Genome Transcription/Translation of Segmented, Negative-Strand RNA Viruses

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Christina Geerts-Dimitriadou

Thesis

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Step by Step

Step by step…
I climbed to the mountain…
And now that I'm standing on the top…
I noticed next to it another one…
even higher…

Christina Geerts-Dimitriadou

To my lovely
husband *Roy* and children *Jannis* and *Eleni*.

To you *my lovely one*,
who your little heart stopped beating too early.

“Life is what is happening to you while you are making other plans.”
“Ζωή είναι ό,τι σου συμβαίνει ενώ κάνεις άλλα σχέδια.”

Translated from the original Dutch:
“*Het leven is wat je gebeurt terwijl je andere plannen maakt*”.

Acda en de Munnik, album “Naar huis”, song “Laat me slapen”.

Abstract

The requirements for alignment of capped RNA leader sequences along the viral genome during influenza transcription initiation ("cap-snatching") have long been an enigma. Previous work on *Tomato spotted wilt virus* (TSWV) transcription initiation has revealed that this virus displays a preference for leaders with increasing base complementarity to the 3'-ultimate residues of the viral RNA template. Assuming that cap-snatching is a highly conserved mechanism, it is tempting to speculate that the findings for TSWV apply to all segmented negative RNA viruses. The research in this thesis aimed to analyze whether similar cap donor requirements applied for *Influenza A virus* transcription initiation as compared to what has been found for TSWV. Indeed, *in vitro* studies demonstrated that influenza transcriptase prefers multiple base-pairing capped leaders. Additionally, the occurrence of "prime-and-realign" during influenza transcription initiation was observed, as well as internal priming at the 3'-penultimate viral residue. The *in vitro* findings were confirmed by similar studies performed during influenza infection of cell cultures. Whereas transcription initiation of TSWV has been relatively well studied, transcription termination has not. It is postulated that transcription termination/translation is triggered by the formation of a hairpin structure. *In cell* experiments support a role of the TSWV hairpin structure in translation.

Subject headings: antiviral drug, capped RNA leader, hairpin structure, *Influenza A virus*, *Tomato spotted wilt virus*, transcription, translation.

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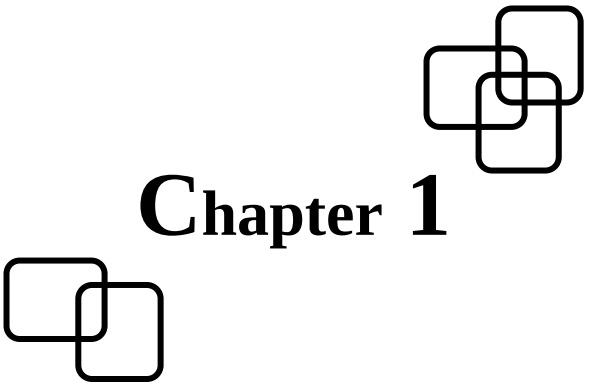
“If we look back to our choices, we should only look in order to learn and not to regret.”

Christina Geerts-Dimitriadou



Chapter 1

General Introduction

Segmented, single-stranded (ss) RNA viruses of negative (-) polarity are classified within the families *Orthomyxoviridae*, *Bunyaviridae*, *Arenaviridae* and a few floating genera (***Fauquet et al., 2005***). They contain well known members of medical as well as agronomical importance (**Table 1-1**). One of the best known examples is influenza orthomyxovirus that causes infections which are responsible annually for considerable morbidity and mortality in humans. Among the bunyaviruses *Rift Valley fever virus* (RVFV), *Crimean Congo hemorhagic fever virus* (CCHFV) and *Tomato spotted wilt virus* (TSWV) are viruses that receive considerable attention. Since the late 1990's RVFV is emerging in East Africa and causes severe disease in cattle and sometimes humans. CCHFV is listed as a potential biological weapon. The virus is found in Eastern Europe, Central Asia, the Middle East, Southern Europe and Africa and in documented outbreaks of CCHFV mortality rates in hospitalized patients range from 10 up to 50%. The plant-infecting bunyavirus TSWV belongs to the top ten of most devastating plant viruses worldwide due to its large host range that includes economically important agricultural and ornamental plants. Arenaviruses include the South American Hemorrhagic fever (HF) viruses and *Lassa fever virus* (LFV) which cause severe human disease and pose a threat as agents of bioterrorism.

Although all these viruses differ from each other in many ways (e.g. pathogenicity, host range, mode of transmission), they do have several features in common. Some of these are highly conserved and play important roles during replication of the viral RNA (vRNA) genome. The genome of (-) RNA viruses is not infectious, in contrast to positive (+) RNA viruses, as it cannot be directly translated into viral proteins to support in ongoing transcription and replication. Instead, their vRNA requires the association with nucleoprotein and RNA-dependent RNA polymerase (RdRp), reconstituted into transcriptionally active, infectious ribonucleocapsids (RNPs), to initiate an infection cycle. In the next paragraphs a brief overview will be presented on the replication cycle of segmented, (-) ssRNA viruses, with emphasis on transcription (initiation and termination) and translation, followed by the scope of this thesis.

TABLE 1-1
Segmented (-) ssRNA viruses.

Family	Genus	Type species	Polarity
Arenaviridae	*Arenavirus*	*Lymphocytic choriomeningitis virus*	Ambisense
Bunyaviridae	*Hantavirus*	*Hantaan virus*	(-)
	Nairovirus	*Dugbe virus*	(-)
	Orthobunyavirus	*Bunyamwera virus*	(-)
	Phlebovirus	*Rift Valley fever virus*	(-) and Ambisense
	Tospovirus	*Tomato spotted wilt virus*	(-) and Ambisense
Orthomyxoviridae	*Influenzavirus A*	*Influenza A virus*	(-)
	Influenzavirus B	*Influenza B virus*	(-)
	Influenzavirus C	*Influenza C virus*	(-)
	Isavirus	*Infectious salmon anemia virus*	(-)
	Thogotovirus	*Thogoto virus*	(-)
	Tenuivirus	*Rice stripe virus*	(-) and Ambisense
	Ophiovirus	*Citrus psorosis virus*	(-)
	Deltavirus	*Hepatitis delta virus*	(-)

Replication and transcription of segmented (-) ssRNA viruses

Once segmented (-) ssRNA viruses have entered the cell by receptor-mediated endocytosis, the viral envelope fuses with the endosome to release transcriptionally-active RNP complexes into the cytoplasm. The RNPs are composed of vRNA, viral nucleoprotein (N), and the viral RdRp and form the initiating complex for the synthesis of progeny vRNA (RNPs). The vRNA is first transcribed into mRNA (+ strand) to allow for the synthesis of larger amounts of viral proteins (N and RdRp) that are required for replication and subsequent encapsidation of progeny RNA into RNPs. Once viral proteins are synthesized, the vRNA will serve as template for the synthesis of full-length antigenomic (or viral-complementary, vc) RNA, which subsequently serves as template for the synthesis of progeny vRNA. The latter becomes encapsidated by N and small amounts of RdRp to form transcriptionally-active RNPs. These then become enveloped, which usually involves budding through one of the cellular membranes. Viruses of the large family of arthropod-born *Bunyaviridae*, whose members are primarily restricted to animals (***Elliott, 1990***), obtain their lipid envelop from intracellular membranes, i.e. the Golgi complex, which requires a final secretion from the cell after transport of the viruses within vesicles to the plasma membrane. For the plant-infecting bunyaviruses, *in casu* TSWV, particle assembly also takes place at the Golgi complex, but particles formed do not become secreted from plant cells. Instead they accumulate and are retained in large endoplasmic reticulum (ER)-derived

vesicles until uptake and further spread by its trips insect vector occurs. Members of the *Orthomyxoviridae* and *Arenaviridae* obtain their envelop by budding of RNPs from the plasma membrane from where virus particles are released and able to initiate infection of neighboring cells.

Most of the segmented (-) ssRNA viruses replicate in the cytoplasm of infected host cells, with the exception of orthomyxoviruses which replicate in the nucleus (**Table 1-1**). For the orthomyxoviruses, RNPs need to be transported from the cytoplasm into the nucleus where transcription and replication occur.

The genomes of segmented (-) ssRNA viruses consist of two (*Arenaviridae*), three (*Bunyaviridae*) or six-to-eight RNA segments (*Orthomyxoviridae*). Each RNA segment constitutes a separate replication and transcription unit. The 5'- and 3'-terminal nucleotides of each segment are highly conserved (**Table 1-2**) and show partial inverted complementarity, leading to the formation of a panhandle structure that contains the base-paired terminal ends. Due to the panhandle structure, RNPs reveal a pseudo-circular appearance in electron micrographs (**Figure 1-1**). The highly conserved 5' and 3' ends of genome vRNAs contain the promoter regions for replication and transcription.

Reverse genetic studies using *Bunyamwera virus* (BUNV) replicons have revealed that the ability of the genomic RNA termini to base-pair is an important requirement of bunyavirus replication (***Barr and Wertz, 2005***). In addition, detailed analysis of the first 20 nt of the 5' and 3' ends of the BUNV S segment showed that sequence complementarity and structure play a significant role in transcription as well (***Kohl et al., 2004***). Mutational analysis of the *Uukuniemi virus* (UUKV) putative promoter element surprisingly reveals major differences compared to studies carried out on the *Influenza A virus* (***Flick et al., 1996, id. 1999***) and the RVFV (***Prehaud et al., 1997***) promoter regions. In UUKV, neither single point substitutions nor changing of panhandle base-pairs within the putative promoter element altered the resulting promoter activity (***Flick et al., 2002***). On the other hand, using a similar strategy the putative element of the influenza A promoter plays an important role during the viral polymerase interaction, either as a stabilizing double-stranded promoter element (***Flick et al., 1996; Fodor et al., 1994, id. 1995; Kim et al., 1997***) or as a regulatory element for viral mRNA transcription rates (***Fodor et al., 1998***).

For influenza virus, several models exist for genome transcription based on the formation of specific folding structures within the 5' and 3' viral termini. The most simplified model describes formation of a panhandle structure due to base-pairing of the 5' and 3' ends (***Hsu et al., 1987***). A so called “Fork” model (***Fodor et al., 1994, id. 1995***), describes a single-stranded conformation for nucleotides 1-10 at the 5' end of the vRNA and nucleotides 1-9 at the 3' end, and a double-stranded element for nucleotides 11-13 and 10-12 at the 5' and 3' end of the vRNA, respectively. The “Corkscrew” model (***Flick et al., 1996, id. 1999***) suggests formation of short stem-loop structures within both termini upon binding to polymerase, and involves base-pairing of nucleotides at position 2 and 9, and 3 and 8 of each genomic end and exposure of the intermediate positions 4-7 as a single-

stranded tetra-loop RNA structure. One of the latest models describes a combination of the "Fork" and the "Corkscrew" models but only proposes a functional stem-loop structure at the 5' end and not at the 3' end of the vRNA (***Pritlove et al., 1999***).

TABLE 1-2

Sequence of the 3' UTRs of ten members of segmented (-) ssRNA viruses.

Genus	Virus	RNA	3'-terminal sequence
Arenavirus	LCMV	L	***AGGAUC***UUCG***GUGCG***-3'
		S	***AGGAUC***CACU***GUGCG***-3'
		L	***AGGAUC***CUCG***GUGCG***-3'
		S	***AGGAUC***CACU***GUGCG***-3'
Hantavirus	HTNV	L	AGGGA***GUCUACUACUA***-3'
		M	UCGGU***GUCUACUACUA***-3'
		S	AGGGA***GUCUACUACUA***-3'
Nairovirus	DUGV	L	AUUGA***UGUCUUUGAGA***-3'
		M	AGGUA***UGUCUUUGAGA***-3'
		S	ACGUU***UGUCUUUGAGA***-3'
Orthobunyavirus	BUNV	L	GUAGG***AGUACACUACU***-3'
		M	UCGGU***AGUACACUACU***-3'
		S	UGUGG***AGUACACUACU***-3'
Phlebovirus	RVFV	L	UUGGGCGC***CUUUGUGU***-3'
		M	UGCACCGU***CUUUGUGU***-3'
		S	CUAGGGAG***CUUUGUGU***-3'
Tospovirus	TSWV	L	GUUACCU***GAUUGCUCU***-3'
		M	UUGCACU***GAUUGCUCU***-3'
		S	UUGACAC***GAUUGCUCU***-3'
Influenzavirus A *Influenzavirus B* *Influenzavirus C*	*Influenza A virus* *Influenza B virus* *Influenza C virus*		***CCUGCUUUUGCU***-3' ***CCUGCUUCUGCU***-3' ***CCUGCUUCUGCU***-3'
Tenuivirus	RSV	RNA 1	UUUUCCUCUG***GACUUUGUGU***-3'
		RNA 2	GUUAUACCCA***GACUUUGUGU***-3'
		RNA 3	UUAUUACCCA***GACUUUGUGU***-3'
		RNA 4	GAUAUGCCCU***GACUUUGUGU***-3'

Sequence of the 3' UTR with complementarity to the 5' UTR is in **bold** and *italics*. Abbreviation: UTR, untranslated region. Virus abbreviation: BUNV, *Bunyamwera virus*; DUGV, *Dugbe virus*; HTNV, *Hantaan virus*; LACV, *La Crosse virus*; LCMV, *Lymphocytic choriomeningitis virus*; RSV, *Rice stripe virus*; RVFV, *Rift Valley fever virus*; TSWV, *Tomato spotted wilt virus*.

Although replication of segmented (-) ssRNA viruses initiates from the 3' end of the genome vRNA and antigenome RNA to make full-length copies, genome transcription initiates only on the vRNA template with the exception of the arena-, phlebo- and tospoviruses. These viruses (additionally) contain one or more genome segments with an ambisense coding strategy and require bi-directional transcription of the v- and vcRNA strands for the expression of their genes. Whereas replication is initiated right at the 3' end of the vRNA template, transcription in contrast requires a short capped RNA primer for viral mRNA synthesis (***Buchmeier et al., 2001; Lamb and Krug, 2001; Schmaljohn and Hooper, 2001***).

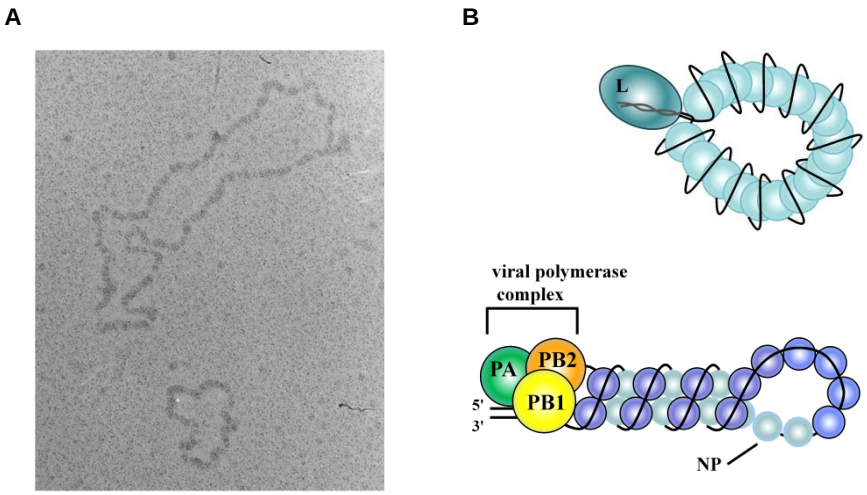


Figure 1-1 (A) Electron micrograph of TSWV RNPs. (B) Schematic presentation of the pseudo-circular RNPs of TSWV (upper diagram) and influenza virus (lower diagram).

Transcription (initiation and termination) and translation strategies

The RdRp of segmented (-) ssRNA viruses does not contain methyltransferase activity, and thus is not able to cap its own transcripts. To render translatable RNA transcripts, these viruses provide their mRNAs with a cap structures from host cellular mRNA molecules, by a process called “cap-snatching” (***Bouloy et al., 1978***). Viral transcripts thus can be distinguished from (anti)genomic RNA strands by the presence of a non-viral capped RNA leader sequences at their 5' ends. During cap-snatching, as being discovered and described first for influenza virus (***Beaton and Krug, 1981; Bouloy et al., 1978; Caton and Robertson, 1980; Dhar et al., 1980; Plotch et al., 1979, id. 1981***), the

viral polymerase steals capped RNA leader sequences from nascent host cell RNA polymerase II transcripts in the nucleus (for influenza virus) or from mature transcripts in the cytoplasm (for arena- and bunyaviruses) by endonucleolytic cleavage and uses them as cap donors/primers to initiate genome transcription **(Figure 1-2**).

The capped leaders vary in size from 1-7 nt in arenaviruses (***Garcin and Kolakofsky, 1992; Meyer and Southern, 1993***), 7-25 nt in hantaviruses (***Garcin et al., 1995***), nairoviruses (***Jin and Elliott, 1993b***), orthobunyaviruses (***Bishop et al., 1983; Bouloy et al., 1990; Dobie et al., 1997; Jin and Elliott, 1993a***), phleboviruses (***Collett, 1986; Simons and Pettersson, 1991***), tospoviruses (***Duijsings et al., 2001; Kormelink et al., 1992b; van Knippenberg et al., 2005a***) and 10-13 nt in influenza viruses (***Lamb and Horvarth, 1991; Shaw and Lamb, 1984***).

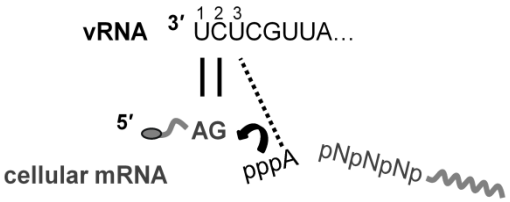


Figure 1-2 **Schematic presentation of TSWV genome transcription initiation.** Cleavage of a cellular mRNA and subsequent use of the capped RNA leader as primer for transcription initiation is shown.

To explain data from transcription initiation and replication studies for *Hantaan virus* (*Bunyaviridae*), an additional "prime-and-realign" mechanism has been proposed (***Garcin et al., 1995***). During this phenomenon, transcription is primed by a capped RNA molecule with a G residue at its 3' end. Priming occurs internally, i.e. on the C residue at position +3 from the 3' end of the vRNA template (3'-AUC…). After extension for a few nucleotides the leader is released and progressive elongation takes place only after backward re-alignment by virtue of the viral 3'-terminal sequence repeats. Although this mechanism remains to be further elucidated, it also explains the presence of repetitive sequences within the 5' leader of viral mRNAs from several phleboviruses (***Prehaud et al., 1997***; ***Simons and Pettersson, 1991***), orthobunyaviruses (***Bishop et al., 1983; Bouloy et al., 1990; Jin and Elliott, 1993a; Vialat and Bouloy, 1992***) and tospoviruses (***Duijsings et al., 1999, id. 2001; van Knippenberg et al., 2005a***).

Only limited information is available on transcription termination for many of the segmented (-) ssRNA viruses. Studies on influenza virus transcription termination have shown that RNA segments of this virus contain a U tract element near the 5' end of the

vRNA template that coincides with the end of transcription (***Li and Palese, 1994; Robertson, 1979; Robertson et al., 1981***) and appears functionally analogous to the U tract within non-segmented (-) RNA viruses (***Poon et al., 1999***). The poly(A) tail of influenza virus mRNAs is synthesized by reiterative copying of this U-tract sequence once the RNA polymerase bounces into the panhandle structure. In contrast, transcription termination for other segmented (-) RNA viruses, such as members of the *Arena*- and *Bunyaviridae*, is not at all clear (***Buchmeier et al., 2001; Schmaljohn and Hooper, 2001***).

In the (-) sense coding segments of these viruses, sequence motifs have been found near or at the 3' end of mRNAs to be involved in transcription termination (***Schmaljohn and Hooper, 2001***). For the phleboviruses RVFV and *Toscana virus* (TOSV), mRNA transcribed from the M RNA segment was found to terminate after a C-rich region in the virus templates (***Collett, 1986; Gro et al., 1997***). For the ambisense coding segments, RNA secondary structures or G-rich like sequence motifs within the intergenic region (IR) appear to terminate the elongation by viral polymerase (***Buchmeier et al., 2001; Schmaljohn and Hooper, 2001***). For arenaviruses, transcription termination clearly involves secondary RNA structures as it was shown for *Tacaribe virus* (TV) (***Iapalucci et al., 1991; Lopez and Franze-Fernandez, 2007***). By using a reverse genetics system, for the first time direct experimental evidence was obtained which supported the idea that transcription termination in TV is related to a single, highly stable hairpin structure that is independent of sequence. This structural element comprises a terminal loop and a stem with variable numbers of uninterrupted base-pairs. Hairpin structures were also predicted at the IR of the ambisense S and M RNA segments of the plant-infecting tospoviruses, indicating that mRNA transcription termination of their ambisense RNA segments might also involve secondary RNA folding structures (mechanisms) similar to those described for arenaviruses (***de Haan et al., 1990; van Knippenberg et al., 2005b***). For the phleboviruses, transcription termination within the ambisense S RNA segment is less clear. Secondary structure prediction analysis suggests that the IR region of the S segment of *Punta toro phlebovirus* forms a stable hairpin structure while transcription termination of its mRNAs maps near the top of this structure (***Emery and Bishop, 1987***). Studies on the ambisense S RNA of *Uukuniemi phlebovirus* (UUKV) demonstrated that both RNA transcripts terminate beyond the IR, i.e. within a sequence that localizes in the 3' end of the other opposite open reading frame (ORF) (***Simons and Pettersson, 1991***). Within this IR region, a short A/U-rich hairpin folding structure is predicted, although this structure does not seem very stable. A similar overlap in the 3' ends of N and NSs (non-structural protein) mRNAs was observed for TOSV phlebovirus (***Gro et al., 1992, id. 1997***). However, no RNA folding structures were observed in the IR of the S RNA segment of RVFV, *Sandfly fever Sicilian virus* (SFSV), and TOSV phleboviruses. Instead, G-rich regions are present in the sequence where transcription termination occurs (***Giorgi et al., 1991***).

Systems for the manipulation of a segmented (-) RNA virus

In order to study the *cis*-and *trans*-acting factors involved in replication and transcription of viral genomes from segmented (-) ssRNA viruses, several *in vitro* and reverse genetics systems have been established. The first system was developed by Luytjes *et al.* (***1989***) and allowed manipulation of *Influenza A virus*. The system was based on the transfection of *in vitro* reconstituted RNP complexes into helper influenza virus-infected cells. Although being the first of its kind, it had a few limitations and disadvantages. First, it required appropriate selection systems to distinguish the modified virus from the helper virus. Secondly, it did not allow identification of *trans*-acting signals required for transcription/replication of the viral genome. Thirdly, the system suffered from the limitation that engineering of all RNA segments was not possible. As an alternative, an influenza virus-free *in vitro* system was developed which was based on the use of recombinant *Vaccinia virus*-expressed PB1, PB2 and PA, and allowed exploration of the influenza virus polymerase complex. Studies using this approach demonstrated that the active polymerase requires sequences located at both the 5' and 3' ends of the viral genome (***Hagen et al., 1994; Tiley et al., 1994***). However, also within this system manipulation of all RNA segments was not possible.

A system that provided this option became available with the development of reverse genetics systems based on minireplicons. These have meanwhile been developed for a majority of the segmented (-) RNA viruses [arenaviruses (***Lee et al., 2000; Lopez et al., 1995***) and bunyaviruses (***Accardi et al., 2001; Blakqori et al., 2003; Dunn et al., 1995; Flick and Pettersson, 2001; Flick et al., 2003a and b; Prehaud et al., 1997***)] in which, a virus-like RNA (minigenome) template containing a reporter gene (e.g. CAT) flanked with the 5' and 3' untranslated regions (UTR) of vRNA is driven from T7- or RNA polymerase I (Pol I)-driven plasmids. Replication and transcription of the RNA template was obtained during co-expression with the viral nucleoprotein and polymerase proteins from RNA polymerase II (Pol II)-driven plasmids or co-infection with helper virus (**Figure 1-3**). In case T7 RNA polymerase driven plasmids are used, the polymerase is provided by co-infection with a T7-recombinant *Vaccinia virus* (vv-T7) (***Accardi et al., 2001; Dunn et al., 1995; Lopez et al., 1995***) (**Figure 1-4**). The minireplicon system mimics replication and transcription of viral genomic RNA and is used as an excellent approach to identify and subsequently mutagenize promoter elements for initiation of both transcription and replication of vRNA segments, but also maps genome packaging signals. In this way, e.g. the panhandle structure of the UUKV phlebovirus S RNA segment was demonstrated to contain the promoter elements for transcription and replication, and that recognition by the viral RdRp involved both the 5' and 3' UTR (***Flick et al., 2002***).

In contrast to the reconstitution of transcriptionally active RNPs from cloned copies of vRNA segments, the rescue of infectious virus particles entirely from cloned

cDNA has only been achieved for a few segmented (-) ssRNA viruses. The first one rescued entirely by reverse genetics was the BUNV (***Bridgen and Elliott, 1996***). Copies of the large (L), medium (M) and small (S) RNA segments were expressed from T7 RNA polymerase driven plasmids, flanked at their 3' end with a ribozyme to generate authentic viral termini. In the additional presence of N and RdRp encoding plasmids and T7 RNA polymerase-expressing *Vaccinia virus*, BUNV particles were obtained. Successful generation of recombinant *Influenza A virus* entirely from cDNAs was achieved with a system in which cDNA constructs for all (8) RNA segments were expressed from RNA Polymerase I driven plasmids in the additional presence of 4 RNA (Pol II) driven constructs coding for the N and polymerase (PA, PB1 and PB2) proteins (***Fodor et al., 1999; Neumann et al., 1999***). This system was a breakthrough in the field of orthomyxovirus research and allowed manipulation of all viral genomic segments simultaneously. In another case, the *Thogoto orthomyxovirus* (THOV) has been rescued by a combination of the T7- and Pol I-driven systems (***Wagner et al., 2001***). Most of the current knowledge on *trans*-acting elements required for viral transcription and replication has been obtained by studies involving the recovery of transcriptionally active RNPs from cloned cDNA.

The limitation of the RNA Pol I system is that transcription exhibits species specificity. For this reason, species specific RNA Pol I promoters have to be used for each different cell type. On the other hand, the T7 RNA polymerase system is based on the vaccinia helper virus which has the potential disadvantage that due to the cytopathogenic effect of *Vaccinia virus* infection the cells can only be studied for a limited amount of time. For this reason, an improved T7 RNA polymerase system has been developed in which T7 expressing cell lines are used that do not require the vaccinia helper virus anymore (***Blakqori and Weber, 2005; Ikegami et al., 2006; Lowen et al., 2004; Sanchez and de la Torre, 2006; de Wit et al., 2007***). A disadvantage of this system is that its success depends on stable cell lines with high levels of T7 polymerase expression.

