophage polymerases for expression of RNA or proteins in yeast and mammalian cells, although there are differences in structure, mode of synthesis, processing and modification of prokaryotic and eukaryotic mRNAs (Chen *et al.*, 1987; Deuschle *et al.*, 1989; Elroy-Stein *et al.*, 1989; Fuerst *et al.*, 1986, 1987 and 1989; Lieber *et al.*, 1989;). Fuerst *et al.* (1986) described a hybrid vaccinia virus/T7 system for transient expression of foreign genes. In this system T7 RNA polymerase is produced by a recombinant vaccinia virus.

We have now combined the convenience of the baculovirus expression vector system with the advantages of T7 RNA polymerase. This hybrid system could be a useful tool, for instance for co-expression of more than one protein or for rapid testing of (cDNA) constructs prior to engineering of viral recombinants. Since baculoviruses replicate in the nucleus of insect cells, two recombinant baculoviruses were constructed, one expressing T7 RNA polymerase in the cytoplasm and the other expressing T7 RNA polymerase targeted to the nucleus by the presence of a nuclear localization signal (NLS). In this paper we show that both recombinants support the transient expression of genes (chloramphenicol acetyltransferase, hepatitis B virus (HBV) precore-, core- and e- antigens) from plasmids

containing a T7 promoter.

Materials and Methods

General.

Cell culture, DNA techniques, SDS-polyacrylamide gel electrophoresis, blotting and immunostaining were done as described by Sambrook *et al.* (1989) and Zuidema *et al.* (1990). Plasmids pAR1173 (Davanloo *et al.*, 1984), pAR3283 (Dunn *et al.*, 1988; Kalderon *et al.*, 1984) and polyclonal rabbit anti-T7 RNA polymerase were kindly provided by Dr F.W. Studier, Brookhaven National Laboratory, Upton, New York, U.S.A.

Construction and isolation of recombinant baculoviruses.

The T7 gene 1 (pAR1173, Davanloo *et al.*, 1984) was cloned as a 2.65 kb *Bam*HI fragment into the unique *Bam*HI site of transfer vector pAcDZ1, resulting in the transfer vector pAcT7-1. The T7 gene 1 plus twelve additional codons (at the N-terminus, pAR3283) coding for the SV40 T antigen nuclear location signal (NLS) (Dunn *et al.*, 1988; Kalderon *et al.*, 1984) were inserted as a *Bam*HI-*Bgl*II fragment into the unique *Bam*HI site of pAcDZ1, yielding transfer vector pAcT7-NLS. *Spodoptera frugiperda* cells (Sf-21) were grown in Hink's medium supplemented with 10% fetal calf serum (FCS)(Vaughn *et al.*, 1977). Sf-21 cells were cotransfected by lipofectin with the recombinant transfervectors pAcT7-1 or pAcT7-NLS and 1 μg BsuI cut viral DNA (AcPAK6) per 10^6 cells according to Kitts *et al.* (1993). The virus collected from the cotransfection supernatant 5 days post transfection was subjected to three rounds of plaque purification according to Zuidema *et al.* (1990). Two recombinant viruses from each of the two transfections containing the T7 gene were selected and further analyzed for expression of T7 RNA polymerase by ELISA and immunostaining. Pure recombinant viruses were then amplified and the titer determined by an end point dilution assay (O'Reilly *et al.*, 1992). Titers of the stocks were $1.1*10^8$ $TCID_{50}$/ml for AcT7-1 and $2*10^8$ TCID/ml for AcT7-NLS, respectively.

Immunofluorescence.

Cells were infected with AcPAK6 and recombinant AcMNPV, attached to a coverslip and grown until 48 hr p.i. Cells were fixed by immersion in 100% acetone at -70 °C, hydrated in phosphate buffered saline (PBS) for 15 min and blocked for 1 hr with PBS containing 1% BSA. The cells were then incubated with rabbit antiserum against T7 RNA polymerase (kindly provided by Dr F.W. Studier, Brookhaven National Laboratories, Upton, New York)

for 1 hr at room temperature. Cells were washed three times with PBS and incubated for 1 hr with fluorescein isothiocyanate-labeled (FITC) goat anti-rabbit antiserum. Cells were washed three times with PBS, covered with glycerine/PBS cityfluor and visualized by fluorescence microscopy.

***In vitro* transcription reactions.**

Sf-21 cells were infected with recombinant AcT7-1. Cells were grown in Hink's medium with 10% FCS and harvested 24 or 48 hr p.i. Cells were washed three times with PBS and finally resuspended in 200 μl PBS, lysed by sonification and used for in vitro transcription. This was carried out at 37 °C for 1 hr in 25 μl reactions containing 1 μg of a BamHI-linearized DNA template containing a T7 promoter, the gene encoding alfalfa mosaic virus RNA4 and 5 μl cell extract 1x T7/T3 transcription buffer (Promega), 30 mM DTT, 40 U RNasin, 1mM of each ATP, CTP, GTP and UTP. Reaction was stopped by phenol/ chloroform extraction and RNA was precipitated with ethanol. RNA was resuspended in 10 μl Tris/HCl-EDTA (10 mM/1 mM) pH=8.0, of which 5 μl was analyzed on a 1% agarose gel, blotted and hybridized to an insert specific probe. As a control T7 RNA polymerase (Promega) was used.

Transient expression of foreign genes

Sf-21 cells were infected with AcT7-1 and AcT7-NLS with a m.o.i. of 10 and transfected using lipofectin with a plasmid containing the CAT gene (pGEM-CAT), the hepatitis B precore-(pET-HBpcAg), core- (pET-HBcAg) and e- (pET-HBeAg) antigens under control of a T7 promoter at t=4 hrs p.i. Cells were harvested at t=64 hrs p.i., lysed and a dilution series was made. CAT expression was assayed by ELISA according to suppliers instruction (Boehringer Mannheim); hepatitis B pre-, core- and e- antigens were assayed by ELISA using specific antisera.

Results

Development of two recombinant baculoviruses expressing T7 RNA-polymerase.

The T7 gene 1 (2.65 kb) (Davanloo *et al.*, 1984) was cloned into the *Autographa californica* multi-nucleocapsid nuclear polyhedrosis virus (AcMNPV) transfer vector pAcDZ1 (Zuidema *et al.*, 1990), downstream of the polyhedrin gene promoter, to produce the recombinant plasmid pAcT7-1 (Fig. 1). Cloning of the T7 gene 1 provided with the coding sequence (36 bp) for the nuclear localization signal of SV40 T antigen (Dunn *et al.*, 1988;

Kalderon *et al.*, 1984) resulted in construct pAcT7-NLS (Fig. 1). The orientation of the inserts was confirmed by restriction enzyme analysis and sequencing of the region spanning the polyhedrin-promoter/T7 polymerase junction. Cotransfection of Sf-21 cells with transfer vector DNA and AcMNPV-PAK6 DNA, linearized with *Bsu*I at the polyhedrin locus (Kitts *et al.*, 1993), produced LacZ-positive recombinant viruses, which were isolated by plaque purification. The recombinants, denoted AcT7-1 and AcT7-NLS, contained the complete T7 RNA polymerase gene in the presence (AcT7-NLS) or absence (AcT7-1) of the nuclear localization signal respectively, as confirmed by restriction enzyme analysis and Southern hybridization analysis (results not shown).

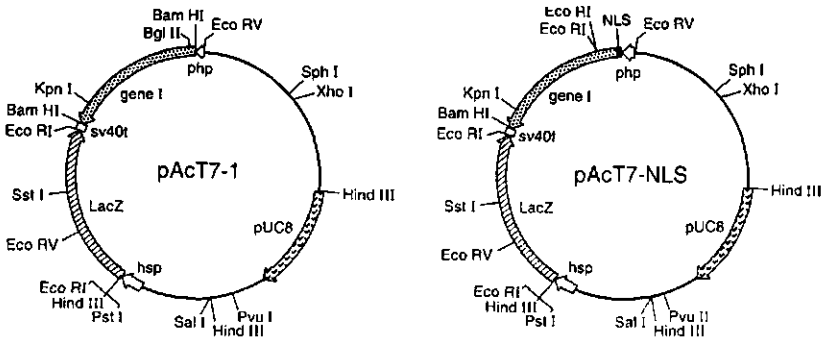


Figure 1. Structure of AcMNPV transfer vectors pAcT7-1 and pAcT7-NLS. The T7 gene 1 was excised from pAR1173 (Davanloo *et al.*, 1984) as a *Bam*HI fragment and inserted into the *Bam*HI site of transfer vector pAcDZ1 (Zuidema *et al.*, 1990), resulting in pAcT7-1. T7 gene 1 containing the nuclear location signal (NLS) from the large SV40 T-antigen (Dunn *et al.*, 1988; Kalderon *et al.*, 1984) (indicated as a black box) was inserted as a *Bam*HI-*Bgl*II fragment in the *Bam*HI site of pAcDZ1, resulting in pAcT7-NLS. Hsp, *Drosophila melanogaster* heat shock promoter; SV40t, terminator sequence of SV40, AcMNPV DNA sequences are indicated as single lines.

T7 RNA polymerase expression.

To demonstrate the expression of T7 RNA polymerase in insect cells, Sf-21 cells infected with recombinants AcT7-1 or AcT7-NLS were harvested and the proteins resolved in a 10% SDS-polyacrylamide gel. Western blot analysis using antiserum against T7 RNA polymerase revealed the presence of a 100 kDa protein in extracts from cells infected with each of the recombinant viruses (Fig. 2A, lanes 1 and 2). The specific reaction products were of the expected size and comigrated with commercial T7 RNA polymerase (Fig. 2A, lane 4). No reaction with uninfected Sf-21 cells or Ac-PAK6 infected cells (Fig. 2A, lanes 3 and 6) was observed.

Activity of baculovirus-expressed T7 RNA polymerase.

An *in vitro* assay was used to demonstrate the activity of the recombinant T7 RNA polymerase. Sf-21 cells were harvested 24 and 48 hr p.i. with AcT7-1. Extracts of infected Sf-21 cells were used for *in vitro* transcription of a linearized plasmid containing

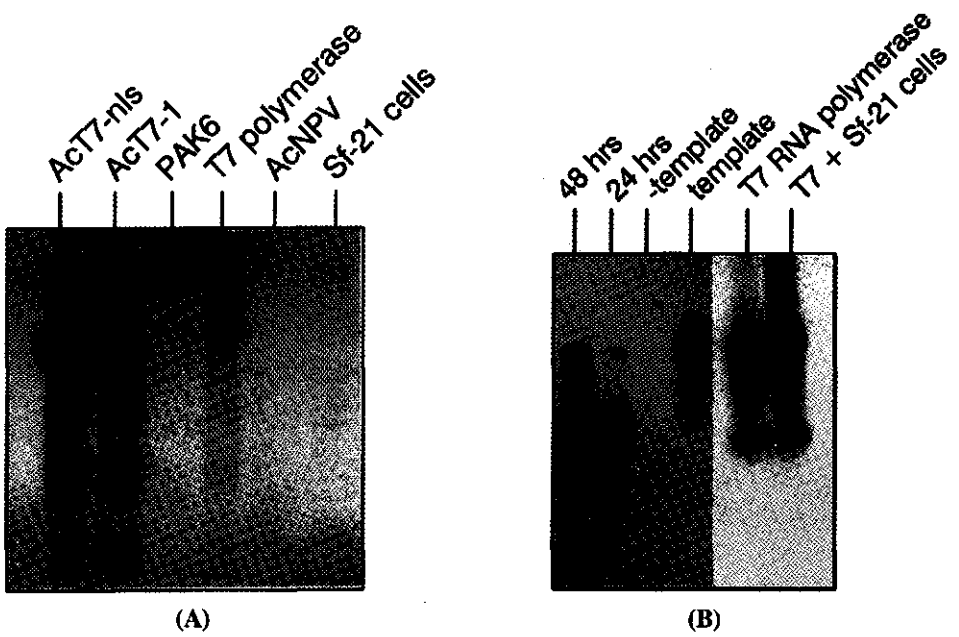


Figure 2. Detection and activity of T7 polymerase produced in AcT7 or AcT7-NLS infected Sf-21 cells. **Panel A:** Immunostaining of extracts from cells infected with recombinant virus expressing T7 RNA polymerase. Sf-21 cells were infected with recombinants AcT7-1 or AcT7-NLS, harvested 60 hrs p.i., subjected to electrophoresis in a 10% SDS-polyacrylamide gel and immunostained with polyclonal rabbit anti-T7 RNA polymerase antibody (second antibody goat anti-rabbit, conjugated with alkaline phosphatase). Lane 1: AcT7-NLS, lane 2: AcT7-1, lane 3: AcMNPV/PAK6, lane 4: commercial T7 RNA polymerase, lane 5: wild type AcNPV, lane 7: uninfected Sf-21 cells; T7 RNA polymerase is indicated with an arrow. **Panel B:** *In vitro* transcription using baculovirus-expressed T7 RNA polymerase. Sf-21 cells infected with AcT7-1 were harvested and extracts were used for *in vitro* transcription of a *Bam*HI linearized plasmid containing a T7 promoter and subsequently analyzed on northern blot (experimental protocol). Lane 1: cells harvested 48 hrs p.i., lane 2: cells harvested 24 hrs p.i., lane 3: no plasmid added to reaction mixture, lane 4: plasmid only (no extract added), lane 5: commercial T7 RNA polymerase, lane 6: mixture of T7 RNA polymerase and Sf-21 cells.

a T7 promoter. Transcripts of the expected size were observed in each case. The highest *in vitro* T7 RNA polymerase activity was detected using cell extracts made at 48 hr p.i. (Fig. 2B).