Despite some disadvantages and limitations with each of these systems, they altogether have enormously boosted the insight into the replication/transcription of segmented (-) ssRNA viruses. Besides this, influenza virus RNA packaging signals meanwhile have been identified and fine mapped (***Liang et al., 2005, id. 2008***), entire virus strains (e.g. 1918 Spanish Flu) have been reconstituted and their pathogenicity can now be studied as well as pathogenicity factors themselves. Furthermore, reassortants can readily be made for fundamental studies and/or to provide in new strains for vaccine production. Finally, the reverse genetics systems may contribute in the development of vectors for therapeutic gene delivery by generating virus-like particles. These particles lack one or more viral genes encoding structural proteins. As result, infectious progeny viruses cannot be generated.

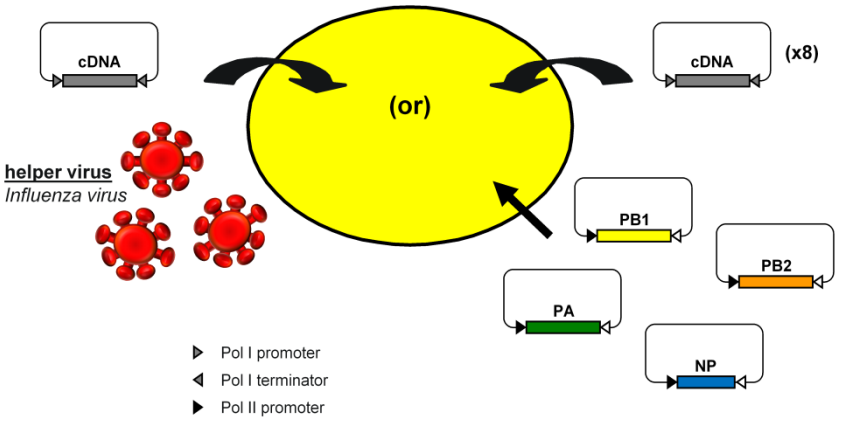


Figure 1-3 **Systems for the generation of *Influenza A virus* from cloned cDNA.** Cells are transfected with (A) a plasmid containing a cDNA that encodes one of the viral RNAs, flanked by RNA polymerase (Pol) I promoter and terminator sequences, or (B) plasmids that encode all eight vRNAs under the control of the RNA Pol I promoter and plasmids encoding the four required viral proteins (PB1, PB2, PA and NP) for vRNA replication and transcription under control of the RNA Pol II promoter. In the first case, influenza helper virus is required for vRNA replication and transcription.

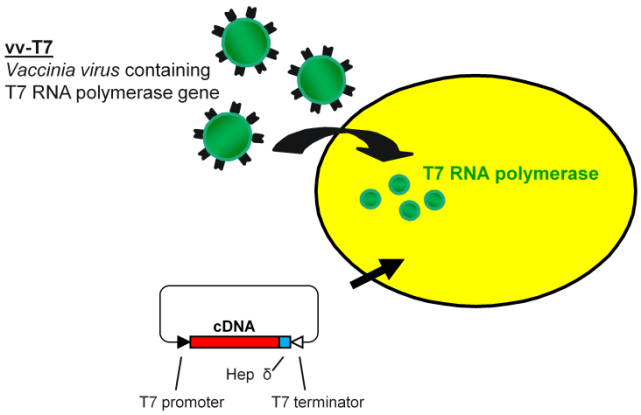


Figure 1-4 ***Vaccinia virus*-T7 (vv-T7) system.** Virus rescue requires the transfection of a cell with plasmids encoding the genome segments along with the support plasmids encoding the viral proteins needed for replication and transcription, all under the control of a T7 promoter. RNA transcripts are produced by the bacteriophage T7 RNA polymerase which is provided by infection with a T7-recombinant *Vaccinia virus*. The exact end of the RNA is specified by self-cleavage of the nascent RNA by the *Hepatitis delta virus* (Hep δ) antigenome ribozyme.

Scope of this Thesis

Recently, extensive studies on transcription initiation of the plant-infecting bunyavirus TSWV have revealed that the virus displays a preference for multiple base-pairing cap donors during transcription initiation (***Duijsings et al., 1999, id. 2001; van Knippenberg et al., 2002, ead. 2005a***). Considering that cap-snatching is a highly conserved mechanism, it is tempting to speculate that the findings for TSWV apply to all segmented (-) ssRNA viruses. However, for many of these viruses, no such information is available, and findings have been limited to the cloning of viral transcripts and elucidation of non-viral leader sequences. For *Influenza A virus* extensive research has been performed on transcription initiation (***Beaton and Krug, 1981; Bouloy et al., 1978; Caton and Robertson, 1980; Dhar et al., 1980; Plotch et al., 1979, id. 1981***), but one major question still has not been answered and has remained a matter of strong debate for a long time: do capped RNA leaders require a base complementarity to the vRNA template to prime transcription. For *Influenza A virus* conflicting reports exist on this issue. While some *in vitro* studies suggest that base-pairing between the capped leader and the viral genome may contribute to the alignment of the leader to the vRNA template (***Plotch et al., 1981; Hagen et al., 1994; Chung et al., 1994***), other studies support the idea, which is favored by most, that priming by capped RNA does not require base-pairing with the influenza vRNA template (***Hagen et al., 1995; Krug et al., 1980***).

To address this issue, first an *in vitro* transcription system was established for influenza based on the use of transcriptionally active purified virus particles, and tested for its capacity to accept an exogenous *Alfalfa mosaic virus* (AlMV) RNA as a source for capped RNA leaders (**Chapter 2**). In **Chapter 3**, various mutant AlMV RNA3 (AlMV3) leaders, differing in i) base complementarity to the vRNA template, ii) residues 5' of potential base-pairing residues or iii) leader length, were tested as cap donors in the *in vitro* assay in pair-wise or multiple competition assays. Based on these findings, less-favored and well favored cap donor molecules were tested for their competitiveness during globin-initiated influenza NS1 gene transcription *in vitro*. To substantiate these findings and confirm that the *in vitro* findings genuinely reflect the cap-snatching mechanism during a natural infection *in vivo*, similar studies were repeated in influenza virus infected cell cultures (**Chapter 4**).

While transcription initiation of TSWV has been well studied, transcription termination from the ambisense S and M genomic RNA segments has not. In this case, transcription termination is most likely triggered by the formation of a folding structure during nascent transcription of the IR. The IR of TSWV contains stretches of highly A- and U-rich sequences which are predicted to fold into a stable hairpin structure. As a result, viral transcripts do not contain a common poly(A) tail, but a 3' predicted (AU-rich) hairpin structure. It is postulated that this hairpin likely acts as a functional equivalent to a poly(A)

tail and hence plays an important role in translation. To test this hypothesis, various N gene-based constructs (**Figure 1-5**) were made and tested in Baby Hamster Kidney (BHK-21) cells for translation efficiency in a vv-T7 expression system (**Chapter 5**).

In **Chapter 6**, the results of the experimental chapters are discussed in the light of reported findings on genome transcription initiation and termination of the animal-infecting bunyaviruses. The commonalities found between TSWV and *Influenza A virus* cap-snatching are discussed and taken as a starting point for a proposed antiviral drug design against a large variety of different segmented (-) ssRNA viruses.

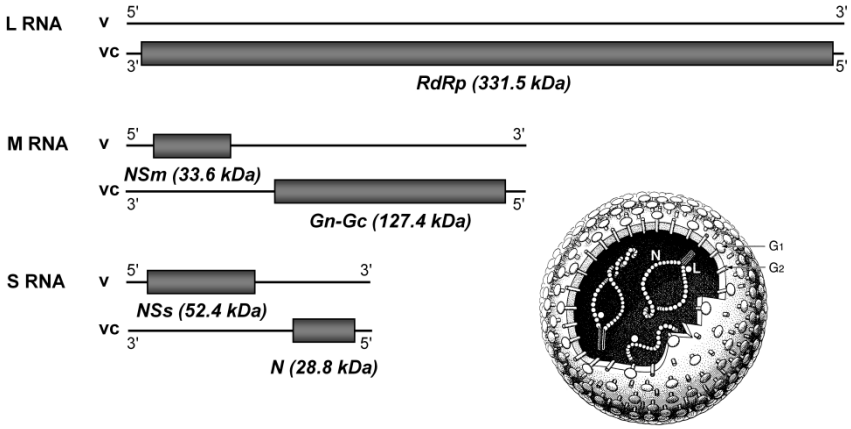
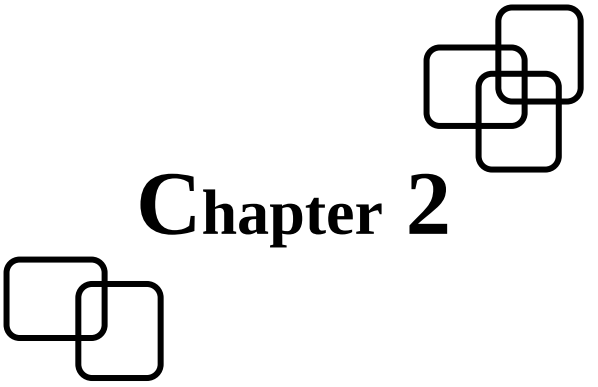


Figure 1-5 ***Tomato spotted wilt virus*** **(TSWV)**. Schematic presentation of TSWV virus particle and its genomic organization. vRNA is viral sense RNA, vcRNA is viral complementary RNA. ORFs are indicated by boxes.

“Education is a guide; knowledge is a key.”

Buddha



Chapter 2

Purified *Influenza A virus* particles support genome transcription *in vitro*

During influenza virus infection, the viral polymerase snatches cellular mRNAs to obtain capped RNA primers for transcription initiation. Here, an *in vitro* transcription system was established based on the use of purified influenza virus particles. *De novo* synthesis of viral RNA was shown only in the presence of Rabbit reticulocyte lysate (RRL) and rendered products that reflected viral genomic RNA segments in size. The requirement for RRL could not be replaced by the addition of RNA as source for capped RNA leaders, neither by elevating the rNTP concentration. The occurrence of *de novo* viral genome transcription was demonstrated by cloning and sequence analysis of viral transcripts containing non-viral leader sequences. The latter were snatched either from α- and β-globin mRNAs present within the RRL, or from *Alfalfa mosaic virus* RNAs that were added as source for capped RNA leaders.

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*I*ntroduction

Influenza A virus is a representative of the *Orthomyxoviridae*, whose members are characterized by a segmented single-stranded (ss) RNA genome of negative (-) polarity. In contrast to positive (+) ssRNA viruses whose genomes are able to initiate infection in the absence of any other viral products, the genomes of (-) ssRNA viruses alone are non-infectious. Initiation of virus transcription and replication requires the viral polymerase complexed with the viral RNA (vRNA) and nucleoprotein into transcriptionally active ribonucleoprotein (RNP) complexes.

The viral polymerase of influenza virus consists of three subunits: PB1, PB2, and PA. It transcribes and replicates the viral genome which consists of eight RNA segments. Synthesis of viral mRNA from the (-) genomic vRNA is dependent on a capped RNA primer mechanism, known nowadays as "cap-snatching" (***Beaton and Krug, 1981; Bouloy et al., 1978; Caton and Robertson, 1980; Dhar et al., 1980; Plotch et al., 1979, id. 1981***). During this mechanism, the viral polymerase binds to the 5' cap structures of cellular mRNAs and cleaves the cellular mRNAs 10-13 nt downstream of the cap (***Li et al., 2001***). The resulting capped RNA leader is used by the viral polymerase to prime transcription. The 5' and 3' ends of the vRNA template have partial inverted complementarity and form a panhandle structure (***Li and Palese, 1994; Robertson, 1979***). During transcription termination the viral polymerase complex starts stuttering once it runs into the panhandle structure, leading to poly-adenylation template by a stretch of 5 to 7 U residues about 17 nt from the 5' end (***Robertson et al., 1981***). In contrast to transcription, replication of the genome does not require a primer. Instead, the viral polymerase initiates RNA synthesis on the 3' residue of the vRNA.

Several studies have been carried out on the transcriptional activity of the polymerase and the requirements for elongation of capped RNA leaders on the vRNA template. The insight into these processes has advanced with the use of various *in vitro* (***Hagen et al., 1994; Tiley et al., 1994***) and reverse-genetics systems (***Fodor et al., 1999; Neumann et al., 1999***), in which many *cis*- and *trans*-acting elements involved in transcription/translation have been analyzed. Luytjes *et al.* (***1989***) established the first system for the manipulation of *Influenza A virus*. *In vitro* reconstituted RNPs were transfected into helper influenza virus-infected cells. RNP complexes were made by incubating synthetic RNA transcripts with purified nucleoprotein (NP) and the three viral polymerase proteins (PB1, PB2 and PA) from influenza virus. The helper virus was used as an intracellular source of viral NP and the polymerase proteins and of the other vRNAs. Alternative influenza virus-free *in vitro* systems have been described exploring the influenza virus polymerase complex expressed by recombinant vaccinia viruses (***Hagen et al., 1994; Tiley et al., 1994***).

Reverse genetics systems based on plasmid DNA for the reconstitution of *Influenza A virus* entirely from cDNAs (***Fodor et al., 1999; Neumann et al., 1999***), have made the vaccinia helper virus superfluous. Transfection of eight plasmids under the control of an RNA polymerase (Pol) I promoter for the production of vRNA and four expression plasmids encoding the three polymerase proteins and NP under the control of an RNA Pol II promoter resulted in the rescue of infectious virus. Hoffmann *et al.* (***2000***) introduced a modification of the RNA Pol I system, the Pol I/II system, in which virus can be generated from only eight instead of 12 plasmids. In this system, the eight genome segments encoding (-) sense vRNA is cloned between the RNA Pol I promoter and terminator transcription unit. This transcription unit is further inserted in the (+) orientation between an RNA Pol II promoter and a transcription termination signal. Transfection of these eight plasmids results in the production of mRNA and (-) sense vRNA generated from the same DNA template. In order to reduce the number of plasmids to be transfected and thus enabling the use of cell lines with low transfectability, an improved Pol I system was also developed (***Neumann et al., 2005***). In this system, plasmids containing up to eight RNA Pol I transcription cassettes were generated.

All these systems have the disadvantage of using a species specific RNA Pol I transcription which limits their use to cells of primate or avian origin. To circumvent this problem, another reverse-genetics system was developed for the rescue of recombinant influenza virus using T7 RNA polymerase (***de Wit et al., 2007***). Expression of T7 RNA polymerase was provided from a contransfected plasmid DNA, a strategy that is applicable in all mammalian cell lines irrespective of their host origin. One potential disadvantage, however, is the strict dependency on cell lines which constitutively express high levels of T7 RNA Polymerase.

So far, the many diverse *in vitro* and *in vivo* reconstitution systems used to study transcription/translation of influenza virus have led to some conflicting results, e.g. the requirement of only one terminal end for endonuclease activity in one system (***Li et al., 2001***), versus both termini in another system (***Cianci et al., 1995; Hagen et al., 1994***).

More recent studies on the plant-infecting bunyavirus *Tomato spotted wilt virus* (TSWV) have shown that extensive base-pairing between capped RNA leaders and vRNA template promote cap donor usage (***Duijsings et al., 2001; van Knippenberg et al., 2005a***). On this point only limited data is available for influenza virus, but also here several studies present conflicting results (***Hagen et al., 1994; Krug et al., 1980; Rao et al., 2003***). Since cap-snatching seems a highly conserved mechanism of transcription initiation for segmented (-) ssRNA viruses, it is not unlikely that the observations earlier made for TSWV apply for influenza virus as well. In order to test this hypothesis and in specific analyze cap donor requirements during influenza virus cap-snatching in further detail, a virus transcription system was required.

The present study describes the establishment of an *in vitro* system that employs purified *Influenza A virus* particles to support the synthesis of viral mRNA. The use of α-

and β-globin mRNAs present in the RRL, and exogenously added wild type (WT) *Alfalfa mosaic virus* RNA3 (AlMV3) as source for capped RNA leaders opens the possibility to exploit the system to study influenza transcription initiation in further detail.

Results

Influenza A transcription in the presence of RRL

To enable an investigation on cap donor requirements during influenza transcription initiation, an *in vitro* transcription assay was established (**Figure 2-1; Materials and methods**) in which purified *Influenza A virus* particles support transcription. It was decided to choose purified mature virus particles for this since these contain the minimal elements to initiate, like in a natural infection cycle, the first transcription steps that are required for ongoing transcription/replication.

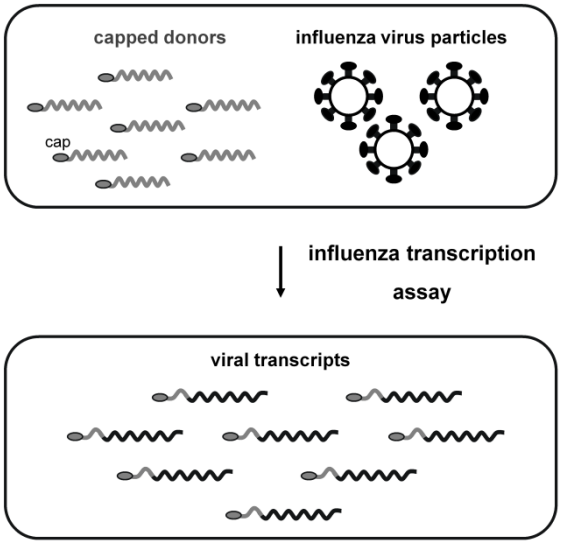

Figure 2-1 **Schematic representation of the methodology.** *In vitro* influenza transcription assay of *de novo* synthesized viral transcripts. As cap donors endogenous globin mRNA or exogenously added AlMV3 transcripts are used.

In vitro reaction products from assays performed in the presence of radionucleotides were resolved by RNA electrophoresis. The results showed that purified virus particles supported the *de novo* synthesis of RNA molecules, but only in the presence of RRL (**Figure 2-2, lanes 1 and 2**). Earlier, this was also observed for several other (-) ssRNA viruses (***Bellocq et al., 1987; Nguyen et al., 1997; van Knippenberg et al., 2002; Vialat and Bouloy, 1992***) in which the requirement of RRL was explained to provide not only in a source of capped RNAs but also factors needed to support in ongoing transcription and prevent premature transcription termination.

When AlMV3 RNAs were used as primers for *in vitro* influenza transcription in the absence of RRL, similar to earlier studies on several other (-) ssRNA viruses (***Bouloy et al., 1980; Li et al., 2001; Plotch et al., 1979, id. 1981; Robertson et al., 1980; van Knippenberg et al., 2002***), no transcriptional activity was observed (**Figure 2-2, lane 4**). The additional increase of rNTP concentrations in the absence of RRL did not recover influenza transcriptional activity either (data not shown).

When in addition to RRL, AlMV3 RNA was provided to the *in vitro* transcription no further increase in transcriptional activity was observed in comparison to the activity observed in the presence of RRL only (**Figure 2-2, lanes 1 and 3**). This was most likely due to the fact that sufficient globin mRNA molecules were present in the RRL to supply in a source of capped RNA for cleavage of 9-13 nt capped RNA leaders. This was strengthened by the observation that the same RRL was previously shown to provide sufficient amounts of globin RNA for cleavage of ~16 nt sized capped globin RNA leaders to initiate *in vitro* genome transcription of TSWV (***van Knippenberg et al., 2002***), in which additional AlMV RNA also did not further increase the yields of TSWV transcription.

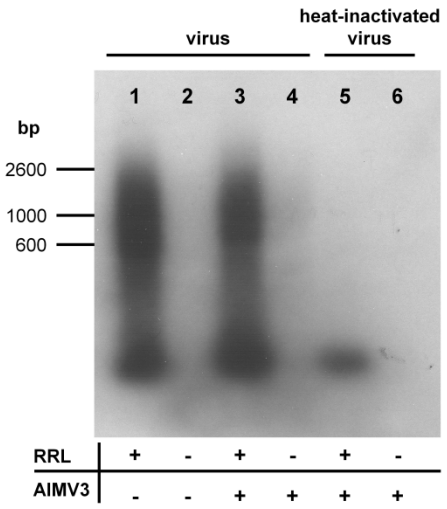

Figure 2-2 Analysis of *in vitro* synthesized influenza mRNA. *In vitro* transcription reactions using [α-^{32}P]CTP radiolabelled nucleotides in the presence/absence of RRL were resolved in a 1% agarose gel and subsequently blotted to Hybond-N membrane. Lanes 1-4: Active virus. Lanes 5-6: heat-inactivated virus. In the absence of RRL, addition of AlMV3 leaders did not recover transcription. RNA size markers are indicated at the left.

RT-PCR analysis of influenza transcripts

To verify that (some of) the *de novo* synthesized RNA molecules were products of genuine transcriptional rather than replicational activity, NS1 RNA molecules from the *in vitro* reaction were reverse transcriptase (RT) PCR cloned and analyzed for the presence of non-viral leader sequences. In view of the expected short (9-13 nt) added leaders, a strategy using the template-switch capacity of Superscript™ II RT was applied to amplify *de novo* synthesized transcripts (**Figure 2-3A; Materials and methods**). In brief, first-strand cDNA was primed using an internal oligonucleotide for the *Influenza* NS1 (or NP) gene in the additional presence of a template-switch oligonucleotide (TS-oligo) (**Figure 2-3A**). Once Superscript II RT reached the 5' end of a mRNA (and not of a genomic RNA strand), its terminal transferase activity attached several C-residues onto the newly synthesized cDNA strand. Subsequently, the TS-oligo, containing a terminal stretch of G residues, paired with the extended first strand cDNA tail and served as an extended template for RT to switch to. As a result, the template switch approach resulted in a DNA copy of the entire 5' leader sequence of viral mRNA transcripts flanked by the TS-oligo sequence. Final PCR amplification of the first-strand cDNA involved a nested NS1 (or NP) primer, and a primer matching the TS-oligo sequence in combination with i) the first 11 nt of the α-globin mRNA leader sequence, ii) the first 6 nt identical in both α- and β-globin mRNA leader sequence**,** or iii) the first 8 nt of the AlMV3 leader sequence.

Cloning and sequencing of the products obtained (**Figure 2-3B, lane 1**) revealed the presence of α- or β-globin RNA leader sequences at the 5' ends of NS1 transcripts, snatched from globin mRNAs present in the RRL (**Table 2-1**), thus confirming the occurrence of *in vitro* transcription. Control reactions using heat-inactivated virus never yielded any PCR product (**Figure 2-3B, lane 2**). Surprisingly, 16 out of the 19 clones analyzed contained α-globin leader sequences in viral transcripts and only 3 (out of 19) β-globin leader (**Table 2-1**). A closer look at those sequences indicated that cleavage of α-globin leaders possibly took place after A_{14} or $A_{14}G_{15}$ in order to allow base-pairing with the UC residues at positions +1 and/or +2 from the 3' end of the vRNA template (3'-UCG…). On the other hand, cleavage of β-globin leaders might have occurred after A_{14} to assist in base-pairing to the ultimate U residue of the influenza template.

The idea of base-pairing is further strengthened by the observation that 5 out of these 19 clones, lacked the first viral A residue, which likely could be due to cleavage after G_9 or G_{10} within the α-globin leader and after G_{13} within the β-globin leader and subsequent internal priming on the 3'-penultimate C residue of the viral template (**Table 2-1**). Additionally, 3 of these leaders showed a repetitive sequence (GCAGC) that probably may have resulted from prime-and-realign.

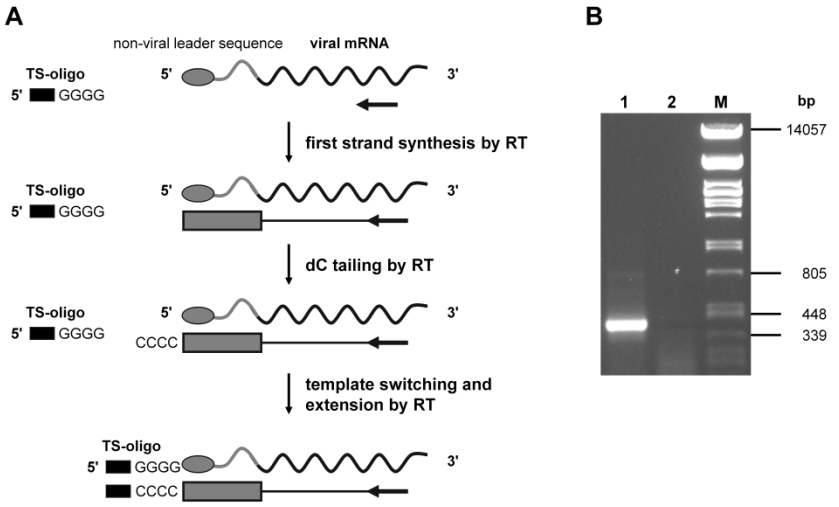

Figure 2-3 **RT-PCR of *de novo* synthesized viral transcripts.** (A) Schematic procedure of RT-PCR amplification of influenza viral transcripts harboring non-viral leader sequences, using the template-switch methodology. (B) RT-PCR analysis of *in vitro* synthesized influenza mRNA in the presence of RRL, and active virus (lane 1) or heat-inactivated virus (lane 2). Reverse transcription was performed using internal primer specific for the *Influenza* NS1 gene, followed by PCR using internal primer for the same gene in combination with a primer specific for the 5' first 6 nt of α- or β-globin mRNA. M: molecular marker.

TABLE 2-1

Distribution and nucleotide sequence of α- and β-globin leaders snatched from globin messengers to prime virus NS1 mRNA transcription *in vitro*.

Capped leader	Retrieved mRNA 5' sequence	# clones
α-globin[a]	5'-ACACUUCUGGUCC***AG***CAAAA	13
	5'-ACACUUCUG***G***CAGCAAAA	1
	5'-ACACUUCU***G***CAGCAAAA	2
β-globin[b]	5'-ACACUUGCUUUUG***A***GCAAAA	1
	5'-ACACUUGCUUUU***G***CAAAA	2

Viral sequence is underlined, while residues that possibly could originate from the globin leader and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*.

9 **14**

[a] α-globin RNA sequence: 5'-ACACUUCUGGUCCAGUCCGA....3'

13

[b] β-globin RNA sequence: 5'-ACACUUGCUUUUGACACAAC....3'

Exogenous AlMV3 capped RNA leaders are used as cap donors for *in vitro* transcription

To test whether the established *in vitro* transcription system would accept an exogenous (manipulable) source of capped RNA, WT AlMV3 was provided to the *in vitro* transcription assay (**Table 2-2**). *De novo* synthesized viral transcripts were RT-PCR cloned using an internal NS1 primer combined with a TS-oligo primer containing only the first 4 AlMV3 nucleotides, and subsequently sequence analyzed for the presence of AlMV3 RNA leader sequences.

TABLE 2-2

WT AlMV3 leader used in assay.