Localization of T7 RNA polymerase in insect cells.

Immunofluorescence was performed to determine the intracellular localization of T7 RNA polymerase in cells infected with recombinants AcT7-1 and AcT7-NLS. Specific labelling with antiserum against T7 RNA polymerase was obtained in nuclei of cells infected with AcT7-NLS (Fig. 3, panel A). This nuclear localization was confirmed by DAPI staining (Fig. 3, panel B). T7 RNA polymerase expressed by AcT7-1 (lacking the NLS), accumulated exclusively in the cytoplasm (Fig. 3, panel C). No immunofluorescence was seen in Ac-PAK6 infected cells (Fig. 3, panel E). This result indicates that the nuclear location signal of the SV40 large-T antigen is not only functional in mammalian and yeast cells but also in insect cells.

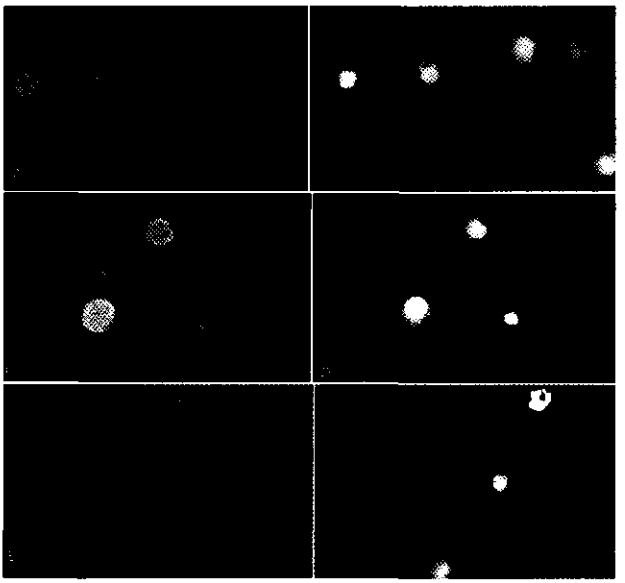


Figure 3. Subcellular location of T7 RNA polymerase expressed by baculovirus recombinants. Sf-21 cells were infected with AcT7-1 or AcT7-NLS, and fixed. T7 RNA polymerase was visualized using rabbit antiserum raised against T7 RNA polymerase and stained with fluorescein-labeled goat anti-rabbit serum. Panels a, c and e are immunofluorescence micrographs, panels b, d and f, respectively, represent DAPI stained nuclei of the same cells. In the immunofluorescence micrographs, AcT7-NLS clearly expressed the T7 RNA polymerase in the nucleus (a) as confirmed by DAPI staining (b). T7 RNA polymerase expressed by AcT7-1 was located in the cytoplasm (c, d), whereas no fluorescence was detected in AcMNPV/PAK6 infected cells (e, f).

Transient expression of foreign genes.

The next step in establishing a transient expression system was to investigate whether (AcT7-1 or AcT7-NLS) infected insect cells could drive the expression of a plasmid-born foreign gene under the control of a T7 promoter. The chloramphenicol acetyltransferase (CAT) gene is commonly used to monitor expression levels. The expression of the CAT gene can be quantitated by measuring the ability of the enzyme in cell extracts to acetylate chloramphenicol in the presence of acetyl CoA, by ELISA (Fig. 4A) or immunostaining (Fig. 4B). Transient expression of the CAT gene depends on expression of T7 RNA polymerase

by the baculovirus/T7 recombinants, intracellular functioning (and localization) of this enzyme, the production of translatable mRNA from a T7 promoter and the synthesis of the prokaryotic CAT.

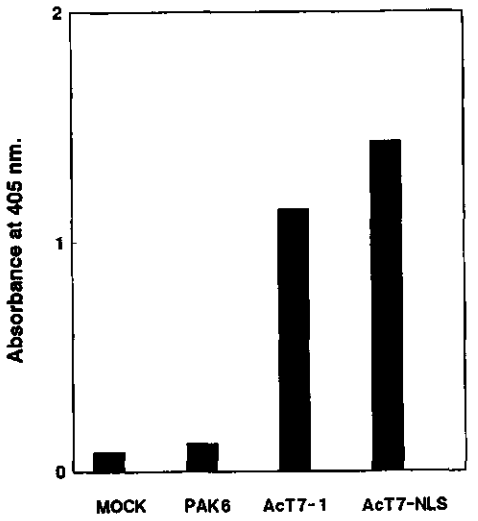
Expression of CAT was only observed from cells infected with AcT7-1 or AcT7-NLS, which express a functional T7 RNA polymerase (Fig. 4A and B). ELISA values obtained from several independent experiments (data not shown) demonstrated that recombinant AcT7-NLS gave consistently a slightly higher expression of CAT than AcT7-1. Under optimum conditions 3.6 ng of CAT was produced per 10^6 AcT7-NLS-infected cells, 64 hrs p.i.

After having established a test system for transient expression using this hybrid baculovirus-T7 RNA polymerase system the feasibility of the system was further analyzed, by expressing a series of clinically relevant antigens. To this end the coding sequences for the three major core proteins of hepatitis B virus (the precore-, core- and e- antigens) were cloned separately into pET vectors and transfected into Sf-21 cells using the AcT7-NLS as T7 polymerase-supplying virus. All three cotransfected constructs were indeed expressed, resulting in HBV antigens which were easily detected by ELISA (Fig. 4C).