Capped leader	5' end sequence of capped leader
	12
WT	5'-GUAUUAAUACCAUUUUCAAAAUAUUCC

Residue potentially base-pairing to the viral template (3'-UCG…) is underlined.

Products of expected size (**Figure 2-4, lane 1**) were obtained and showed to consist of the NS1 transcript preceded with the first 11 nt from the AlMV3 5'-terminal RNA leader sequences (**Table 2-3**). It is of importance to mention that the WT AlMV3 contains a potential single base complementarity to the vRNA template (nucleotide A_{12}). Further analysis of the leader sequence within the NS1 clones showed the presence of repetitive sequences in 1 clone. The RT-PCR control, without any addition of AlMV3 leaders, did not show any product (**Figure 2-4, lane 2**).

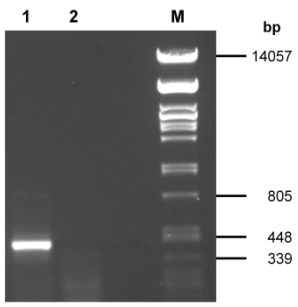

Figure 2-4 RT-PCR of *in vitro* synthesized influenza transcripts. RT-PCR analysis of *in vitro* synthesized influenza mRNA in the presence of RRL, and presence (lane 1) or absence (lane 2) of WT AlMV3 leader. Reverse transcription was performed using internal primer specific for the *Influenza* NS1 gene, followed by PCR using internal primer for the same gene in combination with a TS-oligo primer specific for the AlMV3 leader. M: molecular marker.

The results altogether suggested that the influenza virus transcription system developed was able to accept an exogenous source of synthetic capped RNA leaders which make the system manipulable and a powerful tool to further study the cap donor requirements in further detail. Using synthetic, mutant AlMV3 RNA transcripts, cap donor requirements, and in specific the importance of base-pairing as a selective criterion for cap donor usage, can now be addressed.

TABLE 2-3

Usage of WT leader snatched to prime NS1 mRNA transcription *in vitro*.

Capped leader offered	Retrieved mRNA 5' sequence	# clones
WT	5'-GUAUUAAUACC***A***GCAAAA	6
(500 ng)	5'-GUAUUAAUACC***A***GCAGCAAAA	1

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*.

Discussion

During *in vitro* influenza virus transcription in the presence of RRL, globin messengers were used to prime transcription as expected, as the RRL (although micrococcal nuclease treated) was earlier already shown to contain sufficient amounts of fragmented endogenous cap donors (***van Knippenberg et al., 2002***). Although the requirement of RRL for *in vitro* transcription still remains unknown, it was also observed for several other (-) ssRNA viruses (***Bellocq et al., 1987; Nguyen et al., 1997; van Knippenberg et al., 2002; Vialat and Bouloy, 1992***). Initially the requirement during *in vitro* transcription studies of bunyaviruses suggested a translational dependence, but no actual viral protein products were required. Further analyses using translation inhibitors as well as *in vitro* assays using a nucleotide analogue led to the hypothesis that the RRL was required to provide not only in a source of capped (globin) RNAs but also factors needed to support in ongoing transcription and prevent premature transcription termination (***Bellocq et al., 1987; Vialat and Bouloy, 1992; van Knippenberg et al., 2004***). The observations made here, in which the exchange of RRL for AlMV3 RNA did not recover *Influenza A virus* transcriptional activity *in vitro*, at least implies that the RRL is not solely required to provide in a source of cap donor.

Previous studies have reported that the globin molecules are good primers for influenza virus transcription initiation (***Bouloy et al., 1978; Plotch et al., 1979, id. 1981; Robertson et al., 1980***), and some have even suggested β-globin leaders are more effective primers than α-globin (***Bouloy et al., 1978; Plotch et al., 1979***). Considering that β-globin messengers appear to be more abundant in RRL than α-globin messengers (***Bouloy et al., 1978***), the higher presence of α-globin leader sequences in viral transcripts within a population of randomly cloned α- and β-globin initiated influenza transcripts (**Table 2-1**) was surprising and suggests a preference for the α-globin leader. A further look at both leader sequences revealed a base complementarity of the α-globin, by the $A_{14}G_{15}$ dinucleotide, to the 3'-ultimate residues of the influenza vRNA template compared to the β-globin leader that only contained a single complementary nucleotide (A_{14}) (**Figure 2-5A**). The preferential use of α-globin leaders versus β-globin leaders could thus be explained by its higher base complementarity to the vRNA template. This would be in agreement with earlier observations made for TSWV, and for which base-pairing has been shown a selective criterion for cap donor usage (***Duijsings et al., 2001; van Knippenberg et al., 2005a***).

Interestingly, the α-globin mRNA also contains G residues at positions 9 and 10, which could base-pair with the penultimate C residue of the influenza vRNA template (**Figure 2-5B**). *De novo* transcripts resulting from this internal priming lack the first viral A residue. Cloning of NS1 transcripts in which the A residue indeed showed to be absent thus strengthened the idea that base-pairing plays an important role during influenza transcription initiation. Similar internal priming was observed with the β-globin leaders as well (**Figure 2-5B**). This phenomenon was earlier observed for TSWV mRNAs synthesized *in vitro* (***van Knippenberg et al., 2005a***), where the first one or even the first two viral nucleotides were sometimes missing as a result of internal base-pairing of the capped leader and priming of transcription at the second or even third residue of the viral template. Why internal priming occurs during cap-snatching is still unknown, but in many influenza *in vivo* studies, cloned copies of mRNAs were noticed that had primed on the 3' penultimate C residue of the vRNA template instead on the ultimate U residue (***Beaton and Krug, 1981; Caton and Robertson, 1980; Dhar et al., 1980; Lai et al., 1981; Lamb and Lai, 1980; Lamb and Lai, 1981; Markoff and Lai, 1982; Vreede et al., 2008***).

The presence of repetitive sequences (GCAGC) in 4 out of 26 cloned viral transcripts (**Tables 2-1 and 2-3**) indicates that "prime-and-realign", as first described for *Hantaan virus* and later for transcription initiation of other members of the *Bunyaviridae* and *Arenaviridae* (***Bishop et al., 1983; Bouloy et al., 1990; Duijsings et al., 1999, id. 2001; Garcin et al., 1995; Jin and Elliott, 1993b; Simons and Pettersson, 1991; van Knippenberg et al., 2005a***), also occurs with influenza virus. During prime-and-realign WT AlMV3 leaders offered are likely cleaved by the viral polymerase/endonuclease downstream A_{12}, and after alignment, by virtue of the base-pairing A residue, on the vRNA template become elongated for two nucleotides (GC). Before further elongation proceeds,

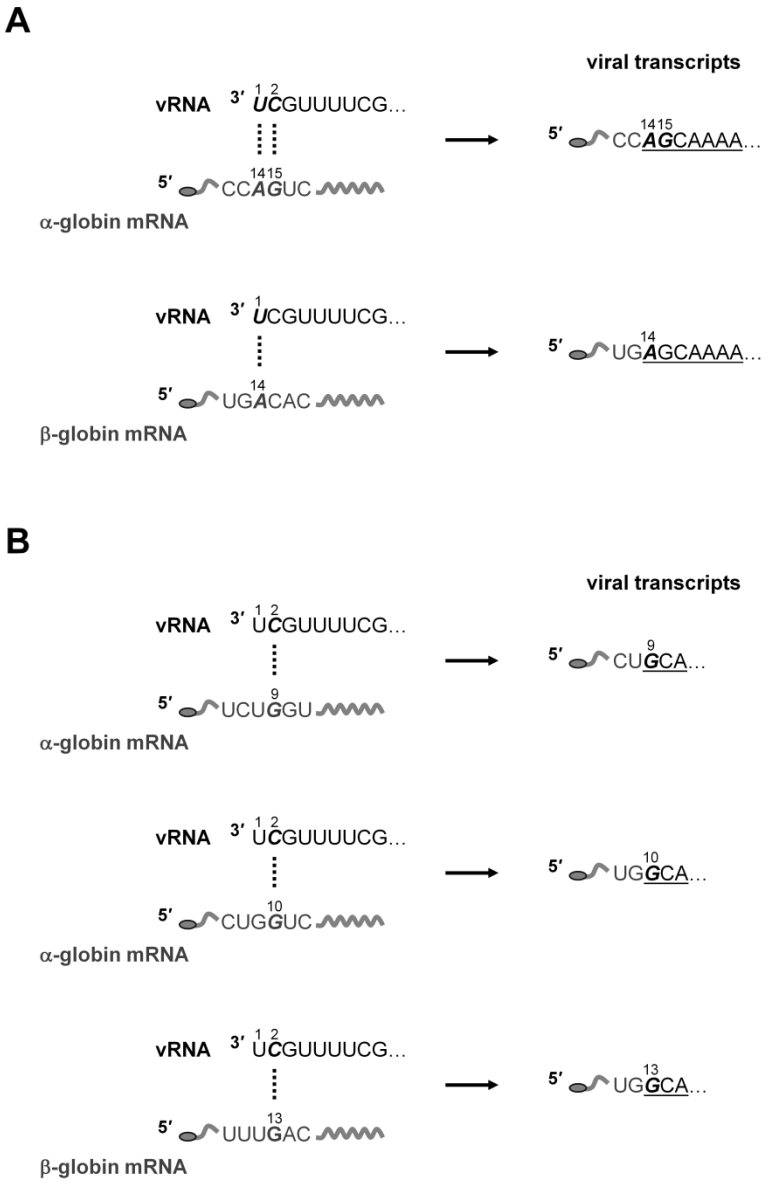

Figure 2-5 **Base-pairing between α- or β-globin leader and the vRNA template.** (A) Schematic representation of single or double base-pairing interactions. (B) The occurrence of internal priming. Influenza sequence is underlined, while residues that possibly could originate from the globin leader and with complementarity to the vRNA template are in **bold** and *italics*. The potential base-pairing between the 3'-terminal A residue of the capped RNA leader and the 3'-terminal U residue of the vRNA template is shown with dotted lines.

the chain realigns backwards on the vRNA template (**Figure 2-6**). Although prime-and-realign has never been observed during influenza transcription, it recently has been reported during replication (***Vreede et al., 2008***) where realignment was observed of a *de novo* synthesized pppApG dinucleotide, templated by nucleotides 4 and 5 from the 3' end of the complementary RNA template, to residues 1 and 2 to allow elongation to full-length vRNA. Prime-and-realign of capped RNA leader sequences during influenza transcription strengthens the occurrence of base-pairing between capped RNA leaders and the vRNA template.

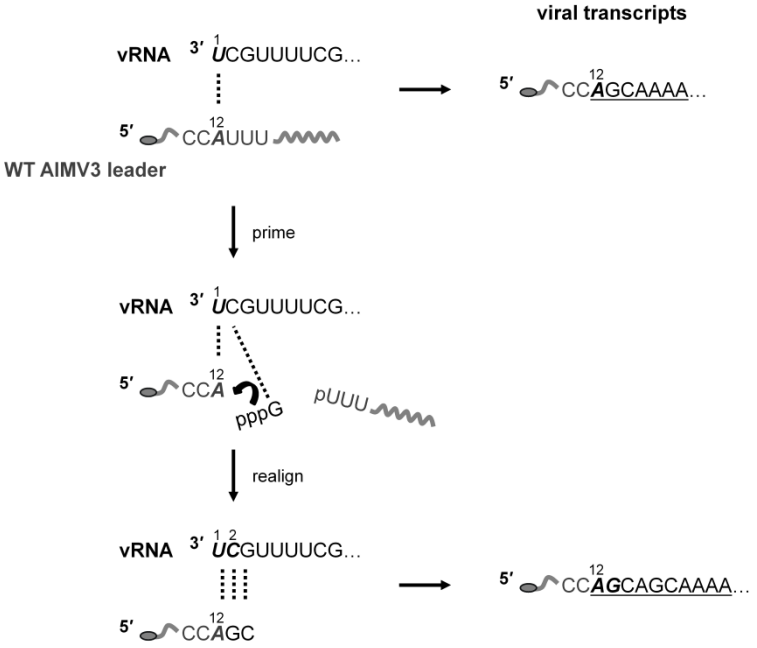

Figure 2-6 **Occurrence of prime-and-realign during initiation of influenza mRNA synthesis.** Influenza sequence is underlined, while residues that possibly could originate from the globin leader and with complementarity to the vRNA template are in **bold** and *italics*. The potential base-pairing between the 3'-terminal A residue of the capped RNA leader and the 3'-terminal U residue of the vRNA template is shown with dotted lines.

Earlier *in vivo* studies on the cap-snatching mechanism of the plant-infecting bunyavirus TSWV have demonstrated a requirement for base complementarity of AlMV-derived capped RNA leaders to the ultimate or penultimate 3' residue of the TSWV vRNA template (***Duijsings et al., 2001***). Similar results have been obtained using an *in vitro* transcription system based on purified TSWV particles supplemented with rabbit RRL (***van***

Knippenberg et al., 2002), suggesting that the *in vitro* system faithfully reflected transcription initiation *in vivo*. Using the same *in vitro* assay, several AlMV-derived (mutant) cap donors were offered in pair-wise competitions, and showed that TSWV prefers capped leaders with a specific leader length and increasing base complementarity to the 3'-terminal residues of the vRNA template, even when offered at relative low amounts compared to others with less base complementarity (***van Knippenberg et al., 2005a***).

The preference of *Influenza A virus* for α-globin leaders over the β-globin (**Table 2-1**) could also be explained due to increased base complementarity of the capped RNA leader to the vRNA template, as earlier observed for TSWV. To investigate whether *Influenza A virus* prefers cap donors with increased base complementarity to the vRNA template, the use of synthetic mutant AlMV3 RNA transcripts can be exploited. To this end, mutant AlMV3 transcripts differing in their complementarity to the 3' end of the vRNA template will be made and pair-wise tested in the *in vitro* influenza virus transcription assay and their use will be quantified.

Materials and methods

Virus

Influenza virus strain A/Puerto Rico/8/34 (H1/N1) was propagated and purified from embryonated chicken eggs. In brief, allantoic fluid was collected and cleared by low speed centrifugation for 10 min at 1000xg and filtrated through a 0.45 µm-pore-size filter. The filtrate was layered onto 0.5 ml 60% (wt/vol) sucrose (in PBS) - 2.0 ml 20% (wt/vol) sucrose (in PBS) step gradients and centrifuged at 150,000xg for 2 h at 4 °C, using a SW41 rotor (Beckmann). Virus particles were recovered from the interface, adjusted to 20% (wt/vol) sucrose (in PBS), and loaded onto the top of a 12 ml 20% to 60% (wt/vol) continuous sucrose gradient (in PBS). After centrifugation at 150,000xg for 16 h at 4 °C in a SW41 rotor (Beckmann), the virus fraction was collected. Purified virus (10-15 µg/µl) was stocked in small aliquots at -80 ^{o}C prior to use in transcription assays.

Construction of WT AlMV3

WT was generated from plasmid pXO32*Nco*P3, which contains a full-length cDNA clone of AlMV3 (***Neeleman et al., 1993***). WT was PCR amplified by using primer AlMV3-Rv (complementary to nucleotides 339-313 of the AlMV3 sequence) and primer WT. Primer sequences are listed in **Table 2-4**.

Amplified PCR fragments were separated by electrophoresis in a 1% agarose gel and purified using the GFX PCR purification kit (Roche). Purified products were restriction enzyme digested with *Bam*HI/*Eco*RI and ligated into pUC19 using T4 DNA ligase (Promega). The nucleotide sequence of individual clones was verified by the dideoxynucleotide chain termination method (***Sanger et al., 1977***) using standard M13 sequencing primers and ultra-high throughput (ABI Prism 3700) DNA sequencer (Greenomics^{TM}, Wageningen University and Research Centre, The Netherlands).

Synthesis of cap-0 RNA leaders

In vitro synthesis of capped RNA leaders was performed by using the Ambion T7 mMESSAGE mMACHINE kit according to the manufacturer's instruction. Transcription was performed in the presence of cap-analog $m^7G^{5'}ppp^{5'}G$. The cap analog:GTP ratio of this solution is 4:1 and results in an 80% yield of capped transcripts.

Prior to transcription each DNA template was linearized with *Eco*RI. T7 promoter sequences were introduced upstream of the AlMV3 leader by PCR amplification in order to allow *in vitro* synthesis of capped leaders.

TABLE 2-4

Primer sequences for WT AlMV3 construct and RT-PCR.

Name	Primer sequence (5'→3')
WT	CCCGGATCC**TAATACGACTCACTATA**GTATTAATACCATTTT CAAAATATTCC
AlMV3-Rv	CCCGAATTCGAAGAGTACGAATTACGCG
TS-oligo	CCCGGATCCGGGGG
α-globin	CCCGGATCCGGGGGACACTTCTGGT
α-/β-globin	CCCGGATCCGGGGGACACTT
AlMV3-leader1	CCCGGATCCGGGGGG**TATTAATA**
NS1UP1	CCCGAATTCGAGTCTCCAGCCGGTC
NS1UP2	CCCGAATTCCGCCTGGTCCATTCTG
NPUP1	CCCGAATTCGAGTCAGACCAGCCGTTGC
NPUP2	CCCGAATTCGCTTGGCGCCAGATTCGCC

Residues which could potentially base-pair to the viral template are underlined; **bold** residues are T7 promoter sequence.

In vitro influenza transcription and AlMV3 leader competition assays

In vitro influenza transcription assays were performed using approximately 10 μg of purified influenza virus particles in a final volume of 25 μl (**Figure 2A**). Assays were done according to van Knippenberg *et al.* (***2002***). These assays contained 4 mM Mg acetate, 1 mM of each NTP, 0.1% NP-40, 0.8 U/μl RNasin, 2.5 μl translation mix (amino acid mixture of 1:1:1 -cys; -lys; -met), and were supplemented by the AP-Biotech RRL system (GE Healthcare) according to the manufacturer's procedures. For visualization of the RNA products, 1 mM of [α-^{32}P]CTP (800 Ci/mmol) was added instead of CTP. The amount of AlMV3 leaders added to the reactions is indicated in the text and tables. After incubation for 2 h at 37 ^{o}C, the reaction mixture was extracted with phenol-chloroform and the RNA ethanol precipitated. Radiolabeled RNA products were resolved by electrophoresis in a 1% denaturing agarose gel and subsequently transferred onto Hybond-N membrane using the TurboBlotter System (Schleicher and Schuell). Radiolabeled RNA synthesized was visualized by exposure of the membrane to autoradiographic film.

Analyses of AlMV3-influenza mRNA sequences

RT-PCR amplification of *de novo* synthesized *Influenza* NP or NS1 mRNAs containing capped 5' sequences derived from AlMV3 was achieved by a strategy during which a known sequence was efficiently incorporated at the end of the cDNA during first strand synthesis, without adaptor ligation (**Figure 2-3A**) (***Schmidt and Mueller, 1999;***

Schramm et al., 2000). First-stand cDNA was synthesized by using the template-switch primer TS-oligo in combination with primer NPUP1 or NS1UP1. PCR amplification was performed using primer α-globin, α-/β-globin or AlMV3-leader1, and a nested primer NPUP2 or NS1UP2. Primer sequences are listed in **Table 2-4**.

PCR products of expected size were purified from a 1% agarose gel using the GFX PCR purification kit (Roche) and cloned into pGEM-T Easy (Promega) according to the manufacturer's procedures, prior to sequence analysis by the dideoxynucleotide chain termination method (***Sanger et al., 1977***) using standard T7 sequencing primer and ABI Prism 3700 sequencer (Greenomics^{TM}).

Acknowledgements

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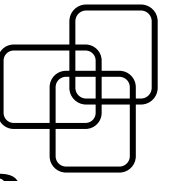
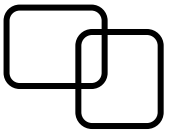
“The worst deaf is the one who doesn’t want to hear.”

Greek proverb



Chapter 3



Base-pairing promotes leader selection to prime *in vitro* influenza genome transcription

The requirements for alignment of capped leader sequences along the viral genome during influenza transcription initiation (cap-snatching) have long been an enigma. In this study, competition experiments using an *in vitro* transcription assay revealed that influenza virus transcriptase prefers leader sequences with base complementarity to the 3'-ultimate residues of the viral template, 10 or 11 nt from the 5' cap. Internal priming at the 3'-penultimate residue, as well as prime-and-realign was observed. The nucleotide identity immediately 5' of the base-pairing residues also affected cap donor usage. Application to the *in vitro* system of RNA molecules with increased base complementarity to the viral RNA template showed stronger reduction of globin RNA leader initiated influenza transcription compared to those with a single base-pairing possibility. Altogether the results indicated an optimal cap donor consensus sequence of ^{7m}G-(N)$_{7-8}$-(A/U/G)-(A/U)-*AGC*-3'.

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Introduction

"Cap-snatching" is the transcription initiation mechanism found among segmented, negative (-) ssRNA viruses as first described for influenza A (***Beaton and Krug, 1981; Bouloy et al., 1978; Caton and Robertson, 1980; Dhar et al., 1980; Plotch et al., 1979, id. 1981***). During this process, the viral transcriptase cleaves m^7G-capped RNA leader sequences from host mRNAs to prime transcription of the viral genome segments (**Figure 3-1**). The development of *in vitro* systems (***Bouloy et al., 1978; Hagen et al., 1994; Plotch et al., 1979***) and reverse genetics approaches (***Fodor et al., 1999; Neumann et al., 1999; Pleschka et al., 1996***) has advanced the insight into *cis*- and *trans*-acting factors involved in cap-snatching.

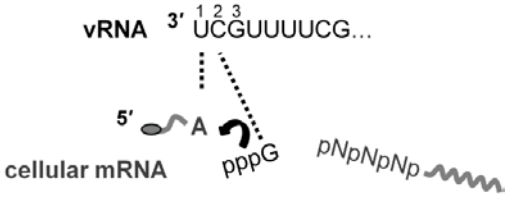

Figure 3-1 Schematic presentation of influenza virus genome transcription initiation. Cleavage of a cellular mRNA and subsequent use of the capped RNA leader in priming genome transcription is shown.

The influenza heterotrimeric polymerase complex consists of subunits, PB1, PB2 and PA. Whereas PB2 recognizes and binds to the 5' cap structure of cellular mRNAs (***Braam et al., 1983; Li et al., 2001***), PB1 has been thought to contain the active site of the polymerase and to cleave bound capped RNAs at a site 10-13 nt downstream of the cap (***Li et al., 2001***). The role of PA in viral transcription and replication has long been unknown but recently the crystal structure of the N-terminal domain of PA (PA_N: residues 1-159) has been determined (***Yuan et al., 2009***).This structure and the presence of a bound magnesium ion by a motif similar to the (P)DX_N(D/E)XK motif characteristic of many endonucleases strongly suggests that PAn holds the endonuclease activity rather than PB1 (***Yuan et al., 2009***). Biochemical and structural studies have shown that the amino-terminal 209 residues of the PA contain the endonuclease activity (***Dias et al., 2009***). Although several reports suggested that specific base-pairing between multiple residues of the 5'- and 3'-terminal end of the viral RNA (vRNA) template is required for activation of the endonuclease (***Cianci et al., 1995; Hagen et al., 1994***), others have suggested that the presence of the 5'-terminal end of the vRNA template is sufficient for cap-dependent endonuclease activity (***Li et al., 2001***).

For *Influenza A virus*, a preference for purine at the 3' end of the donor leader has been proposed (***Beaton and Krug, 1981; Plotch et al., 1981***), while only leaders harboring a 3'-terminal CA dinucleotide would effectively be used as primers (***Beaton and Krug, 1981; Rao et al., 2003; Shaw and Lamb, 1984***). For Bunyamwera and *Dugbe virus* (family *Bunyaviridae*), a strong preference for cleavage after a U or C residue, respectively, has been postulated (***Jin and Elliott, 1993a and b***). In all these cases, however, conclusions were drawn from a limited number of viral mRNAs produced *in vivo* and on basis of the leader sequences only, while the origin of the selected leader and further downstream sequences remained unknown. Hence, it cannot be ruled out that these leaders had been cleaved 1-2 nt downstream of the assumed 3' end and prior to which base-pairing of residues downstream of the snatched leader sequence assists in the alignment of leader on the vRNA template to render a stabilized transcription initiation complex. Indeed some *in vitro* studies on *Influenza A virus* support the idea of base-pairing during leader alignment on the viral template (***Chung et al., 1994; Hagen et al., 1994, id. 1995; Plotch et al., 1981; Chapter 2***). Work by Chung *et al.* (***1994***) already showed the possibility of RNA leaders to directly become elongated on the vRNA template, without the need for additional cleavage. While the involvement of base complementarity of these leaders to the vRNA template was not investigated, these leaders did contain one up to three residues at their 3' terminus with complementarity to the vRNA template to immediately allow elongation without further cleavage. In another study, Hagen *et al.* (***1995***) concluded that the endonuclease cleavage site within an mRNA is completely controlled by the mRNA and does not depend upon interactions with the vRNA template, though the site of initiation is influenced by template-primer interactions. However, a more detailed look at the altered cleavage sites within mutated mRNA leader sequences shows that the altered cleavage sites vary with the possibility to allow initiation/elongation after base-pairing of the leader 3' residues with the vRNA template. In addition to their earlier observed requirement of a vRNA template (harboring the 5' and 3' termini) to activate endonuclease cleavage and transcription (***Hagen et al., 1994***), these data support the idea that base-pairing between nucleotide residues of the snatched leader sequence, and residues further downstream, to the vRNA 3' terminus may play an important role during the alignment and subsequent endonuclease cleavage. This is furthermore supported by the observation of α-globin mRNA leader sequences, containing a higher base complementarity around the endonuclease cleavage site to the viral RNA template, being preferentially used during *Influenza A virus* transcription initiation in comparison to β-globin leader sequences, which only contained a single base-pairing residue around the endonuclease cleavage site (**Chapter 2**).

On the other hand, some studies report cleavage without the presence of the 3' terminal sequence of the vRNA template and the absence of any base complementarity of RNA leaders to the 3' end of the vRNA template (***Krug et al., 1980; Rao et al., 2003***). However, in one of the latest cap-snatching models presented (***Rao et al., 2003***), the

possibility of base-pairing between the 3' terminal A residue with the terminal U of the 3' end of the vRNA template was not excluded.

A “prime-and-realign” mechanism has been proposed for *Hantaan virus* to account for the vRNA polymerase to initiate (anti)genome/mRNA synthesis with GTP (***Garcin et al., 1995***), but a prime-and-realign could also explain the presence of repetitive sequences observed within the leader sequence of viral transcripts from other viruses that apply cap-snatching (***Bishop et al., 1983; Bouloy et al., 1990; Duijsings et al., 2001; Garcin et al., 1995; Jin and Elliott, 1993b; Simons and Pettersson, 1991; van Knippenberg et al., 2005a***), and indicate the involvement of base-pairing between leader and template.