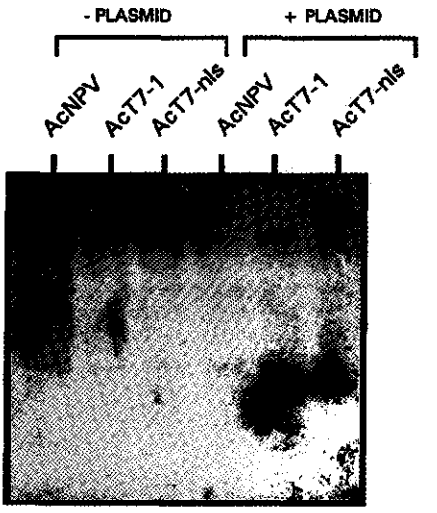
Discussion

In this report the baculovirus expression vector system was tailored for transient expression utilizing the prokaryotic bacteriophage T7 transcription machinery. Functional T7 RNA polymerase, a single subunit enzyme, which is highly specific for its own promoter and having a 5-fold faster elongation rate than *Escherichia coli* RNA polymerase (Chamberlin and Ryan, 1982; Dunn and Studier, 1983), was expressed by recombinant baculoviruses under the control of the polyhedrin promoter. It is shown that this system can effectively drive the transient expression of foreign genes provided with a T7 promoter.

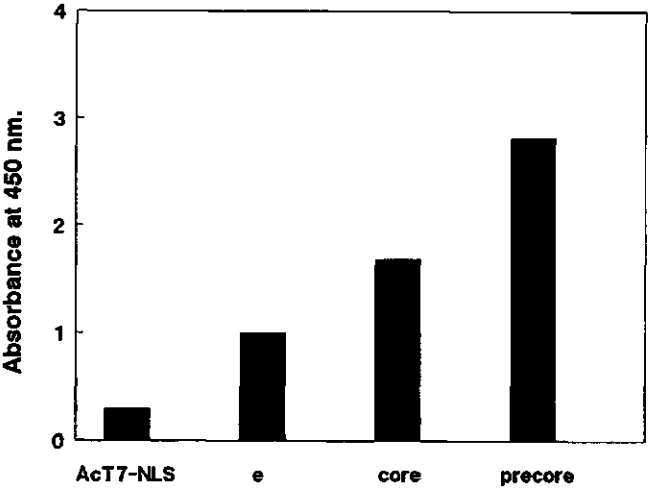
Fuerst and coworkers (1986) were the first to describe this strategy for vaccinia virus in a hybrid vaccinia virus/T7 transient expression system. In a report by Benton *et al.* (1990) the application of T7 RNA polymerase in yeast cells was described. Although intact mRNA was produced in this system no translation of target mRNAs could be detected. In the system described here we show successful translation of insert-specific mRNA in insect cells. Sf-21 cells, infected with recombinant baculoviruses expressing T7 RNA polymerase, are shown to produce CAT, as well as a series of hepatitis B antigens upon transfection with plasmids containing these genes under control of a T7 promoter. These results show that, despite the



(A)



(B)



(C)

Figure 4. Transient expression of CAT and major core antigens of hepatitis B virus (HBV) driven by the baculovirus/ T7 expression system. **Panels A and B:** Transient CAT production. Sf-21 cells were infected with AcT7-1, AcT7-NLS or AcPAK6 and subsequently transfected with a plasmid (pGEMCAT) containing the CAT gene under control of a T7 promoter CAT production was monitored by (A) ELISA and (B) immunostaining. Lanes in panel A: 1, mock infected Sf-21 cells; 2, wild type AcMNPV; 3, AcT7-1; 4, AcT7-NLS. Lanes in panelB: 1 and 4, AcNPV; 2 and 5, AcT7-1; 3 and 6, AcT7-NLS. Lanes 1-3 represent mock transfected cells (- plasmid) whereas lanes 4-6 show cells transfected with pGEMCAT (+ plasmid). **Panel C:** Transient expression of HBV core proteins as followed by ELISA. Three different HBV core antigens, i.e. the precore-, core- and e-antigens, were transiently expressed from pET vectors containing the individual coding sequences, using AcT7-NLS as T7 polymerase donating baculovirus.

absence of cap structures, translatable mRNAs were produced. The various steps leading to the transient expression of foreign, plasmid-born genes in Sf-21 cells using baculovirus recombinant AcT7-NLS are schematically illustrated in Fig. 5. Note that transcription of the foreign gene takes place in the nucleus and that it is anticipated that non-capped mRNAs are produced.

The results obtained with both the CAT and the HBV major antigen genes demonstrate that the baculovirus/bacteriophage T7 expression system now developed can be exploited for

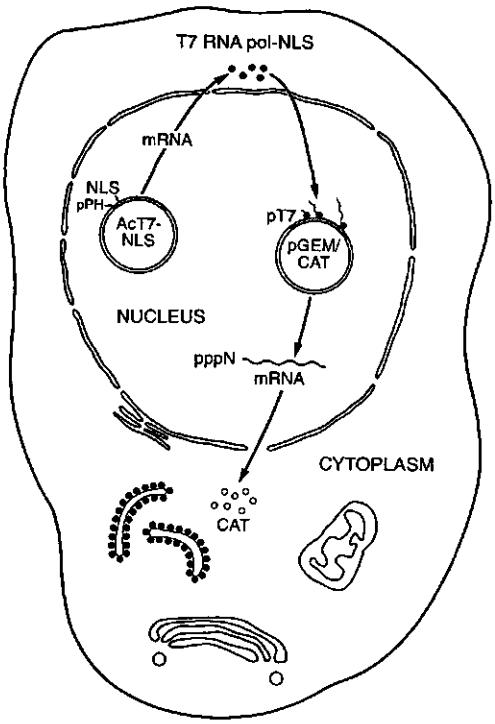


Figure 5. Schematic representation of the baculovirus/T7 transient expression system driving the expression of the chloramphenicol acetyltransferase (CAT) gene. T7 RNA polymerase is produced upon infection of an insect cell with a recombinant AcMNPV containing the coding sequence for T7 RNA polymerase behind the polyhedrin promoter (pPH). The enzyme is directed to the nucleus due to the presence of a nuclear localization signal (NLS). It is anticipated that upon transcription of the foreign gene uncapped mRNAs are produced.

transient expression of desired proteins, including products toxic to insect cells. Furthermore, the system can be applied to assay protein encoding constructs at an early stage prior to engineering of recombinants. It has also become possible now to use recombinant baculoviruses containing a target gene under control of the T7 promoter. This will allow the design of recombinants expressing products, which are inhibitory to AcMNPV replication and expression, upon dual infection of insect cells with AcT7-1 or AcT7-NLS. The exploitation of the T7 RNA polymerase system further enhances the versatility of the baculovirus expression vector system.