Here we set out to analyze *Influenza A virus* cap donor requirements, and more specifically the possible role of base-pairing between donor and template, during transcription initiation. To this end, various *Alfalfa mosaic virus* RNA3 (AlMV3)-derived mutants were tested as cap donors in pair-wise or multiple competitions using the *in vitro* influenza A transcription system as described in **Chapter 2**. The results show that base complementarity to the viral genome template promotes the use of capped leaders and that residues 5' of the base-pairing residues are of importance.

Results

Base complementarity enhances cap donor preference

Since exogenous AlMV3 RNA was shown to be accepted as cap donor in our *in vitro* influenza virus transcription assay (**Chapter 2**), the assay was next exploited to analyze whether base complementarity would enhance cap donor selection. To this end, four variant AlMV3 constructs (**Table 3-1**) differing in their complementarity to the 3' end of the vRNA template, were made and tested in pair-wise competition. Since AlMV3 capped RNA leaders were shown to become cleaved at residue A12 (**Chapter 2**), this residue and nucleotides further downstream, were targeted for mutagenesis to investigate their involvement in the alignment on the vRNA template. The first AlMV3 construct, i.e. wild type (WT), showed a single base complementarity to the vRNA template (nucleotide A_{12}), the second one (denoted Mut-2) was made to contain 2 base-pairing residues ($A_{12}G_{13}$), the third (Mut-3) 3 residues ($A_{12}G_{13}C_{14}$) while the fourth construct (Mut-N_{14}) reflected a genuine influenza A transcript, i.e. a NP mRNA provided with the first 11 nt of the AlMV3 leader (**Chapter 2**), and harboring 14 nt complementary to the NS1 vRNA template. To discriminate between the WT and the three mutant sequences, a marker nucleotide was introduced by changing residue C_{10} into A, G or T respectively (**Table 3-1**).

TABLE 3-1

Wild type (WT) and mutant AlMV3 leaders used in assays.

Capped leader	5' end sequence of capped leader
	12
WT	5'-GUAUUAAUACCAUUUUCAAAAUAUUCC
Mut-2	5'-GUAUUAAUAACAGUUUCAAAAUAUUCC
Mut-3	5'-GUAUUAAUAGCAGCUUCAAAAUAUUCC
Mut-N14	5'-GUAUUAAUAUCAGCAAAAGCAGGGUAG

Residues potentially base-pairing to the viral template (3'-UCG…) are underlined; the marker nucleotide used to discriminate between the different AlMV3 leaders is shaded.

Synthetic, capped RNA transcripts of the constructs were provided to the *in vitro* transcription assay and *de novo* synthesized viral transcripts were RT-PCR cloned using an internal NS1 primer combined with a TS-oligo primer containing only the first 4 AlMV3 nucleotides as described in **Chapter 2**. Clones obtained were sequence analyzed for the presence of the marker nucleotide within the AlMV3 RNA leader sequence.

When WT and Mut-2 were added to the system, all cloned AlMV3-NS1 transcript sequences contained marker nucleotide A (**Table 3-2**), indicative for the presence of the Mut-2 RNA leader sequence. Even when Mut-2 was offered in a ratio 10 times less than WT (**Table 3-2**), all NS1 transcript clones, except of one which had a leader of unknown origin, contained the Mut-2 leader sequence (**Table 3-2**), indicating a strong preference for AlMV3 Mut-2. Next, Mut-N_{14} was tested along with WT. When offered in 1:1 ratio (50 or 500 ng each), 35 out of 39 cloned NS1 transcript sequences contained the marker nucleotide of Mut-N_{14} (**Table 3-2**). When WT and Mut-N_{14} were offered in a 10:1 ratio, still 21 out of 25 contained the Mut-N_{14} leader (**Table 3-2**). The origin of the leader sequence from 4 additional clones could not be identified due to the absence of the marker nucleotide (**Table 3-2**).

TABLE 3-2

Distribution and nucleotide sequence of WT and mutant AlMV3 leaders snatched during pair-wise competition to prime virus NS1 mRNA transcription *in vitro*.

Capped leaders offered	Retrieved mRNA 5' sequence	# clones	Origin leader
WT + Mut-2	5'-GUAUUAAUAAC***AG***CAAAA	31	Mut-2
(1:1; 500 ng each)	5'-GUAUUAAUAAC***AG***CAGCAAAA	1	Mut-2
	5'-GUAUUAAUA***A***GCAAAA	2	Mut-2
WT + Mut-2	5'-GUAUUAAUAAC***AG***CAAAA	9	Mut-2
(10:1; 500 vs. 50 ng)	5'-GUAUUAAUAACGCAAAA	1	Mut-2
	5'-GUAUUAAUA***A***GCAAAA	1	Mut-2
	5'-GUAUUAAU***A***GCAAAA	1	?
WT + Mut-N_{14}	5'-GUAUUAAUAUC***AGC***AAAA	15	Mut-N_{14}
(1:1; 50 ng each)	5'-GUAUUAAUAUU***AGC***AAAA	5	Mut-N_{14}
	5'-GUAUUAAUAUC***AGC***AGCAAAA	3	Mut-N_{14}
	5'-GUAUUAAUACC***A***GCAAAA	3	WT
WT + Mut-N_{14}	5'-GUAUUAAUAUC***AGC***AAAA	11	Mut-N_{14}
(1:1; 500 ng each)	5'-GUAUUAAUAUU***AGC***AAAA	1	Mut-N_{14}
	5'-GUAUUAAUACC***A***GCAAAA	1	WT
WT + Mut-N_{14}	5'-GUAUUAAUAUC***AGC***AAAA	17	Mut-N_{14}
(10:1; 500 vs. 50 ng)	5'-GUAUUAAUAUC***AGC***AGCAAAA	3	Mut-N_{14}
	5'-GUAUUAAUAUGCAGCAAAA	1	Mut-N_{14}
	5'-GUAUUAAUACC***A***GCAAAA	2	WT
	5'-GUAUUAAUACGCAGCAAAA	2	WT
	5'-GUAUUAAU***A***GCAGCAAAA	4	?

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

The data obtained indicate that *in vitro* influenza prefers leader sequences with increasing complementarity to the 3' end of the vRNA template. This was further strengthened by the performance of an additional competition assay in which Mut-2 and Mut-3, only differing in the complementarity to the vRNA template by one residue, was tested in a pair-wise manner (**Table 3-3**). When provided in equimolar amounts, the Mut-3 leader was preferred, being used twice as often as the Mut-2 leader (**Table 3-3**). Surprisingly, when Mut-3 and Mut-N_{14} were offered to the *in vitro* transcription assay in various ratios, no clear preference for the Mut-N_{14} leader was observed (**Table 3-3**).

Further analysis of the leader sequence within all NS1 clones showed the presence of repetitive sequences in 9 clones, likely as a result of prime-and-realign of capped leader sequences as previously observed with WT AlMV3 (**Chapter 2**), indicating again the occurrence of base-pairing during the transcription process.

TABLE 3-3

Distribution and nucleotide sequence of mutant AlMV3 leaders snatched during pair-wise competition to prime virus NS1 mRNA transcription *in vitro*.

Capped leaders offered	Retrieved mRNA 5' sequence	# clones	Origin leader
Mut-2 + Mut-3 (1:1; 500 ng each)	5'-GUAUUAAUAGC***AGC***AAAA	19	Mut-3
	5'-GUAUUAAUAGC***AGC***AGCAAAA	1	Mut-3
	5'-GUAUUAAUAAC***AG***CAAAA	8	Mut-2
	5'-GUAUUAAUA***A***GCAAAA	1	Mut-2
Mut-3 + Mut-N_{14} (1:1; 500 ng each)	5'-GUAUUAAUAUC***AGC***AAAA	12	Mut-N_{14}
	5'-GUAUUAAUAGC***AGC***AAAA	9	Mut-3
Mut-3 + Mut-N_{14} (1:1; 500 vs. 50 ng)	5'-GUAUUAAUAUC***AGC***AAAA	2	Mut-N_{14}
	5'-GUAUUAAUAGC***AGC***AAAA	7	Mut-3

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

Initiation of *Influenza A virus* mRNA synthesis, irrespective of a cap-0 or cap-1 structure

Previously, the influence of different cap structures on influenza transcription initiation *in vitro* was demonstrated suggesting a preference for cap-1 structures (***Bouloy et al., 1980***). However, these studies also showed that AlMV RNA leaders were relatively effective primers for the polymerase reaction as well. The high priming activity of AlMV

RNA appeared not to be due to the presence of cap-1 termini as, in agreement with other reports (***Pinck, 1975***), only mono-methylated cap-0 termini were detected.

To rule out the possibility that the presence of cap-0 structures at the 5' end of AlMV3 RNA affected the use of capped RNA leaders during cap donor competition assays, two pair-wise competition assays were performed with Mut-2 and Mut-3. In the first assay both leader mutants were provided with cap-0 structures and in the second assay with cap-1 structures. After pair-wise competition the results showed that, irrespective of the presence of a cap-0 or cap-1 structure, Mut-3 was clearly preferred (**Table 3-4**) and being used twice as often as Mut-2 as earlier observed (**Table 3-3**). Altogether, these results justified the further use of AlMV3 molecules to analyze leader sequence requirements for influenza virus transcription initiation.

TABLE 3-4

Distribution and nucleotide sequence of leaders having a cap-0 or cap-1 structure snatched during pair-wise competition to prime virus NS1 mRNA transcription.

Capped leaders offered	Retrieved mRNA 5' sequence	# clones	Origin leader
Cap-0 structures	5'-GUAUUAAUAGC***AGC***AAAA	12	Mut-3
Mut-2 + Mut-3	5'-GUAUUAAUAGCGCAAAA	1	Mut-3
(1:1; 500 ng each)	5'-GUAUUAAUAACA***AG***CAAAA	7	Mut-2
Cap-1 structures	5'-GUAUUAAUAGC***AGC***AAAA	12	Mut-3
Mut-2 + Mut-3	5'-GUAUUAAUAACA***AG***CAAAA	6	Mut-2
(1:1; 500 ng each)			

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

Influence of the 3'-terminal nucleotide of the leader

To analyze whether the nucleotide within the sequence, upstream of the base-pairing residues affects leader preference, another set of AlMV3 leader constructs was made that harbored a C (WT), A, G or U at position 11 and a single base-pairing residue A_{12} (**Table 3-5**). Capped transcripts of all four AlMV3 constructs were offered in equimolar amounts to the *in vitro* assay. Surprisingly, 18 out of the 32 clones analyzed contained a G at position 11 (**Table 3-6**), indicating a preference for the $G_{11}A_{12}$ sequence. However, 17 out of these 18 clones, lacked the first viral A residue, likely due to cleavage after G_{11} and internal priming on the 3'-penultimate C residue of the viral template.

TABLE 3-5

Mutant AlMV3 leaders used in multiple competition assay.

Capped leader	5' end sequence of capped leader
	12
$C_{11}A_{12}$ (WT)	5'-GUAUUAAUACCAUUUUCAAAAUAUUCC
$G_{11}A_{12}$	5'-GUAUUAAUACGAUUUUCAAAAUAUUCC
$A_{11}A_{12}$	5'-GUAUUAAUACAAUUUUCAAAAUAUUCC
$U_{11}A_{12}$	5'-GUAUUAAUACUAUUUUCAAAAUAUUCC

Residues potentially base-pairing to the viral template (3'-UCG…) are underlined; the marker nucleotide used to discriminate between the different AlMV3 leaders is shaded.

TABLE 3-6

Usage of leaders with a mutation at position -1 relative to the single base-pairing A_{12} residue snatched to prime NS1 mRNA transcription *in vitro*.

Capped leaders offered	Retrieved mRNA 5' sequence	# clones	Origin leader
$(C/G/A/U)_{11}A_{12}$ (1:1:1:1; 500 ng each)	5'-GUAUUAAUACG***A***GCAAAA	1	$G_{11}A_{12}$
	5'-GUAUUAAUACG***G***CAAAA	9	$G_{11}A_{12}$
	5'-GUAUUAAUACG***G***CAGCAAAA	8	$G_{11}A_{12}$
	5'-GUAUUAAUACA***A***GCAAAA	1	$A_{11}A_{12}$
	5'-GUAUUAAUACA***A***GCAAAA	7	$A_{11}A_{12}$
	5'-GUAUUAAUACU***A***GCAAAA	4	$U_{11}A_{12}$
	5'-GUAUUAAUACC***A***GCAAAA	2	$C_{11}A_{12}$

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

Furthermore, 8 of these leaders showed a repetitive sequence that probably resulted from prime-and-realign. From the remaining 14 (out of 32) clones, 8 clones contained A_{11}, 4 U_{11} and 2 C_{11}. A closer look at the snatched leaders from mutant $A_{11}A_{12}$ showed that 7 out of the 8 clones resulted from cleavage downstream A_{11} rather than A_{12}, giving rise to a CA 3' terminus. Only in 2 (out of 32) clones the leader originated from mutant $C_{11}A_{12}$, after cleavage downstream of A_{12}. Hence, although all 4 single base-pairing AlMV3 leaders were used as cap donor, there was a clear preference for leaders harboring a 3' CGA, primarily leading to internal priming, while a 3' CCA sequence was least preferred. Although explanations as an alternative to the base-pairing hypothesis cannot be excluded, e.g. cleavage after C_{10} and subsequent initiation on the 3'-penultimate residue in case of the 9 clones assigned to $G_{11}A_{12}$, such strategy does not explain the presence of a (non-templated) U residue in 4 clones of $U_{12}A_{12}$.

To determine which nucleotides at positions 10 (2 nt upstream the base-pairing residues) and 11 would promote leader selection without provoking internal priming, a next set of AlMV3 mutant leaders was made, all harboring 3 base-pairing residues (AGC) at their 3' end and variable residues at positions 10 or 11 (**Tables 3-7 and 3-8**). The resulting constructs were referred to as $C_{10}C_{11}A_{12}GC$, $A_{10}C_{11}A_{12}GC$, $G_{10}C_{11}A_{12}GC$ and $U_{10}C_{11}A_{12}GC$, and $C_{11}A_{12}GC$, $A_{11}A_{12}GC$, $G_{11}A_{12}GC$ and $U_{11}A_{12}GC$ respectively.

TABLE 3-7

Mutant AlMV3 leaders used in multiple competition assay.

Capped leader	5' end sequence of capped leader
	12
$C_{10}C_{11}A_{12}GC$	5'-GUAUUAAUACCAGCUUCAAAAUAUUCC
$G_{10}C_{11}A_{12}GC$	5'-GUAUUAAUAGCAGCUUCAAAAUAUUCC
$A_{10}C_{11}A_{12}GC$	5'-GUAUUAAUAACAGCUUCAAAAUAUUCC
$U_{10}C_{11}A_{12}GC$	5'-GUAUUAAUAUCAGCUUCAAAAUAUUCC

Residues potentially base-pairing to the viral template (3'-UCG…) are underlined; the marker nucleotide used to discriminate between the different AlMV3 leaders is shaded.

TABLE 3-8

Mutant AlMV3 leaders used in multiple competition assay.

Capped leader	5' end sequence of capped leader
	12
$C_{11}A_{12}GC$	5'-GUAUUAAUACCAGCUUCAAAAUAUUCC
$G_{11}A_{12}GC$	5'-GUAUUAAUACGAGCUUCAAAAUAUUCC
$A_{11}A_{12}GC$	5'-GUAUUAAUACAAGCUUCAAAAUAUUCC
$U_{11}A_{12}GC$	5'-GUAUUAAUACUAGCUUCAAAAUAUUCC

Residues potentially base-pairing to the viral template (3'-UCG…) are underlined; the marker nucleotide used to discriminate between the different AlMV3 leaders is shaded.

When all 4 leaders differing in position 11 were offered in an equimolar mix, 19 out of 40 NS1 mRNA clones contained A_{11} and 15 U_{11} (**Table 3-9**). Of the remaining clones, 5 contained G_{11} and only 1 C_{11}. Furthermore, in case the leader originated from the G_{11}-containing cap donor, internal priming was only observed in 1 out of 5 clones. When G_{11} was offered individually to the system, 5 out of 22 retrieved NS1 clones showed the absence of the first viral A residue, indicative for internal priming (**Table 3-9**). The lower frequency of internal priming with $G_{11}A_{12}GC$ in comparison to $G_{11}A_{12}$ was likely due to the extra base-pairing residues present in $G_{11}A_{12}GC$ that apparently forced proper alignment along the 3'-terminal residues of the vRNA template.

When all cap donor molecules harboring an altered nucleotide at position 10 were simultaneously offered, no clear preference was found, but C appeared to be inefficient (**Table 3-10**).

TABLE 3-9

Usage of leaders with a mutation at position -1 relative to the triple base-pairing $A_{12}GC$ residues snatched to prime NS1 mRNA transcription *in vitro*.

Capped leader(s) offered	Retrieved mRNA 5' sequence	# clones	Origin leader
$(C/G/A/U)_{11}A_{12}GC$	5'-GUAUUAAUACG***AGC***AAAA	4	$G_{11}A_{12}GC$
(1:1:1:1; 500 ng each)	5'-GUAUUAAUACG***G***CAAAA	1	$G_{11}A_{12}GC$
	5'-GUAUUAAUACA***AGC***AAAA	18	$A_{11}A_{12}GC$
	5'-GUAUUAAUACA***A***GCAAAA	1	$A_{11}A_{12}GC$
	5'-GUAUUAAUACU***AGC***AAAA	15	$U_{11}A_{12}GC$
	5'-GUAUUAAUACC***AGC***AAAA	1	$C_{11}A_{12}GC$
$G_{11}A_{12}GC$	5'-GUAUUAAUACG***AGC***AAAA	16	
(500 ng)	5'-GUAUUAAUACG***AGC***AGCAAAA	1	
	5'-GUAUUAAUACG***G***CAAAA	1	
	5'-GUAUUAAUACG***G***CAGCAAAA	4	

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

TABLE 3-10

Usage of leaders with a mutation at position -2 relative to the triple base-pairing $A_{12}GC$ residues snatched to prime NS1 mRNA transcription *in vitro*.

Capped leaders offered	Retrieved mRNA 5' sequence	# clones	Origin leader
$(C/G/A/U)_{10}C_{11}A_{12}GC$	5'-GUAUUAAUAGC***AGC***AAAA	10	$G_{10}C_{11}A_{12}GC$
(1:1:1:1; 500 ng each)	5'-GUAUUAAUAGC***AGC***AGCAAAA	1	$G_{10}C_{11}A_{12}GC$
	5'-GUAUUAAUAAC***AGC***AAAA	7	$A_{10}C_{11}A_{12}GC$
	5'-GUAUUAAUAUC***AGC***AAAA	11	$U_{10}C_{11}A_{12}GC$
	5'-GUAUUAAUAUC***AGC***AGCAAAA	1	$U_{10}C_{11}A_{12}GC$
	5'-GUAUUAAUACC***AGC***AAAA	1	$C_{10}C_{11}A_{12}GC$

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

Pair-wise competition between a single and a triple base-pairing leader with reciprocal 5' upstream sequences

Based on the results above, single base-pairing AlMV3 leaders harboring a 3' AA were relatively favored but not those harboring a 3' CA. To analyze whether this difference was also found in pair-wise competitions among leaders with triple base-pairing possibilities, thereby underscoring the importance of the nucleotide identity at position 11, two additional competition experiments were performed. In the first one, leaders referred to as $A_{11}A_{12}$ and $C_{11}A_{12}GC$ (**Table 3-11**) were tested and in the second one their reciprocal variants, $C_{11}A_{12}$ (identical to WT) and $A_{11}A_{12}GC$ (**Table 3-11**).

In the first case, 14 out of 18 clones analyzed contained a leader from mutant $C_{11}A_{12}GC$ and 4 from $A_{11}A_{12}$ (**Table 3-12**). In the second case, 18 of the 19 clones contained the $A_{11}A_{12}GC$ sequence and only one $C_{11}A_{12}$ (**Table 3-12**). These results indicate that while nucleotide sequences within the leader are of some importance, extending base complementary to the viral template is the predominant selective criterion.

TABLE 3-11

WT and mutant AlMV3 leaders used in pair-wise competition assays.

Capped leader	5' end sequence of capped leader
	12
$C_{11}A_{12}$ (WT)	5'-GUAUUAAUACCAUUUUCAAAAUAUUCC
$A_{11}A_{12}$	5'-GUAUUAAUACAAUUUUCAAAAUAUUCC
$C_{11}A_{12}GC$	5'-GUAUUAAUACCAGCUUCAAAAUAUUCC
$A_{11}A_{12}GC$	5'-GUAUUAAUACAAGCUUCAAAAUAUUCC

Residues potentially base-pairing to the viral template (3'-UCG…) are underlined; the marker nucleotide used to discriminate between the different AlMV3 leaders is shaded.

TABLE 3-12

In vitro competition of a less preferred (CA) versus more preferred (AAGC) leader and their reciprocal leader variants during priming of NS1 mRNA transcription.

Capped leaders offered	Retrieved mRNA 5' sequence	# clones	Origin leader
$A_{11}A_{12}$ + $C_{11}A_{12}GC$ (1:1; 500 ng each)	5'-GUAUUAAUACC***AGC***AAAA	13	$C_{11}A_{12}GC$
	5'-GUAUUAAUACC***AGC***AGCAAAA	1	$C_{11}A_{12}GC$
	5'-GUAUUAAUACA***A***GCAAAA	2	$A_{11}A_{12}$
	5'-GUAUUAAUAC***A***GCAAAA	2	$A_{11}A_{12}$
$C_{11}A_{12}$ + $A_{11}A_{12}GC$ (1:1; 500 ng each)	5'-GUAUUAAUACA***AGC***AAAA	11	$A_{11}A_{12}GC$
	5'-GUAUUAAUAC***A***GCAAAA	7	$A_{11}A_{12}GC$
	5'-GUAUUAAUACC***A***GCAAAA	1	$C_{11}A_{12}$

Footnote belongs to Table 3-12 (page 55). Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

Leader length requirements for priming influenza transcription

Several publications indicate that the influenza transcriptase generally cleaves at a position 9-15 nt downstream of the cap (***Bouloy et al., 1978; Plotch et al., 1979; Robertson et al., 1980***). To define the optimal leader length of a cap donor, AlMV3 leaders with base-pairing residues at various positions relative to the 5' cap were made and tested. All constructs contained a CAGC sequence embedded within an oligo U stretch, to guarantee proper alignment of the leader along the vRNA template (**Table 3-13**). The initial choice for the CAGC context was made based on the earlier reported and apparent preference of influenza virus for RNA leaders with a 3'-terminal CA (***Rao et al., 2003***).

Leader transcripts were simultaneously offered in equimolar amounts. RT-PCR cloning and sequence analysis of *de novo* synthesized viral transcripts revealed that 9 out of 17 transcripts contained capped leaders originating from $C_9A_{10}GC$, and 5 from $C_{10}A_{11}GC$ (**Table 3-14**), suggesting an optimal leader length of 10-11 nt. Several clones revealed the presence of repetitive sequences, again indicative for prime-and-realign.

TABLE 3-13

Mutant AlMV3 leaders used in multiple competition assay.

Capped leader	5' end sequence of capped leader
	8
C_7A_8GC	5'-GUAUUUCAGCUUUUUUUUUUUUCAAAAUAU
C_8A_9GC	5'-GUAUUUUCAGCUUUUUUUUUUUCAAAAUAU
$C_9A_{10}GC$	5'-GUAUUUUUCAGCUUUUUUUUUUCAAAAUAU
$C_{10}A_{11}GC$	5'-GUAUUUUUUCAGCUUUUUUUUUCAAAAUAU
$C_{11}A_{12}GC$	5'-GUAUUUUUUUCAGCUUUUUUUUCAAAAUAU
$C_{12}A_{13}GC$	5'-GUAUUUUUUUUCAGCUUUUUUUCAAAAUAU
$C_{13}A_{14}GC$	5'-GUAUUUUUUUUUCAGCUUUUUUCAAAAUAU
$C_{14}A_{15}GC$	5'-GUAUUUUUUUUUUCAGCUUUUUCAAAAUAU
$C_{15}A_{16}GC$	5'-GUAUUUUUUUUUUUCAGCUUUUCAAAAUAU
$C_{16}A_{17}GC$	5'-GUAUUUUUUUUUUUUCAGCUUUCAAAAUAU
$C_{17}A_{18}GC$	5'-GUAUUUUUUUUUUUUUCAGCUUCAAAAUAU
	18

Residues potentially base-pairing to the viral template (3'-UCG…) are underlined; the marker nucleotide used to discriminate between the different AlMV3 leaders is shaded.

TABLE 3-14

Usage of RNA leaders varying in length from 7 to 17 nt relative to the position of base complementarity (AGC) during priming of NP mRNA transcription.

Capped leader offered	Retrieved mRNA 5' sequence	# clones
C_8A_9GC	5'-GUAUUUUC***AGC***AGCAAAA	1
$C_9A_{10}GC$	5'-GUAUUUUUC***AGC***AAAA	1
	5'-GUAUUUUUC***AGC***AGCAAAA	8
$C_{10}A_{11}GC$	5'-GUAUUUUUUC***AGC***AAAA	2
	5'-GUAUUUUUUC***AGC***AGCAAAA	3
$C_{11}A_{12}GC$	5'-GUAUUUUUUUC***AGC***AAAA	1
$C_{12}A_{13}GC$	5'-GUAUUUUUUUUC***AGC***AAAA	1

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

Reduced α-globin leader use in the presence of singly or triply base-pairing cap donors

To analyze the potential competitor effect of singly and triply base-pairing leaders on α-globin leader primed transcription, 3 leader constructs ($C_{11}A_{12}$, $C_{11}A_{12}GC$ and $A_{11}A_{12}GC$) (**Table 3-11**), were individually offered in various concentrations to the transcription assay. These leaders differed in their base complementarity to the vRNA template and/or one residue at position 11 just upstream the putative base-pairing residue(s) of the leader sequence. Since the RRL lysate used (AP-Biotech) had been treated with micrococcal nuclease by the manufacturer the exact amounts of residual globin mRNA molecules as source for capped RNA leaders was unknown. As a consequence, the reduction in globin leader initiated viral transcription by various mutant AlMV3 leader RNAs would only provide a relative indicator on the competitor effect of AlMV3 leaders. The relative reduction in α-globin leader NS1 transcription initiation was analyzed by RT-PCR and revealed that increasing amounts of $C_{11}A_{12}$ reduced globin leader initiated NS1 transcription to a lesser extent than $C_{11}A_{12}GC$ and $A_{11}A_{12}GC$ did (data not shown), in other words increasing complementarity of the AlMV3 leader sequence to the vRNA template increased its use and strength as competitor.