In summary it has been shown that the baculovirus/bacteriophage T7 RNA polymerase

system provides a simple and useful tool for rapid expression of genes cloned in T7-promoter containing plasmids. It can now be used for the *in vivo* reconstitution of complex processes which involve an intimate cooperation of multiple factors as has been described for vesicular stomatitis virus employing the vaccinia/bacteriophage T7 system (Pattnaik *et al.*, 1992). A more specific application in the near future would be the reconstitution of the replication and transcription process of tomato spotted wilt virus, a plant virus which has been shown to replicate in insect cells (Wijkamp *et al.*, 1993).

Acknowledgements

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The authors are indebted to dr. F.W. Studier for providing plasmids pAR1173 and pAR3283 containing the T7 RNA polymerase gene, and polyclonal antiserum against T7 RNA polymerase.

CHAPTER 7

General discussion and concluding remarks

With the growing awareness of its economic impact tomato spotted wilt virus (TSWV) has gained increasing interest by virologists during the past ten years. This has led to the elucidation of its genomic organization, coding functions and expression strategy. A major part of these molecular studies have been compiled in the theses of De Haan (1991) and Kormelink (1994). The unravelling of the molecular biology of TSWV also led to the definitive identification of this pathogen as a member of the *Bunyaviridae*, a large family of arthropod-born viruses which were originally supposed to be restricted to the animal kingdom. To date we know that more bunyaviruses are able to infect plants, and these viruses have been classified into a distinct genus, the genus *Tospovirus* (Murphy *et al.*, 1995).

The aim of the Ph.D. research described in this thesis was to gain more insight in the transcription and replication of the tospoviral RNA genome. A crucial viral protein in these processes is, of course, the viral polymerase. As TSWV, in spite of having two ambisense genome segments (De Haan *et al.*, 1990; Kormelink *et al.*, 1992), has all properties typical of negative-strand RNA viruses, it was anticipated that this enzyme was localized in the virus particle. At the onset of the research there were rather contradictory data published with respect to the occurrence and size of a large protein species in purified virus particles. Reported sizes for the putative TSWV polymerase ranged from 110 to 220 kDa (Mohamed *et al.*, 1973; Tas *et al.*, 1977; Peters *et al.*, 1991). With the elucidation of the L RNA sequence (De Haan *et al.*, 1991) the confusion became even worse since the open reading frame in this genome segment would predict a polymerase of 331.5 kDa. Therefore the first goal was to clarify this point and to demonstrate or exclude that the relatively large TSWV polymerase would undergo proteolytic cleavages. By expressing both the 5'-terminal and 3'-terminal parts of the open reading frame of the L RNA in *E. coli*, antibodies were produced which allowed unequivocal detection of the TSWV polymerase. From the results obtained it was concluded that, compared to the polymerases analysed for animal-infecting bunyaviruses, the TSWV polymerase is, indeed, a large protein (330 kDa), which apparently does not undergo further cleavages (Chapter 3). Sequence analysis of another tospovirus, impatiens necrotic spot virus (INSV), indicates that a large polymerase is characteristic for tospoviruses (Chapter 2). Parsimony trees based on the L proteins indicate that, within the

Bunyaviridae family, the genus *Bunyavirus* is more closely related to the tospoviruses, than it is to the other (animal-infecting) genera, *Hanta-* and *Phlebovirus* (Elliott, 1989; Roberts *et al.*, 1995 and Chapter 2), despite their non-overlapping host ranges. This strongly suggests that bunyaviruses have not strictly co-evolved with their hosts but rather that tospoviruses represent bunyaviruses which have relatively recently invaded the plant kingdom. Indeed, comparison of the genetic maps indicates that tospoviruses seem to have acquired one extra gene, the NSm gene (Kormelink *et al.*, 1992; Law *et al.*, 1992), suggesting that tospoviruses represent specialized derivatives of their animal-infecting counterparts. Recent studies in our laboratory provide strong evidence that the product of this additional gene represents the viral "movement protein", involved in viral passage of the cell wall barrier during systemic infection of a plant (Kormelink *et al.*, 1994; Storms *et al.*, 1995), a function not required for infection of animals.

The close relationship between tospoviruses and members of the genus *Bunyavirus* is surprising as tospoviruses share the ambisense nature of their S RNA with members of the genus *Phlebovirus*. Another interesting observation is the highest degree of homology between the putative polymerase (336.9 kDa) encoded by RNA 1 of rice stripe virus (RSV, Toriyama *et al.*, 1994), the prototype of the floating genus *Tenuivirus*, and the L proteins of members of the genus *Phlebovirus* (Chapter 2). Like the tospoviruses tenuiviruses are plant-infecting viruses, but with four to five RNA genome segments of which three segments display an ambisense gene arrangement. The terminal eight nucleotides of each RNA segment of the tenuiviruses are identical to those of members of the genus *Phlebovirus*. Furthermore, there is weak homology between the nucleocapsid proteins of RSV and Punta Toro phlebovirus and, moreover, the putative 94K protein encoded by RNA 2 of RSV also shares homology with the glycoproteins of Punta Toro and Uukuniemi phleboviruses. The significance of the homology of the putative 94K protein is not clear as tenuiviruses seem to lack an envelope. Though tospo- and tenuiviruses are both groups of ambisense plant viruses, their distinct affinities to the *Bunyaviridae* (to the genera *Bunyavirus* and *Phlebovirus*, respectively, see Fig. 8 in Chapter 2) suggests that they descended from the animal bunyaviruses by two independent events.