To further quantify this effect, the AlMV3-NS1/α-globin-NS1 ratio was determined by means of a two step quantitative real-time PCR (**Materials and methods**). A significant, almost 3-fold increase of the AlMV3-NS1/α-globin-NS1 ratio was observed when leader $A_{11}A_{12}GC$ was used compared to $C_{11}A_{12}$ (WT) (**Figure 3-2A**; pair-wise *t*-test

on log transformed data, $T_2 = -7.197$, $P = 0.019$). When all leaders were compared, samples containing $A_{11}A_{12}GC$ (with a triple base complementarity and optimal A residue at position 11) gave the highest AlMV3/α-globin-NS1 ratio, followed by $C_{11}A_{12}GC$ (with a triple base complementarity and a less optimal C residue at position 11) and then by $C_{11}A_{12}$ (with a single base complementarity and less optimal C residue at position 11) (**Figure 3-2B**). There was a significant effect of the leader used on the AlMV3/α-globin ratio (ANOVA on log transformed data, $F_{2,8} = 63.483$, $P < 0.001$), and all three pair-wise comparisons between the three leaders gave a significant outcome with a suitable *post hoc* test (Tukey HSD, $P < 0.010$ for all three comparisons). When only influenza infected material was used as negative control, no viral NS1 transcripts containing cellular capped leaders were PCR amplified with AlMV3 and NS1 primers (**Figure 3-2B**).

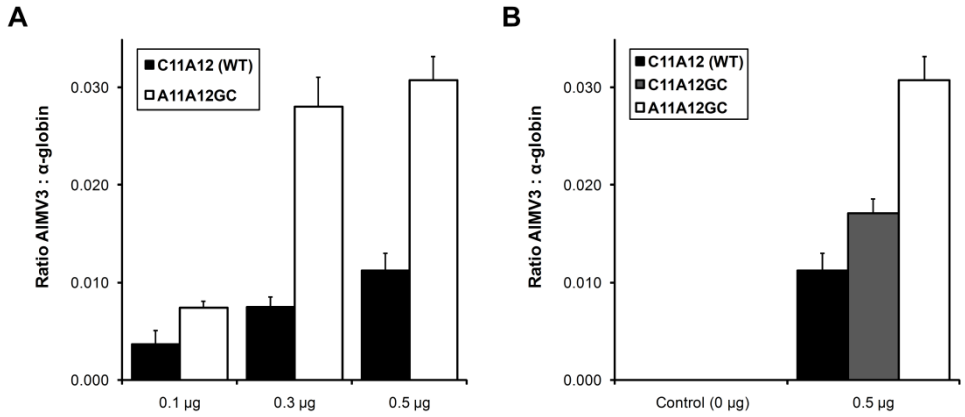

Figure 3-2 Quantification of AlMV3 and α-globin leader initiated NS1 transcription by two-step real-time PCR analysis. *In vitro* synthesized mRNA in the presence of RRL and addition of AlMV3 leader, was reverse transcribed using an internal primer for *Influenza* NS1 gene. Quantitative real-time PCR amplification was performed using a nested primer for the NS1 gene and a primer for the AlMV3 or α-globin mRNA. The geometric mean and standard error of the AlMV3/α-globin-NS1 ratio are given in both panels. (A) Active virus and increasing concentrations of $C_{11}A_{12}$ (WT) or $A_{11}A_{12}GC$ were used (concentrations are indicated in the X-axis). (B) Active virus and 0.5 µg of $C_{11}A_{12}$, $C_{11}A_{12}GC$ or $A_{11}A_{12}GC$ were used. In the case of control, no AlMV3 leader was offered.

Discussion

Although models for influenza are postulated as a paradigm for cap-snatching by all segmented, (-) ssRNA viruses (***Rao et al., 2003***), the matter of base-pairing between capped RNA leader and viral template has remained unresolved. Here, it is shown that *in vitro* influenza transcriptase selects donors with increasing base complementarity to the 3'-ultimate residues of the vRNA template (**Tables 3-1 to 3-4, 3-11, and 3-12**). Competition experiments in which 2, 4 or even up to 11 capped leaders were offered indicated that this preference is influenced, and even overruled when the base-pairing residues were positioned in a less optimal distance to the 5' cap, or when the residues immediately 5' of the base-pairing residues changed. Leaders in which the first base-pairing residue (A) was positioned 10 or 11 nt downstream the cap were most favored (**Tables 3-13 and 3-14**). When alignment of the leader at the 3'-ultimate U residue of the vRNA template occurred (e.g. forced by offering triple base-pairing leaders), there was a preference for an A or U residue 5' of the A_{12} base-pairing residue (**Tables 3-8 and 3-9**).

The presented data indicate that priming by virtue of base-pairing is a preferred determinant for the selection of RNA leaders during transcription initiation (**Tables 3-1 to 3-12**). During this process, base-pairing most likely occurs before cleavage takes place, otherwise the preference for extensive base complementarity cannot be explained. In light of these observations, *de novo* synthesized viral transcripts perfectly match the criteria for preferential use as cap donors. However, selective protection of viral transcripts by the viral polymerase has previously been demonstrated (***Shih and Krug, 1996***) and likely prevents a down regulation of viral transcription due to re-snatching events of viral transcript leaders. The occurrence of base-pairing prior to endonuclease cleavage is also supported by the observation that elongation of short oligoribonucleotides can take place after the first, second or third base-pairing residue of the leader, but not after a fourth. This implies that endonuclease cleavage can take place after the first base-pairing residues but not beyond the third (***Honda et al., 1986***). In case of the latter, an additional cleavage would be required to further trim the RNA leader sequence to render a primer that can be elongated (***Chung et al., 1994***). The absence of a clear viral preference for cap donors with either triple (Mut-3) or multiple (Mut-N_{14}) nucleotide complementarity to the vRNA template would then suggest that residues further downstream of the initial base-pairing ones do not play a major role anymore in the alignment along the viral template.

In only 5 out of 397 cloned viral transcripts the marker nucleotide, to discriminate between leader sequences, was lacking. The reason for the absence of the marker nucleotide is not clear, but might be due to sequencing error, or due to a transcription initiation event that did not involve alignment of a capped RNA leader along the vRNA template by virtue of base-pairing. In case of the latter, the very low number of viral transcripts without a

marker nucleotide would strongly support the importance of a base-pairing requirement of capped RNA leaders during cap-snatching.

A total of 44 out of 397 cloned viral transcripts contained repetitive sequences (GCAGC), indicating the phenomenon of prime-and-realign. This phenomenon is likely intrinsic to the mechanistic model of cap-snatching in which primer alignment involves base-pairing. The reason why some leaders appear to give rise to higher rates of prime-and-realign than others remains unknown. Kawaguchi and Nagata (***2007***) postulated that during replication of the influenza complementary RNA strand, the vRNA polymerase converts from an initiating form that holds the RNA weakly to an elongation form that holds the RNA tightly. In light of these results, we speculate that a similar phenomenon may occur during transcription initiation. During such process, an apparent realignment occurs that in reality represents a new initiation event involving an earlier aborted (weakly bound) primer-initiation complex and resulting in the presence of a repetitive sequence.

The need for base complementarity between cellular leaders and the viral template during influenza cap-snatching has been the source of a long debate due to apparently conflicting data from many earlier studies. Several *in vit*ro studies (***Chung et al., 1994; Hagen et al., 1994; Plotch et al., 1981***) showed that base-pair interactions can contribute for alignment of the leader to the influenza vRNA template, which is further supported by the ability of di- (ApG) and tri-nucleotides to prime transcription *in vitro* (***Honda et al., 1986***). On the other hand, capped ribopolymers lacking a sequence (AG or AGC) complementary to the 3' terminus of the vRNA (3'-UCGU) were still accepted as primers for transcription, which led to the suggestion that base-pair interactions are not required (***Hagen et al., 1995; Krug et al., 1980***). Furthermore, in contrast to an earlier study (***Rao et al., 2003***), the results presented here suggest that influenza does not effectively use CA-terminating sequences as primers for *in vitro* transcription.

The stronger *in vitro* reduction of α-globin RNA leader initiated transcription by triply base-pairing cap donors compared to singly base-pairing variants (**Figure 3-2**) demonstrates the competitiveness of such molecules during transcription initiation. Whether priming by virtue of base-pairing also is a preferred determinant *in vivo*, remains to be analyzed.

Materials and methods

Construction of plasmids

WT was constructed as described previously (**Chapter 2**). AlMV3 mutants were generated as well from plasmid pXO32*Nco*P3 (***Neeleman et al., 1993***). Mut-2 and Mut-3 were PCR amplified by using primer AlMV3-Rv (**Table 2-4**) and primers Mut2 or Mut3 respectively. Mut-N_{14}, an AlMV3-influenza NP mutant which could base-pair over a stretch of 14 nt to the 3' end of the viral NS1 template, was amplified with primers MutN_{14} and NPUP2.

AlMV3 constructs that contained a point mutation at position 11 of the AlMV3 sequence were made by first amplifying pXO32*Nco*P3 using primers AlMV3-Rv and $B_{11}A$ (B = G, A or T). A second amplification was performed with primer T7prom1-AlMV3 for extension of the AlMV3 leader with the T7 promoter sequence. Point mutants of AlMV3 at nucleotide 11 are referred to as $G_{11}A_{12}$, $A_{11}A_{12}$ and $U_{11}A_{12}$ (in which nucleotide at position 11 was changed from C residue into a G, A or T respectively).

Similarly, AlMV3 mutants that contained a mutation at either position 10 or 11 of the AlMV3 sequence, and additionally the 3 base-pairing residues (AGC) were made by first amplification of the same plasmid with primers AlMV3-Rv and D_{10}CAGC (D = C, G, A or T) or E_{11}AGC (E = C, G, A or T) respectively. Second amplification was performed with primer T7prom2-AlMV3 or T7prom1-AlMV3 for AlMV3 constructs with mutation at position 10 or 11 respectively. Point mutants of AlMV3 at 10 or 11 nt with the additional 3 base-pairing residues were referred to as $G_{10}C_{11}A_{12}$GC (in which nucleotide 10 was changed from C into an G, and nucleotides at positions 13 and 14 into a G and C respectively), $A_{10}C_{11}A_{12}$GC and $U_{10}C_{11}A_{12}$GC or $G_{11}A_{12}$GC, $A_{11}A_{12}$GC and $U_{11}A_{12}$GC respectively. $C_{10}C_{11}A_{12}$GC and $C_{11}A_{12}$GC were made by changing the nucleotide U at positions 13 and 14 into a G and C respectively.

Amplified PCR fragments were separated by electrophoresis in a 1% agarose gel and purified using the GFX PCR purification kit (Roche). Purified products were restriction enzyme digested with *Bam*HI/*Eco*RI and ligated into pUC19 using T4 DNA ligase (Promega). The nucleotide sequence of individual clones was verified by the dideoxynucleotide chain termination method (***Sanger et al., 1977***) using standard M13 sequencing primers and ultra-high throughput (ABI Prism 3700) DNA sequencer (Greenomics™).

Constructs for the leader length requirements experiment were made by first amplifying pXO32*Nco*P3 with primer AlMV3-Rv and primers C_7A_8, C_8A_9, C_9A_{10}, $C_{10}A_{11}$, $C_{11}A_{12}$, $C_{12}A_{13}$, $C_{13}A_{14}$, $C_{14}A_{15}$, $C_{15}A_{16}$, $C_{16}A_{17}$, or $C_{17}A_{18}$. Primer T7prom3-AlMV3 was used in the second PCR amplification. For these constructs, capped transcripts were made

directly from the purified PCR fragments without additional cloning. Primer sequences are listed in **Table 3-15**.

Synthesis of cap-0 and cap-1 RNA leaders

Cap-0 AlMV3 leaders were obtained by run-off transcription with Ambion T7 mMESSAGE mMACHINE kit as described in **Chapter 2**.

For the cap-0 versus cap-1 experiments, T7 RNA polymerase transcripts were made in the absence of cap-analog. These uncapped RNA leaders were subsequently provided cap-0 or cap-1 structures by using the ScriptCap™ m^7G Capping System (EPICENTRE Biotechnologies) in the absence or presence of the ScriptCap™ 2'-O-Methyltransferase according to the manufacturer's instruction.

In vitro influenza transcription and AlMV3 leader competition assays

In vitro influenza transcription assays were performed as described in **Chapter 2**.

Analyses of AlMV3-influenza mRNA sequences

RT-PCR and cloning analysis of RNA products was performed as described in **Chapter 2**. In the case of the leader length requirements experiment, primer AlMV3-leader2 (**Table 3-15**) was used instead of primer AlMV3-leader1 during the PCR amplification.

Two-step quantitative real-time PCR analysis

De novo synthesized NS1 transcripts were reverse transcribed into cDNA as described earlier. Afterwards, the first-strand cDNA was applied in a SYBR Green 1-based quantitative real-time PCR according to Zwart *et al.* (***2008***), with minor modifications: an annealing temperature of 50 °C was used and template concentrations were determined using the standard curve method (RotorGene 6.0 software, Corbett Research). Template cDNA was diluted 500-fold. A PCR reaction was performed with the AlMV3-leader1 and NS1UP2 primers to determine the AlMV3-NS1 cDNA concentration in the sample. A separate PCR reaction was performed with the α-globin and NS1UP2 primers to determine the α-globin-NS1 cDNA concentration. The AlMV3-NS1/α-globin-NS1 ratio was determined for each pair of replicates, and three replicates were performed in total. The geometric mean and standard error of mean of the AlMV3-NS1/α-globin-NS1 ratio were then calculated. All further statistical analysis was performed in SPSS 15.0 (SPSS Inc., Chicago, IL). Primer sequences are listed in **Table 2-4**.

TABLE 3-15

Primer sequences for mutant AlMV3 construct.

Name	Primer sequence (5'→3')
Mut2	CCCGGATCC**TAATACGACTCACTATA**GTATTAATAACAGTTTCAAAATATTCC
Mut3	CCCGGATCC**TAATACGACTCACTATA**GTATTAATAGCAGCTTCAAAATATTCC
$MutN_{14}$	CCCGGATCC**TAATACGACTCACTATA**GTATTAATATCAGCAAAAGCAG
$B_{11}A$	**CACTATA**GTATTAATACBATTTTCAAAATATTCC
$D_{10}CAGC$	**CACTATA**GTATTAATADCAGCTTCAAAATATTCC
$E_{11}AGC$	**CACTATA**GTATTAATACEAGCTTCAAAATATTCC
C_7A_8	**CGACTCACTATA**GTATTTCAGCTTTTTTTTTTTTCAAAATATTCCAAT
C_8A_9	**CGACTCACTATA**GTATTTTCAGCTTTTTTTTTTTCAAAATATTCCAAT
C_9A_{10}	**CGACTCACTATA**GTATTTTTCAGCTTTTTTTTTTCAAAATATTCCAAT
$C_{10}A_{11}$	**CGACTCACTATA**GTATTTTTTCAGCTTTTTTTTTCAAAATATTCCAAT
$C_{11}A_{12}$	**CGACTCACTATA**GTATTTTTTTCAGCTTTTTTTTCAAAATATTCCAAT
$C_{12}A_{13}$	**CGACTCACTATA**GTATTTTTTTTCAGCTTTTTTTCAAAATATTCCAAT
$C_{13}A_{14}$	**CGACTCACTATA**GTATTTTTTTTTCAGCTTTTTTCAAAATATTCCAAT
$C_{14}A_{15}$	**CGACTCACTATA**GTATTTTTTTTTTCAGCTTTTTCAAAATATTCCAAT
$C_{15}A_{16}$	**CGACTCACTATA**GTATTTTTTTTTTTCAGCTTTTCAAAATATTCCAAT
$C_{16}A_{17}$	**CGACTCACTATA**GTATTTTTTTTTTTTCAGCTTTCAAAATATTCCAAT
$C_{17}A_{18}$	**CGACTCACTATA**GTATTTTTTTTTTTTTCAGCTTCAAAATATTCCAAT
T7prom1-AlMV3	CCCGGATCC**TAATACGACTCACTATA**GTATTAATAC
T7prom2-AlMV3	CCCGGATCC**TAATACGACTCACTATA**GTATTT
T7prom3-AlMV3	CCCGGATCC**TAATACGACTCACTATA**GTATTT
AlMV3-leader2	CCCGGATCCGGGGGG**TATT**

Residues which could potentially base-pair to the viral template are underlined; **bold** residues are T7 promoter sequence.

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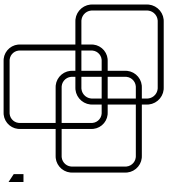
“Don’t escape when you have a problem because there always is a way to solve it.”

Buddha



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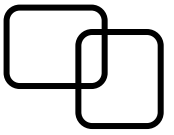

Preferential use of RNA leader sequences during influenza A transcription initiation *in vivo*

In vitro transcription initiation studies revealed a preference of *Influenza A virus* for capped RNA leader sequences with base complementarity to the viral RNA template. Here, these results were verified during an influenza infection in MDCK cells. *Alfalfa mosaic virus* RNA3 leader sequences mutated in their base complementarity to the viral template, or the nucleotides 5' of potential base-pairing residues, were tested for their use either singly or in competition. These analyses revealed that influenza transcriptase is able to use leaders from an exogenous mRNA source with a preference for leaders harboring base complementarity to the 3'-ultimate residues of the viral template, as previously observed during *in vitro* studies. Internal priming at the 3'-penultimate residue, as well as "prime-and-realign" was observed. The finding that multiple base-pairing promotes cap donor selection *in vivo*, and the earlier observed competitiveness of such molecules *in vitro*, offers new possibilities for antiviral drug design.

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Introduction

The *Influenza A virus* genome is composed of eight negative-sense RNA segments (***Palese and Schulman., 1976; Scholtissek et al., 1976***), which can encode up to 11 viral proteins (***Palese and Shaw, 2006***). Upon infection, viral ribonucleoproteins are transported to the nucleus where viral genome transcription and replication take place. Influenza transcription initiation involves m^7G-capped RNA leader sequences which are cleaved from host cellular mRNAs (***Bouloy et al., 1978; Krug et al., 1979; Plotch et al., 1979***). Binding and cleavage of such leaders are catalyzed by the viral polymerase complex, consisting of PB1, PB2 and PA, of which PA has recently been suggested to hold the endonuclease activity rather than PB1 (***Braam et al., 1983; Dias et al., 2009; Li et al., 2001; Shi et al., 1995; Yuan et al., 2009***). This transcription initiation mechanism, known as "cap-snatching", is shared among all other families of segmented, negative (-) ssRNA viruses (***Duijsings et al., 2001; Garcin et al., 1995; Huiet et al., 1993; Jin and Elliott, 1993a and b; Kormelink et al., 1992b***).

While the bunyaviruses and arenaviruses replicate in the cytoplasm and use the available pool of mature mRNAs as source for capped RNA leaders, influenza virus replicates in the nucleus and requires ongoing RNA polymerase II (Pol II) transcription, as demonstrated by the sensitivity to the RNA Pol II inhibitor α-amanitin (***Lamb and Choppin, 1977; Mark et al., 1979***). In the nucleus, the influenza virus RNA polymerase complex interacts with the large subunit of Pol II via its C-terminal domain (CTD), leading to a colocalization of the virus with RNA Pol II transcription sites. It is suggested that these sites may correspond to the earlier described "nuclear cages" or the nuclear matrix function where influenza virus RNA synthesis takes place (***Engelhardt et al., 2005***).

Being confined to RNA Pol II transcription sites, the virus has direct access to locally higher concentrations of cellular capped pre-mRNAs as source to initiate genome transcription. Endonuclease cleavage of these simultaneously leads to premature RNA Pol II transcription termination (***Chan et al., 2006; Engelhardt et al., 2005***). Coupling of viral transcription to RNA Pol II transcription additionally seems logic due to the fact that two of the viral mRNAs are processed by the cellular splicing machinery associated with RNA Pol II transcription.

With few exceptions, endonuclease cleavage by the influenza transcriptase complex generally takes place around 10-13 nt from the 5' cap structure (***Shi et al., 1995***), which appears to represent an optimal size of RNA leader sequence as shown by recent *in vitro* transcription assays in which 11 differently sized leaders were offered in a competition experiment and tested for their use (**Chapter 3**). For several segmented (-) ssRNA viruses, sequence analyses of viral mRNAs have shown a nucleotide preference at the 3' end of the non-viral leader, assumed to reflect a sequence preference for

endonuclease cleavage (***Jin and Elliott, 1993a and b***). For *Influenza A virus* a preference for purine residues has been proposed and suggested sufficient for influenza transcription initiation *in vitro* (***Beaton and Krug, 1981; Krug et al., 1980; Plotch et al., 1981; Rao et al., 2003***). Others have discussed the possibility of base-pair interactions during the alignment of capped leaders to the influenza viral RNA (vRNA) template but experimental data in support of this have been conflicting and inconclusive (***Chung et al., 1994; Hagen et al., 1994; Plotch et al., 1981***).

Recently, we have demonstrated that priming by virtue of base-pairing is a preferred determinant for the selection of leaders during influenza transcription initiation *in vitro* and those nucleotides 5' of the base-pairing residues are of importance. Furthermore, leaders harboring a 3'-terminal CA dinucleotide, and suggested to be the only effectively used primers (***Rao et al., 2003***), appeared to be relatively inefficient cap donors (**Chapter 3**). Whether base-pairing also promotes the selection of capped leaders *in vivo*, however, remains to be questioned.

Here, results are being described from influenza virus transcription initiation experiments in Madin-Darby canine kidney (MDCK) cells which show that, in support of our earlier *in vitro* studies, alignment of capped RNA leader sequences by virtue of base-pairing enhances their use during influenza A transcription initiation.

Results

Influenza A prefers leaders with extended base complementarity to the vRNA template during genome transcription *in vivo*

Previously, we have shown that base complementary to the vRNA template is a preferred determinant for the selection of capped RNA leaders during transcription initiation *in vitro* (**Chapter 3**).

To verify if influenza virus *in vivo* would exhibit a similar preference for leaders with increased base complementarity to the vRNA template, MDCK cells were infected with *Influenza A virus* and co-transfected with wild type (WT) or *Alfalfa mosaic virus* RNA3 (AlMV3)-derived mutants, that differed in complementarity to the 3' end of the vRNA template (**Table 4-1**), and tested for their use as cap donor in pair-wise competition as earlier described (**Chapter 3**).

TABLE 4-1

Wild type (WT) and mutant AlMV3 leaders used in pair-wise assays.

Capped leader	5' end sequence of capped leader
	12
WT	5'-GUAUUAAUACCAUUUUCAAAAUAUUCC
Mut-2	5'-GUAUUAAUAACAGUUUCAAAAUAUUCC
Mut-3	5'-GUAUUAAUAGCAGCUUCAAAAUAUUCC
Mut-N14	5'-GUAUUAAUAUCAGCAAAAGCAGGGUAG

Residues potentially base-pairing to the viral template (3'-UCG…) are underlined; the marker nucleotide used to discriminate between the different AlMV3 leaders is shaded.

Reverse-transcription PCR-cloning of *de novo* synthesized influenza transcripts from infected cells co-transfected with capped RNA from WT and Mut-2 (**Figure 4-1, lane 1**), resulted in the presence of the Mut-2 leader sequence in all cloned AlMV3-NS1 transcript sequences (**Table 4-2**). The WT was also offered in a 1:1 competition with Mut-3, and almost all clones (18 out of 20) had a leader derived from Mut-3 and only 2 (out of 20) a leader from the single base-pairing WT (**Table 4-2**). When WT was offered as cap donor in pair-wise competition with Mut-N_{14} in 1:1 ratio (0.3 μg or 3 μg each), all clones from newly synthesized NS1 transcripts contained the marker nucleotide of Mut-N_{14} (**Table 4-2**). Similarly, when WT and Mut-N_{14} were offered in a 10:1 ratio (3 μg versus 0.3 μg respectively), still all clones contained the Mut-N_{14} leader (**Table 4-2**).

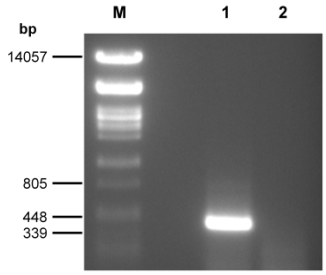

Figure 4-1 **Analysis of *in cell* synthesized influenza mRNA.** RT-PCR analysis of influenza transcripts from virus-infected MDCK cells transfected (lane 1) or not (lane 2) with WT and Mut-2 capped leaders. Reverse transcription was performed using internal primer specific for the *Influenza* NS1 gene, followed by PCR using internal primer for the same gene in combination with a primer specific for the AlMV3 mRNA. M: molecular marker.

When Mut-2 and Mut-3 were pair-wise tested *in cell*, no clear preference for one of the two leaders showed up initially [**Table 4-2, Mut-2 and Mut-3 (a)**], but during a repeated analyses Mut-3 leader was used twice as often as Mut-2 [**Table 4-2, Mut-2 and Mut-3 (b)**]. Furthermore, a closer look at the snatched leaders from Mut-2 showed two potential cleavage sites, i.e. one clone resulted from cleavage downstream A_{10} and the rest of the clones from cleavage downstream of A_{12} in both cases [**Table 4-2, Mut-2 and Mut-3 (a and b)**].

Finally, an experiment was performed in which MDCK cells were infected with *Influenza A virus* and co-transfected with three (mutant) capped leaders, i.e. WT, Mut-2 and Mut-3, in a 1:1:1 ratio. Reverse-transcription PCR-cloning and sequence analysis of *de novo* synthesized influenza transcripts showed that 18 out of 25 clones analyzed contained a leader from Mut-3, while 6 clones contained a leader from Mut-2 and only 1 the WT leader (**Table 4-2**). Altogether these results strongly suggested that, like *in vitro* (**Chapter 3**), influenza virus *in vivo* prefers leaders with extended base complementarity to the vRNA template.

During all experiments, control reactions with influenza infected only MDCK cells did not show any RT-PCR product (**Figure 4-1, lane 2**).

TABLE 4-2

Distribution and nucleotide sequence of WT and mutant AlMV3 leaders snatched during virus NS1 mRNA transcription *in vivo*.