Tospoviral L proteins are significantly larger than their animal infecting counterparts analyzed sofar but similar in size to the tenuiviral putative polymerase. It is tempting to associate the large size of these polymerases with an adaptation to the plant host. The large L protein of tospoviruses may comprise both the RNA synthesizing enzyme activity (core polymerase) and the endonuclease activity, involved in transcription initiation. Such activity is involved in tospoviral genome transcription as sequence analysis of TSWV mRNAs revealed the presence of 12 to 21 extra, nontemplated nucleotides of non-viral origin (Chapter 4). For both Bunyamwera and Rift Valley fever virus the endonuclease activity,

required to obtain the 5' capped leader sequences of the host cell mRNAs, was indeed shown to reside in the L protein (Jin and Elliott, 1993; Lopez *et al.*, 1995). For influenza virus, for which this process of transcription initiation (commonly referred to as "cap-snatching") was first described (for review see Krug, 1981), it was recently shown that the PB2 subunit is the actual endonuclease (Shi *et al.*, 1995). However, association with the other two enzyme subunits, PB1 (the core polymerase) and PA seems to be required for PB2 to function. For both La Crosse and Germiston bunyaviruses *in vitro* transcription experiments demonstrated the presence of an endonuclease acitivity in purified virions, which was shown to be methylated cap-dependent (Patterson *et al.*, 1984; Vialat and Bouloy, 1992). Indirect evidence for this phenomenon has been obtained by sequence analysis of the 5' ends of viral mRNAs (Chapter 4). Similar results were reported for maize stripe tenuivirus (Huiet *et al.*, 1993), followed by the observation that viral mRNAs (containing 5' non-viral sequences) of rice hoja blanca virus, another member of the tenuivirus group, were immunocaptured with an antiserum directed against methylated caps (Ramirez *et al.*, 1995). Accumulating evidence indicates that all segmented negative or ambisense stranded RNA viruses, irrespective of their animal or plant host, appear to have this remarkable mechanism for mRNA synthesis. For TSWV, sequence analysis of the 5' ends of N mRNAs at the endonucleolytic site indicated a slight preference for an U residue at the -1 position (Chapter 4). During cap-snatching, the viral polymerase is believed to bind to the capped 5' end of a host cell mRNA, to cleave this end off and to use it as a primer for mRNA synthesis. The general heterogeneity in leader sequences observed probably reflects the variation in host mRNAs which serve as a source of the capped primers. However, for some members of the *Bunyaviridae* a marked base preference for the cleavage site has been reported (Jin and Elliott, 1993; Garcin *et al.*, 1995). One possible explanation for this phenomenon is that the preferred sequence at the 3' end of the primer represents a cleavage preference of the viral endonuclease. Alternatively, Jin and Elliott (1993) suggested a polymerase slippage model similar to that proposed to account for the apparently nontemplated pppG present at the 5' ends of arenavirus genomes (Garcin and Kolakofsky, 1990; Garcin and Kolakofsky, 1992). The data obtained for hantaan virus mRNA initiation supported this model which was dubbed "prime-and-realign" mechanism (Garcin *et al.*, 1995). The highly speculative consequences of this proposed model extend to the initiation of genome replication of hantavirus postulating a role for the endonuclease. The proposed difference between the arenavirus and hantavirus mechanisms is that the endonuclease activity of the hantaviral polymerase would cleave the unpaired pppG at the -1 position. The nucleotide preference at the 3' end of the primers used for initiation of tospoviral mRNA synthesis is not as striking as described for Dugbe virus and Hantaan virus, indicating that this mechanism might not apply to TSWV.

Transcriptase activity has been detected in detergent-disrupted preparations of several

members of the *Bunyaviridae*. The recently observed *in vitro* transcriptase activity of TSWV (Adkins *et al.*, 1995), and that of INSV (Chapter 5) is completely manganese dependent, a feature shared with Lumbo virus and Uukuniemi virus. For both La Crosse and Germiston virus (Vialat and Bouloy, 1992) a translational dependency of transcription has been reported, which at least for La Crosse virus seems to be cell-type dependent (Raju *et al.*, 1989). Remarkably, in the absence of reticulocyte lysate Germiston virus polymerase activity could be detected only by elevating the incubation temperature and increasing the magnesium concentration or replacement by manganese. In contrast to these observations the results of Adkins *et al.* (1995) revealed that the effect of wheat germ extract could be substituted by the addition of CTP suggesting that concomitant translation is not required for TSWV. The results presented in Chapter 5 demonstrate the major drawback of this *in vitro* system, i.e. it is not manipulatable and will be of limited value for identifying template requirements or proteins involved.

The construction of a full-length cDNA copy of the L RNA (Chapter 3) is a first step towards a manipulatable reversed genetics system. Unfortunately, upon expression of the cloned full-length cDNA employing the baculovirus expression system, only a 67 kDa protein was detected, which specifically reacted with the L-n antiserum described in Chapter 3. Attempts to repair the 80 basepairs deletion which was subsequently deleted in the cDNA, failed, probably due to instability of the newly formed construct, suggesting intramolecular recombination. Similar observations have been reported for rice stripe virus RNA 1 by Toriyama and coworkers (1994). A possible deleterious effect of the protein on host cell metabolism cannot be ruled out. The construction and subsequent expression of a full-length cDNA of the INSV L RNA may provide a feasible alternative.

It is clear that further studies on the tospoviral RNA transcription/replication process are hampered by the lack of a cloned, functional L gene. The remaining TSWV genes have all been cloned and successfully expressed in the baculovirus expression system (Kormelink, 1994). All other tools for studying the role of individual proteins in transcription/replication are thus available. For Bunyamwera virus (Jin and Elliott, 1991) and Rift Valley fever virus (Lopez, 1995) it was shown that after transfection of transcriptase-depleted nucleocapsids in cells infected with a recombinant vaccinia virus, expressing the L protein, transcriptase activity was restored. Analysis of the transcription process using a synthetic genome-like RNA revealed that at least the N and the L protein are required and sufficient to reconstitute the transcriptase activity. Furthermore, amino acid substitutions in the conserved polymerase domain of the L protein of Bunyamwera virus abolished polymerase activity (Jin and Elliott, 1992) as was assessed by employing a vaccinia virus/bacteriophage T7 transient expression system (Fuerst *et al.*, 1986). The development of this system has greatly improved the possibilities to study the *cis*- and *trans*-acting factors involved in the transcription/replication

process and has been used intensively over the last years.

Tospoviruses replicate in their insect host (Wijkamp *et al.*, 1993), which provided the rationale for the development of a baculovirus/bacteriophage T7 transient expression system as described in Chapter 6. Analogous to the previously mentioned vaccinia virus/ bacteriophage T7 system, this system directs the transient co-expression of genes, cloned under control of the T7 promoter, in an insect cell background. Although successful expression of foreign genes was obtained, the system could not be tested for transcription/replication of TSWV due to the lack of a functional full-length L protein. It is therefore crucial to obtain a translationally functional cDNA clone from either TSWV or INSV L RNA in the near future. The baculovirus/bacteriophage T7 expression system may provide a useful tool for selecting those cDNAs from which functional L protein is expressed. Moreover, a rapid method for testing amino acid substitutions affecting polymerase activity would be available, obviating the need to produce recombinant baculoviruses. The expression of a functional transcriptase complex will open the way for analysis of all *cis*-acting elements involved in transcription/replication and packaging. This process can be studied by using defective interfering particles (DIs, for a review see thesis of Resende, 1993) as a model template, as has been described for vesicular stomatitis virus (Pattnaik *et al.*, 1992), or using a synthetic RNA as described for Bunyamwera virus by Dunn *et al.* (1996).