Capped leaders offered	Retrieved mRNA 5' sequence	# clones	Origin leader
WT + Mut-2 (1:1; 3 µg each)	5'-GUAUUAAUAAC***AG***CAAAA	15	Mut-2
WT + Mut-3 (1:1; 3 µg each)	5'-GUAUUAAUAGC***AGC***AAAA	17	Mut-3
	5'-GUAUUAAUAGC***AGC***AGCAAAA	1	Mut-3
	5'-GUAUUAAUACC***A***GCAAAA	2	WT
WT + Mut-N_{14} (1:1; 0.3 µg each)	5'-GUAUUAAUAUC***AGC***AAAA	12	Mut-N_{14}
WT + Mut-N_{14} (1:1; 3 µg each)	5'-GUAUUAAUAUC***AGC***AAAA	23	Mut-N_{14}
	5'-GUAUUAAUAUGCAGCAAAA	1	Mut-N_{14}
	5'-GUAUUAAUAUGCAAAA	1	Mut-N_{14}
WT + Mut-N_{14} (10:1; 3 µg resp 0.3 µg)	5'-GUAUUAAUAUC***AGC***AAAA	6	Mut-N_{14}
Mut-2 + Mut-3 (a) (1:1; 3 µg each)	5'-GUAUUAAUAGC***AGC***AAAA	14	Mut-3
	5'-GUAUUAAUAAC***AG***CAAAA	13	Mut-2
	5'-GUAUUAAUA***A***GCAAAA	1	Mut-2
Mut-2 + Mut-3 (b) (1:1; 3 µg each)	5'-GUAUUAAUAGC***AGC***AAAA	19	Mut-3
	5'-GUAUUAAUAAC***AG***CAAAA	10	Mut-2
	5'-GUAUUAAUA***A***GCAAAA	1	Mut-2
WT + Mut-2 + Mut-3 (1:1:1; 3 µg each)	5'-GUAUUAAUAGC***AGC***AAAA	18	Mut-3
	5'-GUAUUAAUAAC***AG***CAAAA	6	Mut-2
	5'-GUAUUAAUACC***A***GCAAAA	1	WT

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

Nucleotide/-s close to the 3' terminus of the leader do not play an essential role during cap-snatching *in vivo*

Previous studies suggested that only leaders harboring a 3'-terminal CA dinucleotide are effectively used as primers during transcription initiation (***Rao et al., 2003***). However, in these studies capped leaders were singly used and subsequently comparatively analyzed while these differed in length and sequence context relative to the

first A base-pairing residue. To analyze whether nucleotides within the leader sequence positioned upstream of the first base-pairing residue to the vRNA template indeed would affect the efficiency of cap donor usage, a transcription assay was performed in which the use of competing leaders during influenza transcription initiation was quantified. To this end, transcripts of four mutant AlMV3 constructs, harboring three base-pairing residues (AGC) and a variable nucleotide at position 11, were simultaneously offered to influenza virus infected MDCK cells and analyzed for their use. The AlMV3 mutants were referred to as $C_{11}A_{12}GC$, $G_{11}A_{12}GC$, $A_{11}A_{12}GC$ and $U_{11}A_{12}GC$ (**Table 4-3**) in which the nucleotide residue at position 11 also served as marker nucleotide to discriminate between leaders derived from these mutants.

TABLE 4-3

Mutant AlMV3 leaders used in multiple competition assay.

Capped leader	5' end sequence of capped leader
	12
$C_{11}A_{12}GC$	5'-GUAUUAAUACCAGCUUCAAAAUAUUCC
$G_{11}A_{12}GC$	5'-GUAUUAAUACGAGCUUCAAAAUAUUCC
$A_{11}A_{12}GC$	5'-GUAUUAAUACAAGCUUCAAAAUAUUCC
$U_{11}A_{12}GC$	5'-GUAUUAAUACUAGCUUCAAAAUAUUCC

Residues potentially base-pairing to the viral template (3'-UCG…) are underlined; the marker nucleotide used to discriminate between the different AlMV3 leaders is shaded.

When transcripts of all four AlMV3 constructs were offered in equimolar amounts to influenza virus infected cells, cloned NS1 gene transcripts harboring the AlMV3 leader sequence revealed a G residue at position 11 in 12 out of 33 clones, an A residue in 9 clones, a U residue in 8 clones and a C residue in 4 clones (**Table 4-4**). A detailed look at the nucleotide sequence of clones with the leader sequence of $G_{11}A_{12}GC$ revealed that the first viral A residue was lacking in 9 out of these 12 clones, likely due to endonuclease cleavage immediately after the G residue and subsequent internal priming on the 3'-penultimate C residue of the vRNA template. The presence of a repetitive sequence in one of these clones furthermore indicated the occurrence of a prime-and-realignment event.

In case the leader sequence originated from the construct with an A at position 11, 1 out of 9 clones lacked an A residue at position 12 (**Table 4-4**), likely due to endonuclease cleavage downstream A_{11} and subsequent alignment on the vRNA template by a single base-pairing interaction with the ultimate 3' U residue of the vRNA template.

TABLE 4-4

Usage of leaders with a mutation at position -1 relative to the triple base-pairing $A_{12}GC$ residues snatched to prime NS1 mRNA transcription *in vivo*.

Capped leaders offered	Retrieved mRNA 5' sequence	# clones	Origin leader
$(C/G/A/U)_{11}A_{12}GC$	5'-GUAUUAAUACG***AGC***AAAA	3	$G_{11}A_{12}GC$
(1:1:1:1; 3 µg each)	5'-GUAUUAAUACG***G***CAAAA	8	$G_{11}A_{12}GC$
	5'-GUAUUAAUACG***G***CAGCAAAA	1	$G_{11}A_{12}GC$
	5'-GUAUUAAUACA***AGC***AAAA	8	$A_{11}A_{12}GC$
	5'-GUAUUAAUACA***A***GCAAAA	1	$A_{11}A_{12}GC$
	5'-GUAUUAAUACU***AGC***AAAA	8	$U_{11}A_{12}GC$
	5'-GUAUUAAUACC***AGC***AAAA	4	$C_{11}A_{12}GC$

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

We next determined whether AlMV3 leaders with three base-pairing residues and a variable residue at position 10, and referred to as $C_{10}C_{11}A_{12}GC$, $G_{10}C_{11}A_{12}GC$, $A_{10}C_{11}A_{12}GC$ and $U_{10}C_{11}A_{12}GC$ (**Table 4-5**), were affected in their relative usage during influenza transcription initiation *in vivo*. When transcripts of all four constructs were offered in a multiple competition, 6 out of 11 NS1 mRNA clones contained G_{10}, 4 contained A_{10} and only 1 contained U_{10} (**Table 4-6**). No clones were retrieved with C_{10}. In 1 out of the 4 clones containing the leader from $A_{10}C_{11}A_{12}GC$ the absence of C_{11} within the leader sequence implied the occurrence of an endonuclease cleavage immediately downstream A_{10}.

Although the results altogether did not reveal a strong preference for a specific nucleotide residue at position 10 or 11, capped RNA leaders harboring a C residue at these positions always were most poorly used.

TABLE 4-5

Mutant AlMV3 leaders used in multiple competition assay.

Capped leader	5' end sequence of capped leader
	12
$C_{10}C_{11}A_{12}GC$	5'-GUAUUAAUACCAGCUUCAAAAUAUUCC
$G_{10}C_{11}A_{12}GC$	5'-GUAUUAAUAGCAGCUUCAAAAUAUUCC
$A_{10}C_{11}A_{12}GC$	5'-GUAUUAAUAACAGCUUCAAAAUAUUCC
$U_{10}C_{11}A_{12}GC$	5'-GUAUUAAUAUCAGCUUCAAAAUAUUCC

Residues potentially base-pairing to the viral template (3'-UCG…) are underlined; the marker nucleotide used to discriminate between the different AlMV3 leaders is shaded.

TABLE 4-6

Usage of leaders with a mutation at position -2 relative to the triple base-pairing $A_{12}GC$ residues snatched to prime NS1 mRNA transcription *in vivo*.

Capped leaders offered	Retrieved mRNA 5' sequence	# clones	Origin leader
$(C/G/A/U)_{10}C_{11}A_{12}GC$	5'-GUAUUAAUAGC***AGC***AAAA	6	$G_{10}C_{11}A_{12}GC$
(1:1:1:1; 3 µg each)	5'-GUAUUAAUAAC***AGC***AAAA	3	$A_{10}C_{11}A_{12}GC$
	5'-GUAUUAAUAAGCAAAA	1	$A_{10}C_{11}A_{12}GC$
	5'-GUAUUAAUAUC***AGC***AAAA	1	$U_{10}C_{11}A_{12}GC$

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

Pair-wise competition between a singly and a triply base-pairing leader with reciprocal 5' upstream sequences

All results so far support the idea that base-pairing residues within a leader sequence are of importance during influenza transcription initiation *in vivo* and nucleotides upstream these residues at positions -1 and -2 (i.e. in our constructs at position 10 or 11) are of lesser importance, although a C at these positions was not favored. To further substantiate these findings a singly base-pairing leader ($A_{11}A_{12}$) was offered to influenza virus infected MDCK cells in pair-wise competition with a triply base-pairing leader ($C_{11}A_{12}GC$) (**Table 4-7**), and in a second one their reciprocal variants, $C_{11}A_{12}$ (identical to WT) and $A_{11}A_{12}GC$ (**Table 4-7**).

TABLE 4-7

WT and mutant AlMV3 leaders used in pair-wise competition assays.

Capped leader	5' end sequence of capped leader
	12
$C_{11}A_{12}$ (WT)	5'-GUAUUAAUACCAUUUUCAAAAUAUUCC
$A_{11}A_{12}$	5'-GUAUUAAUACAAUUUUCAAAAUAUUCC
$C_{11}A_{12}GC$	5'-GUAUUAAUACCAGCUUCAAAAUAUUCC
$A_{11}A_{12}GC$	5'-GUAUUAAUACAAGCUUCAAAAUAUUCC

Residues potentially base-pairing to the viral template (3'-UCG…) are underlined; the marker nucleotide used to discriminate between the different AlMV3 leaders is shaded.

In the first experiment, 10 out of 14 NS1 transcript clones analyzed contained a leader from mutant $C_{11}A_{12}GC$ and 4 from $A_{11}A_{12}$ (**Table 4-8**). In the second experiment with their reciprocal leader variants, 13 of the 16 clones contained the $A_{11}A_{12}GC$ sequence and 3 the $C_{11}A_{12}$ (**Table 4-8**). In both experiments the triple base-pairing leaders were favorably used versus the single ones, and only in case of C at position 11 seemed somewhat reduced in its usage. The outcome of these experiments clearly indicate that base-pairing between the leader and the viral template enhances the use of leader sequences during influenza transcription initiation *in vivo* whereas residues at positions -1 and -2 to a lesser extent.

TABLE 4-8

In vivo competition of a less preferred (CA) versus more preferred (AAGC) leader and their reciprocal leader variants during priming of NS1 mRNA transcription.

Capped leaders offered	Retrieved mRNA 5' sequence	# clones	Origin leader
$A_{11}A_{12}$ + $C_{11}A_{12}GC$ (1:1; 3 µg each)	5'-GUAUUAAUACC***AGC***AAAA	10	$C_{11}A_{12}GC$
	5'-GUAUUAAUACA***A***GCAAAA	1	$A_{11}A_{12}$
	5'-GUAUUAAUAC***A***GCAAAA	3	$A_{11}A_{12}$
$C_{11}A_{12}$ + $A_{11}A_{12}GC$ (1:1; 3 µg each)	5'-GUAUUAAUACA***AGC***AAAA	7	$A_{11}A_{12}GC$
	5'-GUAUUAAUAC***A***GCAAAA	4	$A_{11}A_{12}GC$
	5'-GUAUUAAUAC***A***GCAGCAAAA	1	$A_{11}A_{12}GC$
	5'-GUAUUAAUACA***AGC***AAAGCAAAA	1	$A_{11}A_{12}GC$
	5'-GUAUUAAUACC***A***GCAAAA	3	$C_{11}A_{12}$

Influenza sequence is underlined, while residues that possibly could originate from the RNA leader offered and with complementarity to the vRNA template (3'-UCG…) are in **bold** and *italics*; the marker nucleotide of each mutant is shaded.

Discussion

Influenza viral mRNA synthesis is initiated by cap-snatching; a mechanism in which the endonuclease cleaves capped RNAs to ensure the required primers for viral mRNA synthesis (***Beaton and Krug, 1981; Bouloy et al., 1978; Caton and Robertson, 1980; Dhar et al., 1980; Plotch et al., 1979, id. 1981***). Recently, we observed that influenza virus *in vitro* exhibits a strong preference for capped RNA leaders harboring 3'-terminal residues with complementarity to the vRNA template (**Chapter 3**). Here we demonstrate that also during an infection in MDCK cells the influenza viral polymerase complex favors capped RNA leaders that match this criterion. Priming is not restricted to the ultimate viral template residue but can also take place internally, i.e. on the penultimate viral (C) residue, leading to absence of the first viral A residue in transcript sequences. A pair-wise competition of singly and triply base-pairing leaders with reciprocal 5' upstream sequences showed that the presence of less favorable residues in the leader sequence is overruled by the presence of downstream base-pairing residues, and again strengthens the importance of base complementarity (**Table 4-8**). Since not only for *Influenza A virus* but also for a completely different virus, the plant-infecting *Tomato spotted wilt virus* (TSWV) (***Duijsings et al., 1999, id. 2001; van Knippenberg et al., 2002, ead. 2005a***), the presence of base complementarity within the snatched leader sequence appears a preferred determinant for the selection of capped RNA leaders, it is likely that this applies to all other families of segmented (-) ssRNA viruses. For influenza virus, base complementarity is optimally positioned around 10-11 nt from the cap structure of the leader (**Chapter 3**) whereas for TSWV, likely due to a difference in the conformational folding of the viral transcription complex, is more optimal when positioned around 16 nt from the cap structure (***Duijsings et al., 2001***). The preference of influenza virus for leaders with base complementarity at positions 10-11 probably also explains why mutant $G_{11}A_{12}GC$ (**Table 4-4**) more often leads to (internal) priming by $G_{11,}$ as observed by the absence of the first viral A residue, instead of alignment and priming by the (less optimally positioned) $A_{12}GC$.

Whereas the *in vitro* pair-wise competition between Mut-2 and Mut-3 (**Chapter 3**), as well as the *in vivo* multiple competition between WT, Mut-2 and Mut-3 (**this Chapter**) clearly showed a two- to three-fold increased use of Mut-3 over Mut-2, it is interesting to note that in one out of two *in vivo* pair-wise competition experiments between Mut-2 and Mut-3 no clear preference for one of the two capped RNA leaders was observed (**Table 4-2**). This could be caused by other factors that may influence the selection of leaders during transcription initiation. In light of this, it is interesting to mention that experimental evidence supports the formation of specific folding structures within the 5' and 3' viral termini as a requirement for genome transcription, which could as well affect the thermodynamics of primer alignment on the vRNA template. The most simplified model

describes formation of a panhandle structure due to base-pairing of the 5' and 3' ends (***Hsu et al., 1987***), but others have described a so-called "Fork" model (***Fodor et al., 1994, id. 1995***), a "Corkscrew" model (***Flick et al., 1996, id. 1999***) or a combination of both (***Pritlove et al., 1999***). While the requirement of both 5'- and 3'-terminal ends for cap-dependent endonuclease cleavage by the viral polymerase complex still is a matter of debate (***Cianci et al., 1995; Hagen et al., 1994; Lee et al., 2003***), and one might discuss which of the predicted stem-loop folding structures genuinely exists *in vivo*, the proposed involvement of a secondary structure on the 3' end of the vRNA template could affect the competitiveness of Mut-3 during a pair-wise testing with Mut-2 (**Table 4-2**). This could, likewise, explain the absence of a beneficial effect of extended base complementarity of Mut-N14 during a pair-wise competition *in vitro* with Mut-3 (**Chapter 3**).

When revisiting the published 5' non-viral leader sequence data from previous *in vivo Influenza A virus* studies (**Table 4-9**) in view of our proposed model, 9 out of 15 transcripts may have resulted from priming of capped RNA leaders on the 3'-ultimate U residue by means of single (or even multiple) base complementarity. The remaining 6 clones showed the absence of the first viral A residue (***Beaton and Krug, 1981; Caton and Robertson, 1980; Dhar et al., 1980; Lai et al., 1981; Vreede et al., 2008***), likely due to internal priming of a G-terminated leader on the 3'-penultimate C residue of the vRNA template. For *Influenza B virus*, on the other hand, 10 out 19 transcripts may have resulted from priming of leaders on the vRNA template and 9 out 19 from internal priming (**Table 4-10**) (***Briedis et al., 1982; Briedis and Lamb, 1982; Briedis and Tobin, 1984; Shaw et al., 1982; Shaw and Lamb, 1984***). Furthermore, the 5' non-viral leader sequence data from *in vivo Influenza A virus* studies (**Table 4-9**) do not indicate a preference for capped RNA leaders with a 3' CA-terminus, in support of our *in vitro* and *in vivo* findings (**Chapter 3 and this Chapter**), whereas those from *Influenza B virus* show a C residue upstream the base-pairing (A or G) residue in 14 out of 19 clones analyzed (**Table 4-10**). This indicates a preference of *Influenza B virus* for such leaders, and could be confirmed using capped RNA leader competition assays similarly as being described in this paper.

Influenza virus steals capped RNA leaders from nascent RNA in the nucleus. There, the virus is confined to RNA Pol II transcription sites through binding of its RNA polymerase complex to the large subunit of Pol II via its CTD, which allows the virus direct access to higher concentrations of cellular capped pre-mRNAs as source to initiate genome transcription. The benefit for the virus is obvious but brings up the question whether the viral polymerase, while being attached to the RNA Pol II CTD, is limited to nascent RNA as substrate for capped leaders. Our data shows that the influenza virus during transcription/replication in the nucleus is able to accept leaders from an exogenous source. Whether this involves the RNA Pol II bound viral polymerase complex, or the viral polymerase at sites distinct from this (nuclear cages or the nuclear matrix function**)** (***Engelhardt et al., 2005***), remains unclear.

TABLE 4-9

Host-derived sequences at the 5' ends of influenza A mRNA reported in earlier publications on sequencing of cloned cDNAs.

Retrieved *Influenza A virus* mRNA 5' sequence	vRNA	References
5'-AAGUAG***A***GCAAAA	NS	Dhar *et al.*, 1980
5'-ACCGGAGGGAGCAA***A***GCAAAA	HA	Dhar *et al.*, 1980
5'-AUCCUUUUGCA***A***GCAAAA	NS	Lamb and Lai, 1980
5'-AUCUUUUGCA***A***GCAAAA	NS	Dhar *et al.*, 1980
5'-ACCAGCAGA***A***GCAAAA	NA	Vreede *et al.*, 2008
5'-AAGGCUUGU***A***GCAAAA	NA	Markoff and Lai, 1982
5'-AGUGUUCGC***A***GCAAAA	HA	Dhar *et al.*, 1980
5'-AUUUACAGCUC***A***GCAAAA	NA	Vreede *et al.*, 2008
5'-AACAAACUUC***A***GCAAAA	M	Lamb and Lai, 1981
5'-CGGAAGGCCUCG***G***CAAAA	NS	Beaton and Krug, 1981
5'-AGUGUCUCCGCG***G***CAAAA	NA	Lai *et al.*, 1981
5'-CUGGUUGCGG***G***CAAAA	NS	Dhar *et al.*, 1980
5'-CAACAAGAUG***G***CAAAA	NA	Vreede *et al.*, 2008
5'-GUUCAUCAUCCCU***G***CAAAA	M	Caton and Robertson, 1980
5'-GAAGCGCUUCC***G***CAAAA	NA	Vreede *et al.*, 2008

Influenza sequence is underlined, while the first residue base-pairing to the viral template and with possible origin from the RNA leader is in **bold** and *italics*; the non-viral 3'-terminal nucleotide at position -1 relative to the potential first base-pairing residue which might influence the use of the leader during cap-snatching process is shaded. The proteins encoded by the cloned segments are also indicated.

During the past years, a number of reports have appeared in which RNA molecules were shown to down-regulate influenza vRNA replication/transcription *in vitro*. Small RNA molecules (47 nt) containing the conserved termini of influenza inhibited most of the CAT expression from a NS-CAT RNA molecule in their recombinant replication/transcription system (***Luo et al., 1997***). In another study, a pool of capped oligonucleotides (4-9 nt in length) was designed, too short to prime transcription, but long enough to bind (sequester) the viral polymerase and these were shown to be potent inhibitors of cap-dependent transcription (***Chung et al., 1994***). Uncapped RNA molecules lacking sequence complementarity to the 3' end of the influenza vRNA template, and with diminished secondary structure, have also been shown to be potent inhibitors of the transcription primed by ApG or globin mRNAs (***Krug et al., 1980***). Although in all cases described an inhibitory effect of specific RNAs on replication/transcription has been observed, there are no reports on possible antiviral effects of these molecules during influenza infection *in vivo*. Whether such oligos would work *in vivo* remains to be analyzed, especially when considering that they would have to compete for the polymerase with naturally and possibly more favorable abundant cap donors.

TABLE 4-10

Host-derived sequences at the 5' ends of influenza B mRNA reported in earlier publications on sequencing of cloned cDNAs.

Retrieved *Influenza B virus* mRNA 5' sequence	vRNA	References
5'-AAUAGCAG***A***GCAGAA	NA	Shaw and Lamb, 1984
5'-AGAGGAUUGCUUGC***A***GCAGAA	M	Shaw and Lamb, 1984
5'-CUUGCCUUGC***A***GCAGAA	M	Shaw and Lamb, 1984
5'-CAGAUGUAUGC***A***GCAGAA	M	Shaw and Lamb, 1984
5'-CUCUUGUGUGC***A***GCAGAA	NA	Shaw and Lamb, 1984
5'-ACCUCCCUCGGC***A***GCAGAA	M	Shaw and Lamb, 1984
5'-UCAUUCUGGC***A***GCAGAA	NS	Shaw and Lamb, 1984
5'-AGACACACGC***A***GCAGAA	NA	Shaw *et al*., 1982
5'-CAGCCGCCGC***A***GCAGAA	M	Shaw and Lamb, 1984
5'-CAGUUCGGCGC***A***GCAGAA	NS	Shaw and Lamb, 1984
5'-AAGCGGCUGGUCG***G***CAGAA	NP	Briedis and Tobin, 1984
5'-CUAGGGCG***G***CAGAA	M	Shaw and Lamb, 1984
5'-AGCAGCUCCU***G***CAGAA	M	Briedis *et al*., 1982
5'-CUUGCUUUU***G***CAGAA	NA	Shaw and Lamb, 1984
5'-CAGAGGAC***G***CAGAA	NS	Shaw and Lamb, 1984
5'-AGUCUCUCUCGC***G***CAGAA	NS	Briedis and Lamb, 1982
5'-CCACCACCGC***G***CAGAA	M	Shaw and Lamb, 1984
5'-AGACGGCGCGC***G***CAGAA	M	Shaw and Lamb, 1984
5'-AAAACCCCCCACC***G***CAGAA	M	Shaw and Lamb, 1984

Influenza sequence is underlined, while the first residue base-pairing to the viral template and with possible origin from the RNA leader is in **bold** and *italics*; the non-viral 3'-terminal nucleotide at position -1 relative to the potential first base-pairing residue which might influence the use of the leader during cap-snatching process is shaded. The proteins encoded by the cloned segments are also indicated.

The recently observed stronger *in vitro* reduction of α-globin RNA leader initiated transcription by triply base-pairing cap donors compared to singly base-pairing variants (**Chapter 3**), and the confirmation that also *in vivo* influenza virus strongly prefers cap donor molecules with extended base complementarity to the vRNA template, not only demonstrates the competitiveness of such molecules during transcription initiation but also holds a promise, once made dysfunctional, in their use as antiviral RNA compounds.

Materials and methods

Virus and Construction of plasmids

Influenza A virus and AlMV3 plasmids used have been described previously (**Chapter 2 and 3**).

Synthesis of capped RNA leaders

In vitro synthesis of capped RNA leaders was performed by using the Ambion T7 mMESSAGE mMACHINE kit according to the manufacturer's instruction.

Cell culture

MDCK cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco® Invitrogen, Breda, The Netherlands) supplemented with 10% FBS, 100 $U \cdot ml^{-1}$ penicillin, and 10 $\mu g \cdot ml^{-1}$ streptomycin. The cells were cultured in a 5% CO_2 atmosphere at 37 ^{o}C.

Influenza transcription *in vivo*

Cells were grown in DMEM (without FBS and antibiotics) at 60% to 70% confluence in a 6-well plate at 37 °C and infected with *Influenza A virus* at a multiplicity of infection (m.o.i.) of 3-10. After 2 h the medium was removed, cells were washed and subsequently transfected with the indicated amounts of wild type (WT) or (mutant) AlMV3 RNA leaders using Lipofectamine reagent (Invitrogen, Breda, The Netherlands) according the manufacturer's instructions. After 4 h the transfection, medium was removed and substituted for supplemented DMEM. Cells were harvested after 17 h and total RNA was extracted with Trizol® (Invitrogen, Breda, The Netherlands), phenol-chloroform and subsequently ethanol precipitated. AlMV3 leader initiated influenza mRNA transcripts were RT-PCR amplified and sequence analyzed as previously described (**Chapter 2**). Healthy and virus infected only MDCK cells were used as controls.

Acknowledgements

The authors thank Dr. ***Gorben P. Pijlman*** for critical reading of the manuscript.

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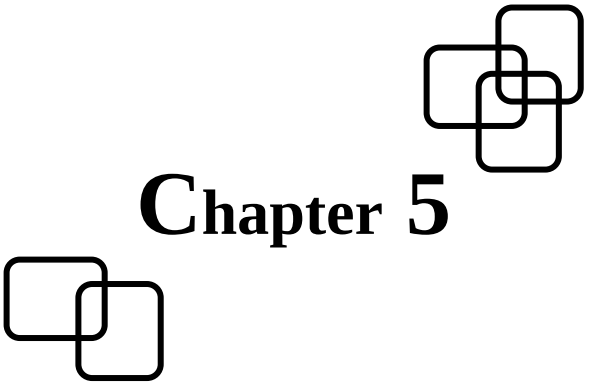
“The good lad always knows of an alternate path.”

Greek proverb



Chapter 5

Analysis of the *Tomato spotted wilt virus* S RNA-encoded hairpin structure in translation

Transcripts of *Tomato spotted wilt virus* (TSWV) generally possess a 5' cap structure followed by an untranslated region (UTR) and at its 3' end a UTR containing a sequence that is predicted to fold into a stable hairpin structure. Due to the lack of a poly(A) tail, the predicted hairpin structure is postulated to act as an functional equivalence. In the present study, the role of the predicted hairpin structure in translation was investigated by expression analysis of various *Renilla* luciferase sensor constructs containing modified 3' and/or 5' UTR sequences of the TSWV nucleocapsid (N) gene. The results showed good luciferase expression levels in the presence of the 3' UTR including the predicted hairpin structure, and a loss of expression when the hairpin sequence was removed. Constructs containing the entire 3' UTR, but lacking the 5' UTR still rendered good expression levels, but when in addition the entire 3' UTR was exchanged for that of the non-structural (NSs) gene transcript, containing the complementary hairpin folding sequence, the loss of luciferase expression could only be recovered by providing the 5' UTR sequence of the NSs transcript. Luciferase activity remained unaltered when the hairpin structure sequence was swapped for the analogous one from *Tomato yellow ring virus*, another distinct tospovirus. The addition of viral N and NSs proteins further increased luciferase expression levels from hairpin structure containing constructs. The results suggest a role for the predicted hairpin structure in translation in concert with the viral N and NSs proteins.

A slightly modified version of this chapter has been submitted for publication.
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Introduction

The *Bunyaviridae* are a large and diverse family of primarily animal-infecting viruses that are characterized by spherical enveloped virus particles of 80-120 nm in diameter and having a segmented negative (-) strand tripartite RNA genome. Based on molecular, serological and biological (host) features, members of this family are divided into five genera, i.e. *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus* (**Table 1-1; Chapter 1**) of which the latter contain the plant-infecting members. Most bunyaviruses are arthropod-borne and include causal agents for several important so-called "emerging infections" in human, livestock and plant pathogens, such as *La Crosse* and *Rift Valley fever virus* which cause encephalitis in human and epizootics in livestock respectively (***Elliott, 1997***).

Tomato spotted wilt virus (TSWV) is the representative of the plant-infecting tospoviruses and ranks among the top ten of economically most important plant viruses worldwide (***Mumford et al., 1996***). The TSWV genome comprises three single-stranded RNA segments (**Figure 1-5; Chapter 1**), and distinguishes from the other *Bunyaviridae* members as two out of its three genomic segments contain an ambisense gene arrangement. Only the large (L) segment is of entire negative polarity and encodes an RNA-dependent RNA polymerase (RdRp; L protein). The medium (M) segment codes in ambisense arrangement for a precursor of the envelope glycoproteins (Gn and Gc) and for the cell-to-cell movement protein (NSm). The small (S) segment contains, like the M RNA, two non-overlapping open reading frames (ORFs) on opposite strands, coding for the nucleocapsid (N) and non-structural (NSs) protein respectively. The NSs has been shown to be involved in suppression of gene silencing (***Bucher et al., 2003; Takeda et al., 2002***). All three segments are encapsidated by the N protein and together with small amounts of the RdRP form transcriptionally active ribonucleoproteins (RNPs), the templates for RNA synthesis (replication and transcription) by the L protein (**Figure 5-1**) (***Goldbach and Peters, 1996***).

Within each genomic RNA segment, the first 8 nucleotides of both termini are complementary and conserved. Depending on the segment, terminal complementarity extends for up to 65 nucleotides (***de Haan et al., 1989, id. 1990; Kormelink et al., 1992a***). These complementary regions are thought to base-pair intramolecularly to form stable panhandle structures *in vivo*, as evidenced by the observed circular conformation of viral RNPs in electron micrographs (***de Haan et al., 1989; Kellmann et al., 2001; Kitajima et al., 1992; Martin, 1964***). The remainder internal regions of the untranslated regions (UTRs) are unique to each segment.

The two non-overlapping genes of each ambisense RNA segment are located on opposite strands and separated by a large intergenic region (IR). The IR contains stretches of highly A- and U-rich sequences which are predicted to fold into a stable hairpin

structure. Genes encoded by the ambisense RNA segments become expressed by the synthesis of subgenomic messenger RNA (mRNA) molecules, a process that is being initiated by cap-snatching from host cellular mRNAs (***Kormelink et al., 1992b; van Poelwijk et al., 1996***). Whereas the requirements for cap-snatching have been studied in quite some details for TSWV (***Duijsings et al., 1999, id. 2001; van Knippenberg et al., 2002, ead. 2005a***) and shown to similarly apply for influenza (**Chapters 2, 3 and 4**), not much is known yet on TSWV transcription termination and translation initiation. All bunyavirus transcripts lack a common poly(A) tail, and sequences that signal transcription termination have not been identified yet. Transcription termination for the ambisense encoded genes is thought to involve formation of the hairpin structure in the same manner as prokaryotic transcription termination occurs (***Iapalucci et al., 1991***). This is being supported by the observation that 3′ end mapping of viral mRNAs revealed the presence of the entire hairpin structure encoding sequence (***van Knippenberg et al., 2005b***).

Eukaryotic mRNAs possess a 5' cap structure and a 3' poly(A) tail that are involved in bridging the 3' and 5' ends of the mRNA (***Gallie, 1998, id. 2002***). This circularization supports efficient translation of mRNA, presumably by facilitating recycling of the ribosomal subunits from the 3' end back to the 5' end. While bunyavirus mRNAs lack a poly(A) tail, it is not unlikely that such role is assigned to a structural sequence that functionally acts as an equivalent. For the TSWV ambisense encoded genes, the 3' hairpin structure could thus have an additional role as functional analog of a poly(A) tail, and support efficient translation.

To test this hypothesis here, various N gene-based constructs were made and tested in Baby Hamster Kidney (BHK-21) cells for translation efficiency in a *Vaccinia virus*-T7 (vv-T7) expression system. These constructs differed in their 3' termini, i.e. with mutations in the sequence of the predicted hairpin structure. For quantification purposes, the viral N gene was swapped for the *Renilla* (REN) luciferase gene. Results from this analysis are shown and suggest a role of the TSWV hairpin structure in translation, during which the 5' UTR may act in concert with the hairpin structure.

Results

TSWV S segment 3' UTR is required for translation

To analyze the involvement of the predicted hairpin structure within the IR region of the ambisense TSWV S RNA segment in translation of the S RNA-encoded N and NSs transcripts, translation studies were performed on variants of a model template that reflected authentic viral mRNAs (**Figure 5-1**). To this end, a copy of an N mRNA molecule was made, preceded with an *Alfalfa mosaic virus* RNA 3 (AlMV3) leader sequence and at its 3' end flanked with the predicted hairpin structure sequence (**Materials and methods**). This construct was fused at its 3' end with a ribozyme sequence, to create 3' ends that would most closely mimic authentic viral transcripts. The entire construct was cloned in a T7 promoter-terminator cassette. For (sensitive) quantification purposes, the viral N gene was next swapped by the REN luciferase gene (**Figure 5-2A**). This construct, marked pREN-$H^{A/U\text{-}rich}$, was used for the construction of variants in which the predicted hairpin structure sequence was (partially) deleted, mutated, or exchanged for another tospovirus hairpin sequence and subsequently analyzed for translation efficiency using the vv-T7 system. The first set of variants from pREN-$H^{A/U\text{-}rich}$ differed at the 3' termini, i.e. either lacked the entire predicted hairpin structure sequence (construct pREN-NoH, **Figure 5-2B**), or instead contained a poly(A) tail coding sequence of 40 nucleotides (pREN-polyA, **Figure 5-2B**).

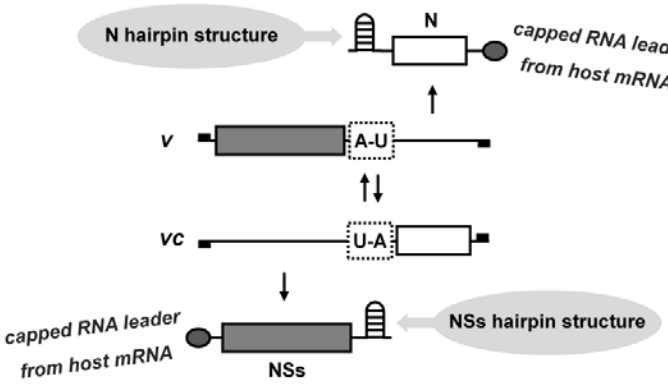

Figure 5-1 Structural features within the S RNA segment.

BHK-21 cells were first infected with vv-T7 and then co-transfected with the aforementioned mutant REN luciferase constructs and a firefly (FF) luciferase plasmid (pIRES-FF) as internal control. While the REN luciferase gene, flanked with the 5' and 3' UTR of the N gene and including the sequence for the hairpin structure, showed good expression levels, no luciferase expression was observed when the hairpin structure sequence was deleted from this REN construct (**Figure 5-2B**). When the REN sensor construct from which the hairpin was deleted, was being provided a 3' poly(A) tail-encoding sequence (pREN-polyA), high levels of luciferase activity were recovered again (**Figure 5-2B**). To exclude that differences in luciferase expression were not due to differences or even absence of transcription, transcriptional expression of all mutant REN constructs was verified by semi-quantitative RT-PCR and revealed similar levels (data not shown). Altogether, the results indicate the requirement/importance of the TSWV hairpin sequence for translation.

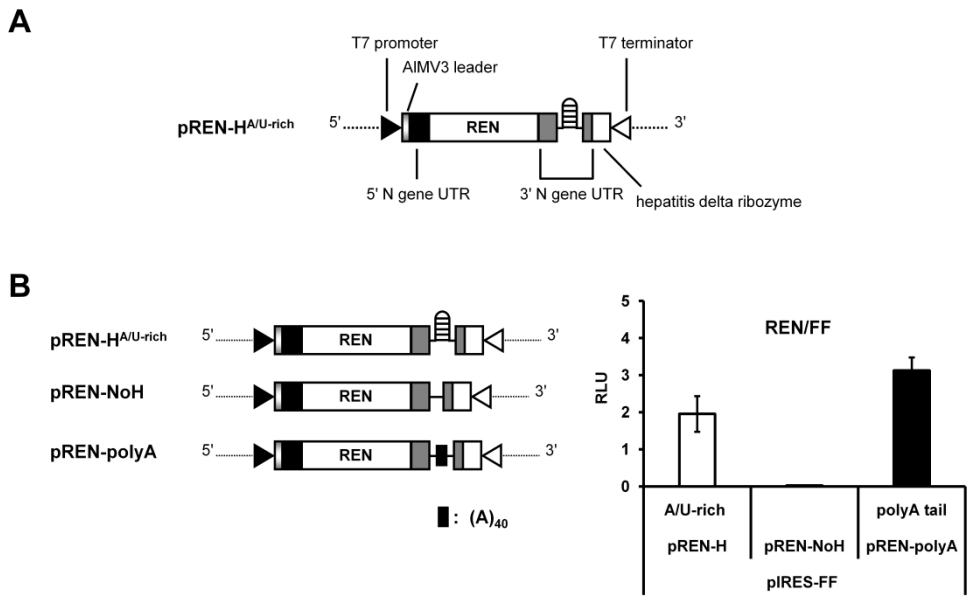

Figure 5-2 **Analysis of the hairpin structure sequence in translation.** Schematic presentation of TSWV-N (REN) and derived templates with modifications at the 3' UTR (A and B). (C) Luciferase activity monitored from REN constructs transfected to BHK-21 cells. Cells were infected with vv-T7 and subsequently co-transfected with 100 ng of the indicated REN constructs and 0.5 ng of the FF luciferase expression plasmid (pIRES-FF) as internal control. The relative luciferase expression (REN/FF) was measured after 23 h post transfection. Error bars indicate standard deviations from the means of three replicate experiments.

Requirement of 5' and 3' UTR interaction for translational enhancement

In order to determine whether the observed translation of pREN-H$^{A/U\text{-}rich}$ required the presence of a hairpin structure at the 3' end of the mRNA or just the IR sequence, a reporter construct was produced (pREN-H$^{A/U^{*}\text{-}rich}$) that contained the reverse complementary copy of the IR sequence encoding the hairpin structure (**Figure 5-3**). While this construct now contained a different sequence at its 3' end, i.e. the sequence normally involved in transcription termination of the ambisense S RNA encoded NSs gene, it still was able to fold into a similar hairpin structure (***Zuker, 2003***) (**Figure 5-3, A and B**). The construct was transfected to *Vaccinia virus* infected BHK-21 cells and its translational activity was measured. As a control, transcriptional expression of all mutant REN sensor constructs was verified by semi-quantitative RT-PCR and revealed similar transcription levels (data not shown). Although both hairpin constructs, pREN-H$^{A/U\text{-}rich}$ and pREN-H$^{A/U^{*}\text{-}rich}$, are highly AU-rich and share structural homology to each other (**Figure 5-3A**), a reduction in luciferase activity was observed with the reverse complementary pREN-H$^{A/U^{*}\text{-}rich}$ construct (**Figure 5-3C**).

To enhance the recruitment of ribosomes during translation of eukaryotic mRNAs, the 5' and 3' UTR sequences may also directly interact and lead to the formation of a pseudo-circularized mRNA molecule. The reduction in luciferase activity of pREN-H$^{A/U^{*}\text{-}rich}$ could thus be due to the presence of heterologous 5' and 3' UTR sequences, i.e. the 5' UTR originating from the TSWV N gene and the 3' hairpin encoding IR from the TSWV NSs gene, and their inability to interact. To analyze whether the 3' hairpin sequence acts in concert with the 5' UTR, the latter sequence was either removed from the REN sensor hairpin constructs or replaced by the 5' NSs UTR (**Figure 5-4**).

Luciferase expression analysis revealed no change in the activity of the N-based hairpin construct lacking the 5' UTR or having the 5' NSs UTR. However, the previous loss of reporter activity observed with the NSs-based hairpin construct harboring the 5' N UTR (pREN-H$^{A/U^{*}\text{-}rich}$), and recovery after the addition of the 5' NSs UTR (**Figure 5-4**) indicated that the presence of both 5' and 3' UTR sequences of the NSs gene was required to enhance translation. The observation that the N gene hairpin structure encoding 3' UTR sequence could be replaced by its reverse complement, able to fold into a similar structure, pointed towards the requirement for the hairpin structure rather than a specific nucleotide sequence.

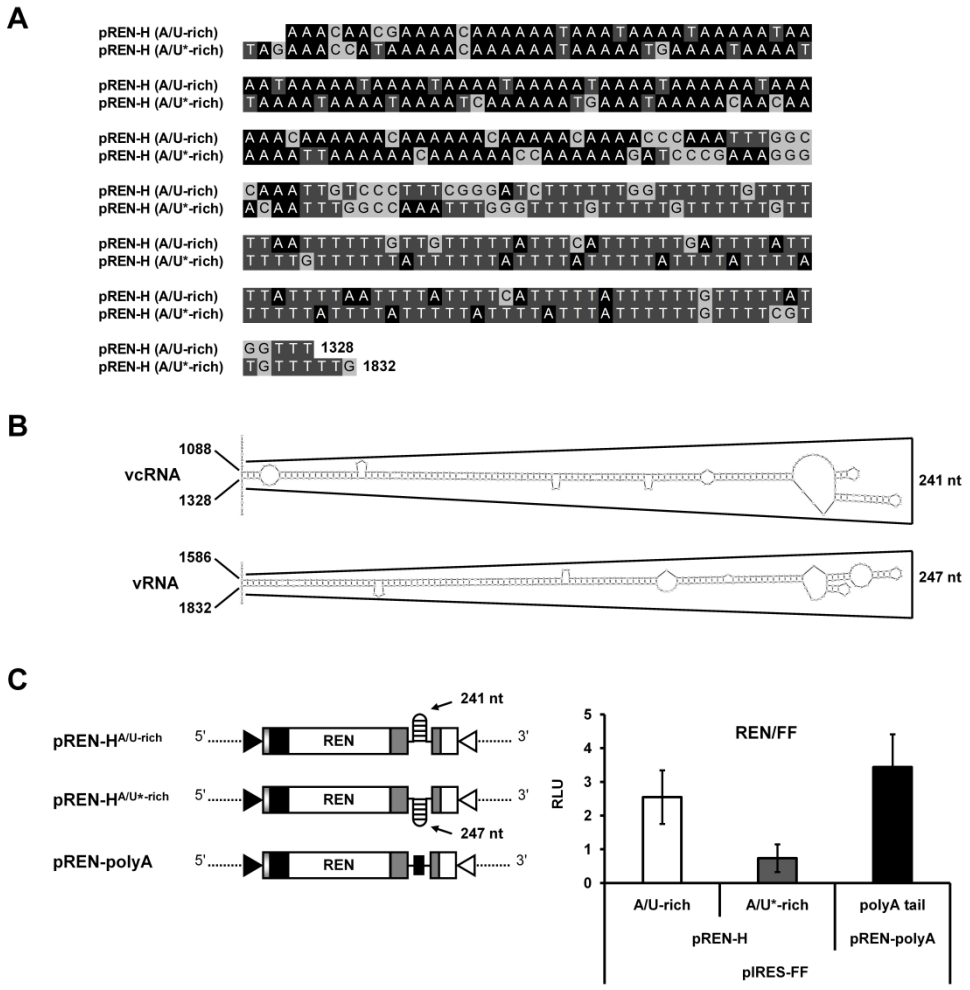

Figure 5-3 **Requirement of the 3' UTR of TSWV mRNAs in translation.** (A) Sequence alignment of the TSWV N gene 3' UTR (pREN-H$^{A/U\text{-}rich}$) and its reverse complement (pREN-H$^{A/U^*\text{-}rich}$). (B) Mfold predictions of the highly AU-rich sequence in the viral sense RNA (vRNA) flanking the 3' end of the NSs ORF (pREN-H$^{A/U^*\text{-}rich}$, panel A), and the analogous sequence in the viral complementary RNA (vcRNA) flanking the 3' end of the N ORF (pREN-H$^{A/U\text{-}rich}$, panel A). (C) Luciferase activity measured from BHK-21 cells infected with vv-T7 and subsequently co-transfected with 100 ng of expression REN constructs (pREN-H$^{A/U\text{-}rich}$, pREN-H$^{A/U^*\text{-}rich}$, or pREN-polyA) and 0.5 ng of pIRES-FF as internal control. The relative luciferase expression (REN/FF) was measured after 23 h post transfection. Error bars show the standard deviations from the means of three replicate experiments.

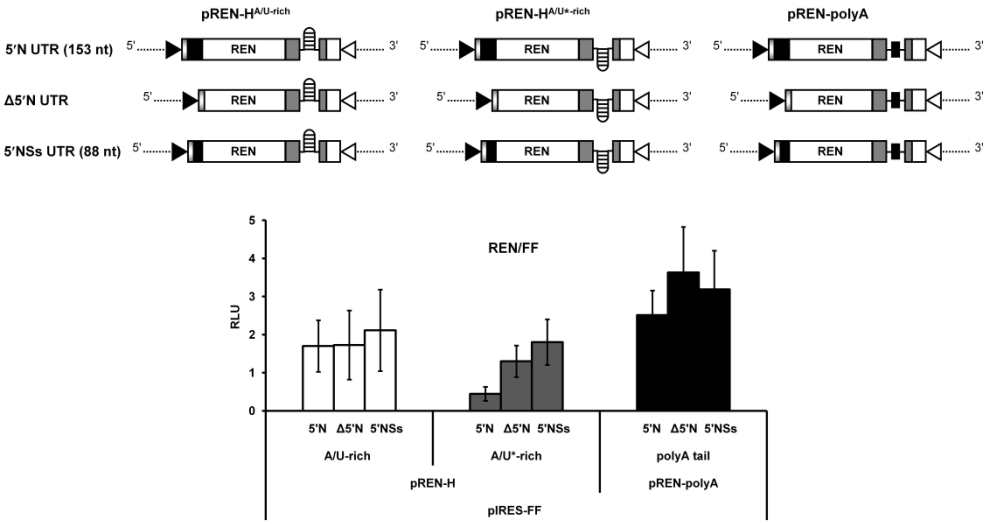

Figure 5-4 Requirement of the 5' UTR sequence in translation. BHK-21 cells were infected with *Vaccinia virus*, and subsequently co-transfected with 100 ng of the indicated REN constructs and 0.5 ng of pIRES-FF as internal control. The relative luciferase expression (REN/FF) was measured after 23 h post transfection. Error bars show the standard deviations from the means of three replicate experiments.

Rescue of TSWV translation by an IR-encoding hairpin structure sequence from a distinct tospovirus

To further substantiate the finding that the hairpin structure, rather than a sequence specific element within the IR, is involved in translation of the TSWV S RNA-encoded N and NSs genes, the hairpin structure sequence within the TSWV N-gene 3' UTR was exchanged for the one from a completely distinct tospovirus, i.e. *Tomato yellow ring virus* (TYRV). Whereas the TYRV S RNA encoded hairpin structure sequence from the N gene is also AU-rich, it is a bit larger in size than the one from TSWV (**Figure 5-5, A and B**). When the TSWV hairpin structure sequence from pREN-H$^{A/U\text{-rich}}$ was replaced by the one from TYRV (construct pREN-TYRV H), no difference in luciferase activity was observed in BHK21 cells between the two hairpin constructs (**Figure 5-5C**).

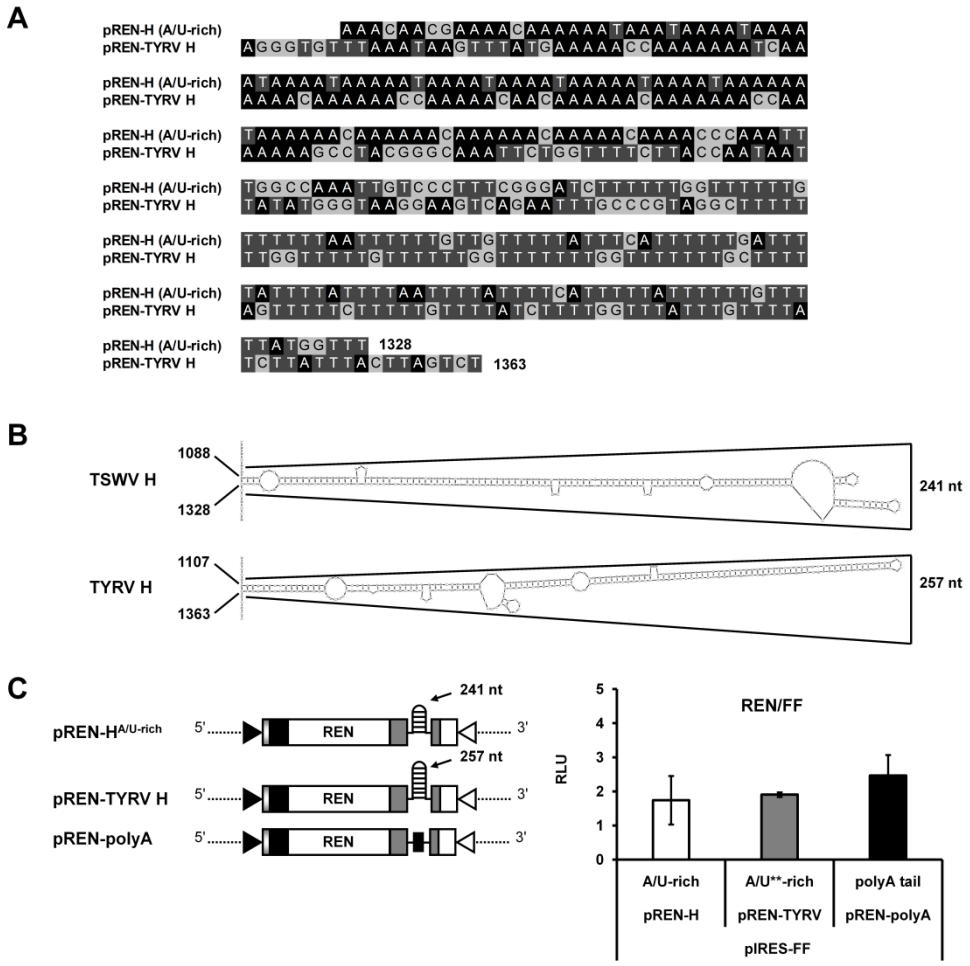

Figure 5-5 **Comparison of the predicted hairpin structure sequence from TSWV (N gene transcript) with the analogous one from TYRV.** (A) Alignment of the TSWV and TYRV N-based hairpin structure sequence. (B) Predicted hairpin structure at the 3' end of the N gene of TSWV respectively TYRV. (C) BHK-21 cells were infected with *Vaccinia virus* and transfected with 100 ng of either pREN-H$^{A/U\text{-}rich}$, pREN-TYRV H, or pREN-polyA. In addition to the REN construct, 0.5 ng of pIRES-FF was added as internal control. After 23h, the cells were lysed and assayed for relative luciferase activity. Error bars show the standard deviations from the means of three replicate experiments.

The A-rich stretch of the predicted hairpin structure suffices for translation

A close look at the IR showed that the first half of the predicted hairpin structure sequence contained stretches rich in A residues followed by a second half rich in U residues (**Figure 5-6**). Due to this sequence arrangement, it could not be excluded that only the first half of the predicted hairpin sequence mimicked a natural poly(A)-tail. To analyze this possibility, two mutants of pREN-H$^{A/U\text{-rich}}$ were made, referred to as pREN-halfH$^{A\text{-rich}}$ and pREN-halfH$^{U\text{-rich}}$, from which the U- respectively A-rich part was lacking (**Figure 5-7**). Furthermore, mutant pREN-halfH$^{A*\text{-rich}}$ was made, containing the A-rich sequences from the reverse complementary strand of the hairpin, i.e. the A-rich sequence part of the predicted hairpin structure of the NSs gene transcript.

Analyses of these constructs on luciferase expression interestingly revealed a two fold increase of luciferase activity with mutants pREN-halfH$^{A\text{-rich}}$ and pREN-halfH$^{A*\text{-rich}}$ compared to the plasmid containing the entire hairpin structure sequence (**Figure 5-7**), while no activity was measured with mutant pREN-halfH$^{U\text{-rich}}$ (**Figure 5-7**). Transcriptional expression of all REN sensor constructs was verified and observed to be similar based on semi-quantitative RT-PCR (data not shown). These findings supported the possibility that the A-rich sequence within the hairpin structure sequence could assist in translation by mimicking a poly(A) tail.

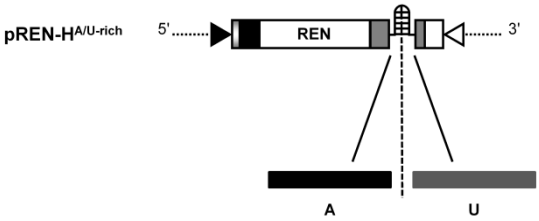

Figure 5-6 Localization of the A- and U-rich part within the predicted hairpin structure sequence.

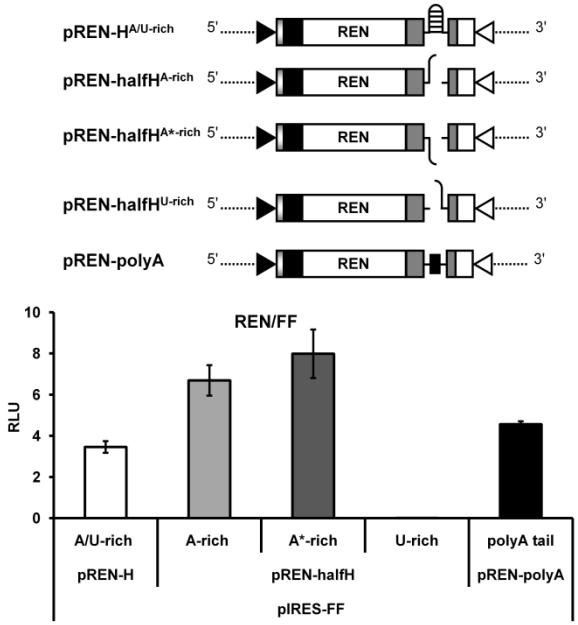

Figure 5-7 **Analysis of the A- and U-rich part of the predicted hairpin structure sequence in translation.** BHK-21 cells were infected with vv-T7 and co-transfected with 100 ng of pREN sensor constructs (pREN-H$^{A/U\text{-}rich}$, pREN-halfH$^{A\text{-}rich}$, pREN-halfH$^{A*\text{-}rich}$, pREN-halfH$^{U\text{-}rich}$, or pREN-polyA) and 0.5 ng of pIRES-FF as internal control. Relative luciferase expression was measured after 23 h post transfection. Error bars show the standard deviations from the means of three replicate experiments.