Summary

The work described in this thesis was aimed at the unravelling of the molecular biology of tospoviruses, with special emphasis on the process of replication of the tripartite RNA genome.

At the onset of the research the complete genome sequence of tomato spotted wilt virus (TSWV), type species of the genus *Tospovirus*, became available. These sequence data indicated that the tospoviruses represent plant-infecting members of the large family of the arthropod-born *Bunyaviridae*. Genome sequence comparisons indicated however that the L RNA segment of TSWV would encode a much larger viral polymerase (331.5 kDa) than, as far as known, its animal-infecting counterparts (reported sizes of 241 to 259 kDa). To verify whether a large polymerase represents a characteristic i.e. genus-specific property of tospoviruses the complete sequence of the L RNA segment of a second tospovirus, impatiens necrotis spot virus (INSV), was elucidated (Chapter 2). These sequence data revealed that the L RNA of INSV appeared to be comparable in size to that of TSWV (8675 nucleotides versus 8897 for TSWV), containing an open reading frame with a predicted size of 330.3 kDa of the INSV polymerase. Therefore the next question to be answered was whether the large primary translation product of the tospoviral L RNA acts as an unprocessed polymerase or whether this protein would undergo some cleavages to obtain smaller, functional replication proteins. Answering this question was even more necessary since the theoretical size of the TSWV L RNA ORF greatly exceeded previously determined sizes (110 to 220 kDa) for a large protein reported to copurify with TSWV particles. To this end both the 5'-terminal and 3'-terminal parts of the ORF in the TSWV L RNA were expressed in *Escherichia coli* and antibodies raised against these regions. Using these tools it could be established that the polymerase (L protein) of TSWV, though significantly larger than that of other bunyaviruses, is present in virus particles (10 to 20 copies per virion) in an unprocessed, full length form (Chapter 3). To allow further analyses of the TSWV polymerase, attempts were made to clone and express the complete L RNA ORF in the baculovirus/insect cell system. In spite of all efforts, only a shorter translation product of 67 kDa was obtained from a baculovirus recombinant containing a complete DNA copy of the TSWV L RNA (Chapter 3). Sequence analysis of the cloned copy revealed a 80 basepairs deletion, resulting in two premature stop codons, which most likely have led to the resulting truncated L protein.

To gain more insight in the "cap-snatching" event which takes place during initiation of tospovirus transcription, nucleoprotein (N) mRNAs were partially purified from TSWV-infected *N. rustica* leaves and cloned (Chapter 4). Sequence analysis of the cloned,

5'-proximate regions of 20 cloned mRNAs showed the presence of extra, non-templated sequences, ranging in length from 12 to 21 nucleotides, confirming our earlier primer extension studies. As these sequences were of non-viral origin a cap-snatching mechanism for tospoviral transcription initiation could thus be definitively identified. None of the host-derived leader sequences analyzed were identical and only limited sequence specificity at the endonucleolytic site was observed (some preference for cleavage at a U residue). During the course of this Ph.D. research, Adkins *et al.* (1995) reported that *in vitro* transcriptase activity was associated with freshly isolated TSWV particles. It was investigated (Chapter 5) whether the reported levels of *in vitro* activity could be further improved and whether this system would lend itself for analysis of the viral proteins involved by e.g. inhibition studies using specific antibodies. Trichloroacetic acid-precipitable products could consistently be obtained after incubation of detergent-disrupted TSWV virions under the assay conditions reported by Adkins *et al.* (1995) and using (α-^{32}P)CTP. No significant improvement in CMP incorporation levels could be achieved by testing variable conditions and varying concentrations of assay components. The reaction products obtained hybridized with clones from all three genomic RNA segments. No discrimination between transcription and replication could be made however, and since none of the available specific antibodies directed against any viral protein had an inhibitory effect, it was concluded that the current *in vitro* system will be of limited value for unravelling the RNA synthesizing process and the role of the individual viral proteins therein.

As a first step towards a manipulatable transcription/replication system, a hybrid baculovirus/bacteriophage T7 vector system was developed for transient expression in insect cells of all factors involved in TSWV genome transcription and replication. The results obtained (Chapter 6) illustrate the potential of the system. Although various foreign genes could successfully be expressed to measurable amounts, the reconstitution of a TSWV transcription/replication complex was hampered due to the apparent impossibility (Chapter 3) to clone the complete polymerase gene. Finally, in Chapter 7 (General discussion and concluding remarks), the results obtained are compared with the data reported for animal-infecting bunyaviruses, leading to a discussion of some evolutionary aspects. Furthermore, suggestions are made to circumvent some of the problems encountered during the course of the studies presented in this thesis.

Samenvatting

Het onderzoek, dat in dit proefschrift beschreven is, was gericht op de opheldering van de moleculaire biologie van tospovirussen, waarbij de nadruk op de transcriptie/replicatie lag. Bij de aanvang van dit onderzoek kwam, dankzij het promotieonderzoek van De Haan (1991) en Kormelink (1994), de volledige nucleotidenvolgorde van het genoom van het tomatebronsvlekkenvirus (Engels:tomato spotted wilt virus, afgekort TSWV) beschikbaar. Uit deze gegevens kwam naar voren dat tospovirussen plant-infecterende leden zijn van de *Bunyaviridae*, een virusfamilie die zich verder beperkt tot het dierenrijk. Uit sequentievergelijkingen bleek dat het L RNA segment van TSWV potentieel voor een groter viraal polymerase (331.5 kDa) codeert dan dat van de andere, dier-infecterende leden van deze familie (gerapporteerde groottes variëren van 241 tot 259 kDa). Een groot polymerase zou kenmerkend voor leden van het genus *Tospovirus* kunnen zijn. Om hier meer inzicht in te verkrijgen werd de volledige basenvolgorde van het L RNA segment van een tweede tospovirus, het "impatiens necrotic spot" virus (INSV), opgehelderd (Hoofdstuk 2). De verkregen data bevestigden dat het L RNA van INSV (8675 nucleodtiden) inderdaad overeen kwam in grootte met dat van TSWV (8897 nucleotiden) en een open leesraam bevatte dat voor het virale polymerase codeert, een eiwit met een bijbehorend molekuulgewicht van 330.3 kDa. Vervolgens werd bestudeerd of dit grote, primaire translatieproduct als geheel als polymerase fungeert of dat dit eiwit, na klieving, resulteert in kleinere functionele eenheden.

Het beantwoorden van deze vraag was des te belangrijker omdat de theoretische grootte van het translatieproduct van het TSWV L RNA veel groter is dan de in de literatuur gerapporteerde groottes van een eiwit (variërend van 110-220 kDa) dat meezuivert met virusdeeltjes. Hiertoe werden zowel het 5'- als het 3'- deel van het open leesraam van het TSWV L RNA tot expressie gebracht in *Escherichia coli* en antilichamen tegen deze eiwitten opgewekt. Met behulp van deze antilichamen kon aangetoond worden dat het L eiwit van TSWV, alhoewel groter (330 kDa) dan dat van andere bunyavirussen, in een intacte vorm in virusdeeltjes aanwezig is (10-20 copieën per virion). Om het polymerase van TSWV verder te onderzoeken werd getracht het complete open leesraam van het L RNA te kloneren en tot expressie te brengen in het heterologe baculovirus/insectecel-systeem. Ondanks verschillende pogingen werd slechts een klein eiwit (67 kDa) verkregen na infectie van insectecellen met een baculovirus recombinant die een complete cDNA kopie van het L RNA bevatte (Hoofdstuk 3). Na analyse van de nucleotidenvolgorde van deze copie bleken twee opeenvolgende stopcodons te zijn ontstaan als gevolg van een deletie van 80 basen, hetgeen resulteert in de vorming van het (te) kleine eiwit.

Om meer inzicht te krijgen in transcriptie-initiatie van tospovirussen, een proces dat ook wel "cap-snatching" wordt genoemd, werden mRNA's coderend voor het N eiwit gezuiverd

uit geïnfecteerde *Nicotiana rustica* bladeren en vervolgens gekloneerd (Hoofdstuk 4). Analyse van het gekloneerde 5'-uiteinde van 20 mRNA's toonde aan dat er additionele, niet virus-gecodeerde nucleotiden aanwezig waren, in lengte variërend van 12 tot 21 nucleotiden. Met de bepaling van de nucleotidenvolgorde van deze "leaders", die van niet-virale oorsprong zijn maar gestolen van gastheer mRNA's, kon definitief vastgesteld worden dat TSWV gebruik maakt van "cap-snatching". De leaders waren niet identiek en er lijkt slechts een lichte voorkeur te zijn voor een U residue op de plaats waar het endonuclease klieft.

Tijdens dit promotieonderzoek werd door Adkins en medewerkers (1995) een *in vitro* transcriptase activiteit beschreven die geassocieerd is met gezuiverde TSWV deeltjes. De experimenten zoals beschreven in Hoofdstuk 5 waren erop gericht om deze activiteit te verhogen en om te onderzoeken of dit systeem geschikt zou zijn om de rol van de verschillende virale eiwitten in de transcriptie/replicatie te ontrafelen, bijvoorbeeld met inhibitiestudies waarbij specifieke antilichamen gebruikt worden. Trichloorazijnzuur-precipiteerbare produkten werden verkregen na incubatie van virusdeeltjes (behandeld met een niet-ionisch detergens) met (α-^{32}P) CTP, waarbij de reactieomstandigheden zoals beschreven door Adkins en medewerkers werden gebruikt. Na het testen van verschillende reactieomstandigheden en verschillende concentraties van componenten van het reactiemengsel werd geen verhoging van de CMP-incorporatie niveau's waargenomen. De verkregen reactieprodukten hybridiseerden met cDNA kloons van alle drie de genomische RNA segmenten. Er kon geen onderscheid gemaakt worden tussen transcriptie en replicatie. Bovendien bleek het niet mogelijk met de beschikbare antilichamen tegen de individuele virale eiwitten remming te verkrijgen waaruit geconcludeerd werd dat het *in vitro* systeem in de huidige vorm niet geschikt is om het RNA synthetiserende proces en de rol van de individuele virale eiwitten daarin te ontrafelen.

Als eerste stap tot het ontwikkelen van een manipuleerbaar systeem, om de rol van alle factoren van belang voor transcriptie en replicatie van TSWV te bestuderen, werd een hybride baculovirus/bacteriofaag T7 transient expressie systeem ontwikkeld. De verkregen resultaten zijn beschreven in Hoofdstuk 6 en laten de mogelijkheden van dit systeem zien. Alhoewel verschillende heterologe genen detecteerbaar tot expressie konden worden gebracht was het helaas niet mogelijk om reconstitutie van een transcriptie/replicatie complex te verkrijgen. Dit hing samen met de kennelijke onmogelijkheid om het volledige polymerase-gen te kloneren (Hoofdstuk 3). Tenslotte wordt in de algemene discussie (Hoofdstuk 7) verder ingegaan op de onderlinge samenhang van de tijdens dit promotieonderzoek verkregen resultaten en de in de literatuur gerapporteerde gegevens met betrekking tot dier-infecterende bunyavirussen. Hierbij worden tevens enige aspecten van de evolutionaire verwantschap van tospovirussen met de dier-infecterende leden van de *Bunyaviridae* en tenuivirussen nader belicht.

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Curriculum vitae

Op 12 oktober 1965 ben ik, Frank van Poelwijk, in Arnhem geboren. Ik behaalde in 1984 het diploma V.W.O. aan het Nederrijn College te Arnhem en begon in hetzelfde jaar een studie Biologie aan de toenmalige Landbouwhogeschool te Wageningen. In 1985 behaalde ik de propadeuse en vervolgde mijn studie met de specialisatie "cel". De doctoraalfase werd afgerond met afstudeeropdrachten in de Moleculaire Virologie en de Moleculaire Genetica. In het kader van een praktijktijd Moleculaire Genetica werd in de periode oktober 1989 - april 1990 onderzoek verricht op de Biophysics and Physiology Department aan de State University of New York, Stony Brook, USA. In augustus 1990 behaalde ik het ingenieursdiploma in de Biologie.

Vanaf september 1990 was ik als onderzoeker in opleiding verbonden aan de vakgroep Virologie van de Landbouwuniversiteit. Onder leiding van Prof. Dr. R.W. Goldbach verrichtte ik onderzoek aan TSWV, waarvan de resultaten beschreven zijn in dit proefschrift. Sinds oktober 1995 ben ik als moleculair viroloog verbonden aan het ID-DLO te Lelystad waar ik aan het swine vesicular disease virus ("blaasjesziekte") werk.

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