Requirement of viral (N and NSs) proteins in translation of hairpin structure sequence containing sensor constructs

While previous experiments indicated an important role of the TSWV hairpin structure sequence in translation, although do not rule out a poly(A)-tail mimicking effect either, the requirement of the viral N and NSs proteins in translation was tested. To this end, the hairpin luciferase constructs pREN-H$^{A/U\text{-}rich}$, pREN-halfH$^{A\text{-}rich}$, and pREN-polyA were expressed in BHK21 cells in the absence or presence of expression constructs pTUG-NSs and/or pTUG-N. As a negative control, a MBP (Maltose binding protein) expression construct was included. As another negative control from which no protein would become expressed, pUC19 vector DNA was used as MOCK. Whereas no change in luciferase activity was observed when the N or NSs gene constructs were added individually (450 ng each), a clear increase was observed in the presence of both proteins (450 ng expression constructs each) (**Figure 5-8A**). When the N and NSs gene constructs were added in a higher concentration (700 ng each), translation was even more strongly enhanced (**Figure**

5-8B). In all samples MBP, N, and NSs protein could be detected at 23 hours of post transfection (**Figure 5-8C**).

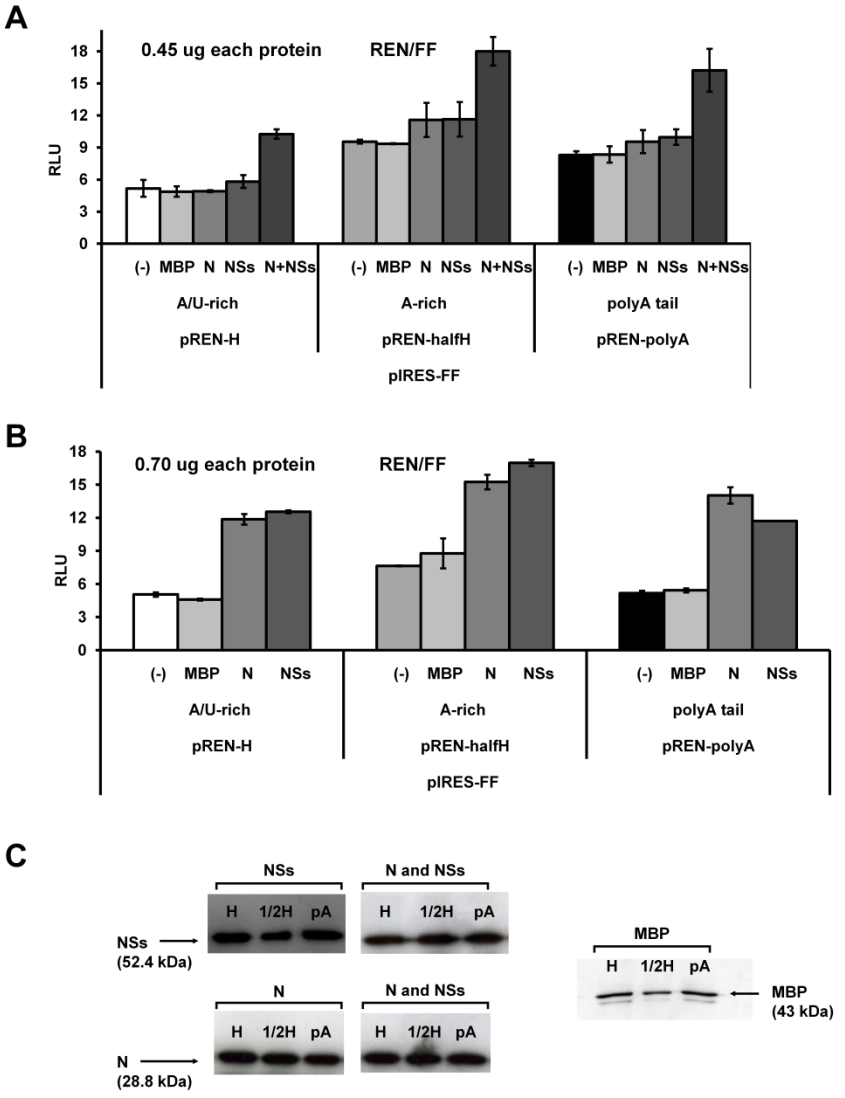

Figure 5-8 **Influence of N and NSs on translation.** BHK-21 cells were infected with *Vaccinia virus* and co-transfected with 100 ng of expression vectors encoding REN luciferase, FF luciferase and MBP, N, NSs, combination of N and NSs, or pUC19 at the amount of 450 ng (A) and 700 ng (B). Luciferase expression was measured 23 h post transfection. The relative luciferase expression is shown, corrected for the internal FF control (REN/FF). (C) Cells were analyzed for expression of MBP, N, or NSs by Western blotting and using antisera specific for MBP, N or NSs respectively. Abbreviation: MBP, Maltose binding protein; N, nucleoprotein; NSs, non-structural protein; H, hairpin; ½H, half hairpin; pA, polyA.

Discussion

The mRNAs from the majority of segmented (-) ssRNA viruses are not polyadenylated as common eukaryotic mRNAs, but instead feature translation enhancing 3' UTRs. In general, the mechanism that allows these viral mRNAs to be efficiently translated in infected cells is poorly understood. In this study, we provide evidence that the 3' hairpin structure sequence of TSWV S RNA-encoded mRNAs constitutes a translation enhancer that mediates efficient translation in the absence of a common poly(A) tail. Substitutions and deletions at the 3' end, as shown in **Figures 5-2 to 5-7**, strongly suggest that the hairpin structure sequence is required for translation. Deletion of the hairpin sequence resulted in diminished translation rates (**Figure 5-2**), while its substitution for another slightly different hairpin encoding sequence, i.e. from its reverse complement or from another distinct tospovirus, still supported translation. The lower levels of translation in case of a REN sensor construct with a hairpin encoding sequence from the reverse complement and its recovery by complementation with the corresponding homologous 5' UTR implies a concerted action, i.e. interaction, of the 5' and 3' UTR (**Figure 5-4**). Due to the possibility to exchange the hairpin structure sequence among different tospoviruses indicates that these viruses share a common protein expression strategy where the hairpin structure rather than particular sequences might be the key feature in translation.

While the TSWV S RNA specific mRNAs are shown to contain the entire hairpin structure sequence at their 3' end (***van Knippenberg et al., 2005b***), stable secondary hairpin structures are predicted at the IR of both highly AU-rich ambisense S and M RNA segments (**Figures 5-3B and 5-9**). It is not unlikely that these structures are also involved in mRNA transcription termination similar to what has been described for other ambisense RNA viruses. For *Tacaribe arenavirus*, it was shown that transcription termination is related to a small stable GC-rich hairpin structure and independent of a specific nucleotide sequence (***Iapalucci et al., 1991; Lopez and Franze-Fernandez, 2007***). A termination mechanism based on formation of a hairpin structure is also suggested for *Lymphocytic choriomeningitis arenavirus* (LCMV) (***Meyer and Southern, 1993***). Transcription termination of LCMV mRNAs has been suggested to be reminiscent of rho-independent transcription in prokaryotes, in which termination also occurs at several positions 3' of a GC-rich hairpin structure (***Platt, 1986; Reynolds et al., 1992; Reynolds and Chamberlin, 1992***). For the *Uukuniemi phlebovirus* transcription termination of the S RNA specific mRNAs was reported to similarly occur 3' of an AU-rich intergenic hairpin structure (***Simons and Pettersson, 1991***). On the other hand, the 3' ends of the mRNAs of *Punta toro phlebovirus* seemed to map near to the top of a 200 base-pair predicted AU-rich hairpin structure (***Emery and Bishop, 1987***).

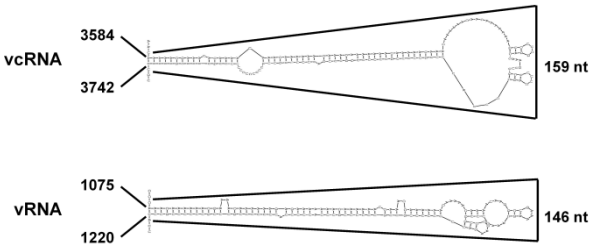

Figure 5-9 **RNA folding predictions of TSWV M segment**. Mfold predictions of the highly AU-rich IR in the vRNA flanking the 3' end of the NSm ORF, and the analogous sequence in the vcRNA flanking the 3' end of the G precursor ORF. Abbreviation: vRNA, viral sense RNA; vcRNA, viral complementary RNA.

The overall efficiency in terms of transcription and translation by the TSWV hairpin constructs raises the question of why the viral segments carry sequences that apparently can fold into structures while the transcripts can also do with the A-rich stretch only (**Figure 5-7**). Although a clear answer to this question has not been provided, one can speculate that such a structure provides stability of the mRNAs for exonuclease cleavage. Considering the fact that among the ambisense RNA segments from the bunyaviruses (genus *Phlebovirus* and *Tospovirus*) and arenaviruses, besides AU-rich also GC-rich hairpin folding structures are found involved in transcription termination and present in viral transcripts, strongly supports the idea that the RNA folding structure is the primary element required for translation and in which the A-rich stretches from AU-hairpin folding structures can additionally support translation in a concerted poly(A) tail binding protein (PABP)-dependent manner. For cellular mRNAs harboring a common PABP supports circularization and thereby i) stabilizes the ribonucleoprotein complex involved in translation initiation, ii) helps protect the mRNA from decay, iii) mediates ribosome recycling, and/or iv) promotes preferential translation of full-length messages (***Bailey-Serres, 1999; Borman et al., 2000; Gallie, 1998, id. 2002***). For the ambisense arenaviruses and bunyaviruses some or all of these activities may be facilitated by a hairpin folding structure, as a functional equivalence to the poly(A) tail. During such translation strategy, the hairpin structure may assists in circularization of the mRNA through specific binding of a viral proteins for efficient translation, prior to the sequential recruitment of additional translation initiation factors to the 5' end of the mRNA. Functional replacement of a poly(A) tail-PABP complex has been reported for many plant-infecting RNA viruses, e.g. *Tobacco mosaic virus* and *Alfalfa mosaic virus* (***Dreher and Miller, 2006; Fabian and White, 2004; Gallie, 1998, id. 2002; Gallie and Kobayashi, 1994; Leonard et al., 2004; Matsuda and Dreher, 2004; Meulewaeter et al., 2004; Neeleman et al., 2001***). For TSWV, such model is supported by the observation that the viral NSs and N proteins enhanced the

translation of REN sensor constructs containing a 3' hairpin structure. In this process the N and NSs proteins may likely act as a functional analog of PABP. The involvement of NSs in translation is not completely surprising, since the NSs proteins of several bunyaviruses have previously been implied to play some role in translation (***Blakqori et al., 2009; Blakqori and Weber, 2005; Bridgen et al., 2001; Di Bonito et al., 1999; Simons et al., 1992***). Since the TSWV NSs protein has recently been shown to exhibit affinity to short (si- and mi-) and long double stranded (ds)RNA (***Schnettler et al., 2010***), it is tempting to hypothesize that NSs binds to the hairpin structure to prevent its recognition and subsequent degradation in plants by RNaseIII dicer-like (DCL) proteins, while simultaneously supporting translational enhancement of viral transcripts by circularization. The involvement of the viral N protein in translational enhancement is more intriguing and so far has only been reported similarly for *Hantaan bunyavirus* N protein (***Mir and Panganiban, 2008***). Although speculative, the N protein could be required in binding to the 5' cap-structure to prevent decapping of the mRNA by decapping enzymes and thereby stabilizing viral transcripts. This hypothesis is being supported by the affinity of *Hantaan bunyavirus* N for cap-structures in cytoplasmic processing bodies (P bodies), discreet cytoplasmic foci which serve in mRNA degradation as well as cellular storage sites for mRNA (***Balagopal and Parker, 2009; Brengues et al., 2005; Mir et al., 2008***).

The observation that only the A-rich part of the hairpin structure suffices for translation, even leaves the possibility that the AU-rich hairpin structures rely on a concerted action of viral proteins and PABP. A concerted action (interaction) would also explain why both N and NSs proteins, vice versa, stimulate translation of REN sensor constructs containing a poly(A) tail. A concerted action of viral proteins and PABP in translational enhancement is not unique as this has been earlier reported for influenza virus, where the viral NS1 protein was found to interact with PABP and also with elF4G (eukaryotic translation initiation factor) (***Aragón et al., 2000; Burgui et al., 2003; Marión et al., 1997; Qiu and Krug, 1994; Salvatore et al., 2002; Yángüez and Nieto, 2011***). Whether PABP indeed plays an (essential) role during translation of TSWV transcripts from the ambisense S and M RNA segments remains to be analyzed, e.g. by performing translation studies in a PABP-knock down environment.

Materials and methods

Viruses

Recombinant *Vaccinia virus* MVA-T7 (attenuated *Vaccinia virus* containing a copy of the T7 RNA polymerase gene) (***Sutter et al., 1995; Wyatt et al., 1995***) was used for T7 RNA polymerase driven expression of cDNA constructs. TSWV (***De Avila et al., 1992***) was propagated in *Nicotiana benthamiana* plants. Inoculum to infect plants was obtained by grinding systemically infected plant leaves in a 0.5% Na_2SO_3 solution. The inoculum was applied by softly rubbing the leaves using carborundum powder.

Construction of plasmids

To investigate the role of the TSWV hairpin structure in translation initiation, different constructs were made. TSWV constructs were generated from plasmid pTOS-S Dual (***Duijsings, 2001***), by using PCR amplification and cloning procedures. Briefly, this plasmid consists of a full-length DNA copy of the viral complementary strand of the ambisense S RNA cloned into a pUC19 plasmid containing a T7 promoter-terminator cassette. Luciferase marker genes were added by replacing the N and NSs genes with the REN and FF luciferase gene respectively to allow sensitive detection and quantification of translational activity.

pREN-$H^{A/U\text{-}rich}$, pREN-NoH and pREN-polyA were PCR amplified from pTOS-S Dual by using primer TSWV S-hepδ to remove the NSs-FF ORF and primers TSWV S-H, TSWV S-NoH and TSWV S-pA respectively. Deletion of the NSs-FF ORF from the expression plasmids allowed the use of the FF luciferase pIRES-FF vector as internal control. To further mimic the authentic transcripts, all expression plasmids were re-amplified by using primers AlMV3-N-Fr and T7-AlMV3-Rv in order to add an AlMV3 RNA 3 leader immediately after the T7 promoter.

Amplified PCR fragments were separated by electrophoresis in a 1% agarose gel and purified using the GFX PCR DNA and Gel Band purification kit (GE Healthcare, Buckinghamshire, UK). Purified products were restriction enzyme digested with *Msc*I and re-ligated using T4 DNA ligase (Invitrogen). The nucleotide sequence of individual clones was verified by the dideoxynucleotide chain termination method (***Sanger et al., 1977***) using standard M13 sequencing primers and ultra-high throughput (ABI Prism 3700) DNA sequencer (Greenomics™, Wageningen University and Research Centre; The Netherlands).

A reverse complementary hairpin sequence of pREN-$H^{A/U\text{-}rich}$, referred to as pREN-$H^{A/U*\text{-}rich}$, was made as follows. pREN-$H^{A/U\text{-}rich}$ was re-amplified with primers H-*Spe*I-Fr and H-*Nco*I-Rv. A second amplification of pREN-$H^{A/U\text{-}rich}$ was performed with primers Rluc*Spe*I-Fr and Rluc*Nco*I-Rv to obtain the appropriate vector for inserting the reverse

complement hairpin. Both hairpin and vector were *Spe*I/*Nco*I digested, purified and subsequently ligated together. For the TYRV hairpin construct (pREN-TYRV H), the hairpin sequence of TYRV was PCR amplified using primers TYRV-Fr and TYRV-Rv. The fragment obtained was ligated into the backbone of *Spe*I/*Nco*I digested and purified pREN-H $^{A/U*\text{-rich}}$. A mutant of pREN-H$^{A/U\text{-rich}}$ (pREN-halfH$^{A\text{-rich}}$) from which the U-rich part of the hairpin was removed, was made by *Msc*I digestion of plasmid pREN-H$^{A/U\text{-rich}}$, GFX purification and re-ligation. In a similar way, mutant pREN-halfH$^{A*\text{-rich}}$ was made by *Msc*I digestion of pREN-H$^{A/U*\text{-rich}}$. To obtain pREN-halfH$^{U\text{-rich}}$**,** the U-rich fragment of the hairpin was excised from pREN-H$^{A/U\text{-rich}}$ by *Msc*I digestion, and after purification subsequently ligated into *Msc*I digested pREN-NoH.

For analysis of the 5' UTR, the luciferase plasmids pREN-H$^{A/U\text{-rich}}$, pREN-H$^{A/U*\text{-rich}}$, and pREN-polyA were modified. Therefore, the plasmid-specific 5' UTR (148 nucleotides) of the N-REN ORF was removed by PCR re-amplification using primers UTRdel-Fr and UTRdel-Rv. The resulting constructs were referred to as pREN-H$^{A/U\text{-rich}}$ Δ5'UTR, pREN-H$^{A/U*\text{-rich}}$ Δ5'UTR, and pREN-polyA Δ5'UTR and expressed a luciferase transcript without 5' UTR. For exchanging the 5' N UTR with the 5' NSs UTR, pREN-H$^{A/U\text{-rich}}$, pREN-H$^{A/U*\text{-rich}}$, and pREN-polyA were re-amplified with primer NSsUTR-Fr and NSsUTR-Rv harboring part of the 88 nt 5' NSs UTR. These modified REN constructs were referred to as pREN-H$^{A/U\text{-rich}}$ 5'NSsUTR, pREN-H$^{A/U*\text{-rich}}$ 5'NSsUTR, and pREN-polyA 5'NSsUTR.

The ORF coding for the TSWV N protein was RT-PCR amplified using primers pN-Fr and pN-Rv and cloned into the *Bam*HI site of pTUG3, resulting in plasmid pTUG-N. The ORF for the TSWV NSs protein was cloned into pTUG3 as a *Bam*HI fragment from pAc33DZ1/NSs (***Kormelink et al., 1991***), resulting in pTUG-NSs. Additionally, the MBP ORF was PCR amplified from a donor vector with primers MBP-Fr and MBP-Rv and cloned into pTUG3 as a *Bam*HI fragment. Plasmid pUC19 was used as a MOCK in the experiments involving the N and NSs proteins. All constructs were checked by restriction analysis and sequencing. Primer sequences are listed in **Table 5-1**.

Cell culture and infection/transfection

BHK-21 cells were maintained as a monolayer in Glasgow MEM (GMEM; Gibco® Invitrogen, Breda, The Netherlands), supplemented with 1× Tryptose phosphate broth solution (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands), 10% FBS, 100 U/ml·penicillin, and 100 µg/ml streptomycin. The cells were cultured in a 5% CO_2 atmosphere at 37 C.

Cells grown in serum-free GMEM were inoculated at 37°C with *Vaccinia virus* at a multiplicity of infection (m.o.i.) of 10. After 1 hour post infection, the medium was removed and the cells washed once with serum-free GMEM. Transfection of cells was performed with indicated amounts of plasmid DNA by using Lipofectin transfection

reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. The transfection performed for 5 h at 60-70% confluence in 24-well plates.

Protein isolation and luciferase reporter gene assays

Protein extracts were isolated using passive lysis buffer (Promega) in accordance with the manufacturer's protocol. Luciferase expression levels were determined 23 hours after transfection, using the Dual-Luciferase® Reporter Assay (Promega). Luciferase Reporter assays were carried out by co-transfecting vaccinia infected cells with plasmids containing REN luciferase and FF luciferase under the control of T7 promoter. The transfection with the FF luciferase served as control and the luciferase activity was detected using a TD 20/20 Luminometer (Turner Design) of Promega. The relative light unit (RLU) was calculated for each sample by normalization of the REN luciferase against the FF luciferase levels.

TABLE 5-1

Primer sequences.

Name	Primer sequence (5'→3')
TSWV S-hepδ	CCCCTGGCCAGCTCTGGCCGGCATGGTCCC
TSWV S-H	CCCCTGGCCACTCTTTCAAATTCCTCCTGTC
TSWV S-NoH	CCCCTGGCCAGCACAACACACAGAAAGCAAAC
TSWV S-pA	CCCCTGGCCAT(40)GCACAACACACAGAAAGCAAAC
AlMV3-N-Fr	CATTTTCAGAGCAATCGTGTCAATTTTGTGTTC
T7-AlMV3-Rv	GTATTAATACTATAGTGAGTCGTATTAGGATCCCCGG
H-SpeI-Fr	ACTAGTTAACAAAAACAACGAAAACAAAAAATAAATA
H-*Nco*I-Rv	CCATGGTAGTAGAAACCATAAAAACAAAAAATAAAAATG
RlucSpeI-Fr	CCGGACTAGTGACAGGAGGAATTTGAAAGAG
Rluc*Nco*I-Rv	CATGCCATGGTTTTATTATTTATTAAGCACAACACAC
TYRV-Fr	CCGGACTAGTTGTAAGTCAGTAATAAAGGAGTACTAGTTTAGA
TYRV-Rv	CATGCCATGGATCAGTGTGTTTAAGTTTTATCTGTCTATCA
UTRdel-Fr	ACGAGCTAGCCACCATGACT
UTRdel-Rv	CGGCTAGCGAAAATGGTATTAATACTATAGTGAGTCGT
NSsUTR-Fr	AGAAAATCACAATACTGTAATAAGAACACAGTACCAATAACCA GCTAGCCACCATGACTTCGA
NSsUTR-Rv	AAGTGAGGTTTGATTATGAACAAAATTCTGACACAATTGCTCTG AAAATGGTATTAATACTATAGTG
pN-Fr	CCCGGATCCATGTCTAAGGTTAAGCTCACTAAGG
pN-Rv	CCCGGATCCTCAAGCAAGTTCTGCGAGTTTTG
MBP-Fr	CGGGATCCATGAAAATCGAAGAAGGTAAACTG
MBR-Rv	CGGGATCCCTAGGATCCGAATTCTGAAATCCT

Western blot analysis

Protein expression was characterized after plasmid transfection by Western blot analysis. Lysate prepared for the Dual-Luciferase® Reporter Assay was separated by 12% SDS/PAGE gel. After electrophoresis, proteins were semi-dry blotted to Immobilon-P transfer membranes (Millipore Corporation, Bedford, USA) using a Bio-Rad Mini-PROTEAN system. For MBP, a specific MBP-rat primary and goat alkaline phosphotase conjugated secondary antibody were used. The protein-IgG complexes were visualized with NBT-BCIP as substrate (Roche) according to the manufacturers' procedures. For visualization of viral proteins, specific antibody for the N or NSs protein was used conjugated to alkaline phosphotase and CSPD as substrate (Roche) according to the manufacturer's procedures.

Folding predictions

The RNA folding prediction was performed using MFold 3.5 (***Zuker, 2003***). The most energetically stable secondary structure is shown.

Acknowledgements

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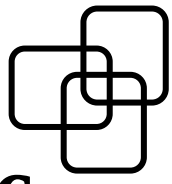
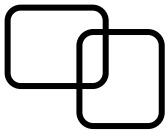
"*When a question has no correct answer, there is only one honest response.*
The grey area between yes and no.
Silence."

Dan Brown, "THE DA *V*INCI CODE".



Chapter 6



General Discussion

In this Thesis, *cis*- and *trans*-acting factors involved in transcription/translation of segmented, negative (-) single-stranded (ss) RNA viruses were studied with specific emphasis on two issues. The first one aimed to analyze whether similar cap donor requirements applied during influenza A genome transcription initiation (cap-snatching) relative to what has earlier been discovered for the plant-infecting *Tomato spotted wilt virus* (TSWV) (**Chapters 2, 3 and 4**). The second one aimed to analyze the involvement of 5' and 3' untranslated regions (UTR), the latter containing a predicted hairpin structure sequence, of TSWV mRNAs in transcription/translation (**Chapters 5**).

During influenza transcription initiation studies, novel insights into the cap-snatching mechanism have been provided by *in vitro* and *in cell* studies (**Chapters 2, 3 and 4**). The viral transcriptase seems to prefer capped host mRNA leaders with increasing base complementarity to the vRNA template. The results obtained led to the proposed model for influenza transcription initiation as schematically presented in **Figure 6-1**. This model proposes that the viral polymerase first selects capped leaders of the host mRNA according to (extensive) base-pairing to the vRNA template. After selection the polymerase bound to the cap structure of a capped RNA leader (further onwards referred to as cap donor molecule), cleaves the desired leader 10-12 nucleotides downstream the cap structure. Finally, the remaining capped leader base pairing with the viral RNA is elongated to give rise to a full-length viral mRNA. The same criteria for cap donor selection have previously been demonstrated for TSWV (***Duijsings et al., 2001; van Knippenberg et al., 2005a***), suggesting that the proposed model most likely applies to all members of the segmented (-) ssRNA viruses. The finding that multiple base-pairing promotes cap donor selection and the possibility to use such leaders as model molecules for antiviral drug design will be further discussed in this chapter.

It is now well established that poly(A) tails of eukaryotic mRNAs regulate both mRNA turnover and translation. Transcripts of segmented (-) ssRNA viruses lack a common poly(A) tail, and thus have likely developed mechanisms to generate an alternative structure or modifications to the 3' mRNA end that act as a substitute for a poly(A) tail. Viral transcripts from the bunyavirus TSWV have been well characterized and the 3' end of the S RNA encoded gene transcripts were shown to contain a UTR sequence predicted to fold into a hairpin structure. Translation studies on a large set of REN-sensor constructs containing (partial) deletions or nucleotide changes within this 3' UTR but also the 5' UTR sequence, have indicated an important role for the hairpin structure in translation (**Chapter**

5). This process seems to require a concerted action with the viral N and/or NSs protein as a kind of PABP-analog, in which an additional enhancement by PABP after binding to the A-rich sequence of the hairpin structure can also not be excluded. However, most of the RNA segments from the segmented (-) ssRNA viruses, including the bunyaviruses, are of entire negative polarity and features required for translational enhancement of their transcripts are still unknown. In this chapter the results from our translational analysis of TSWV ambisense encoded gene transcripts will be discussed in light of a model for translation of transcripts from (-) RNA segments that seemingly lack a hairpin folding structure.

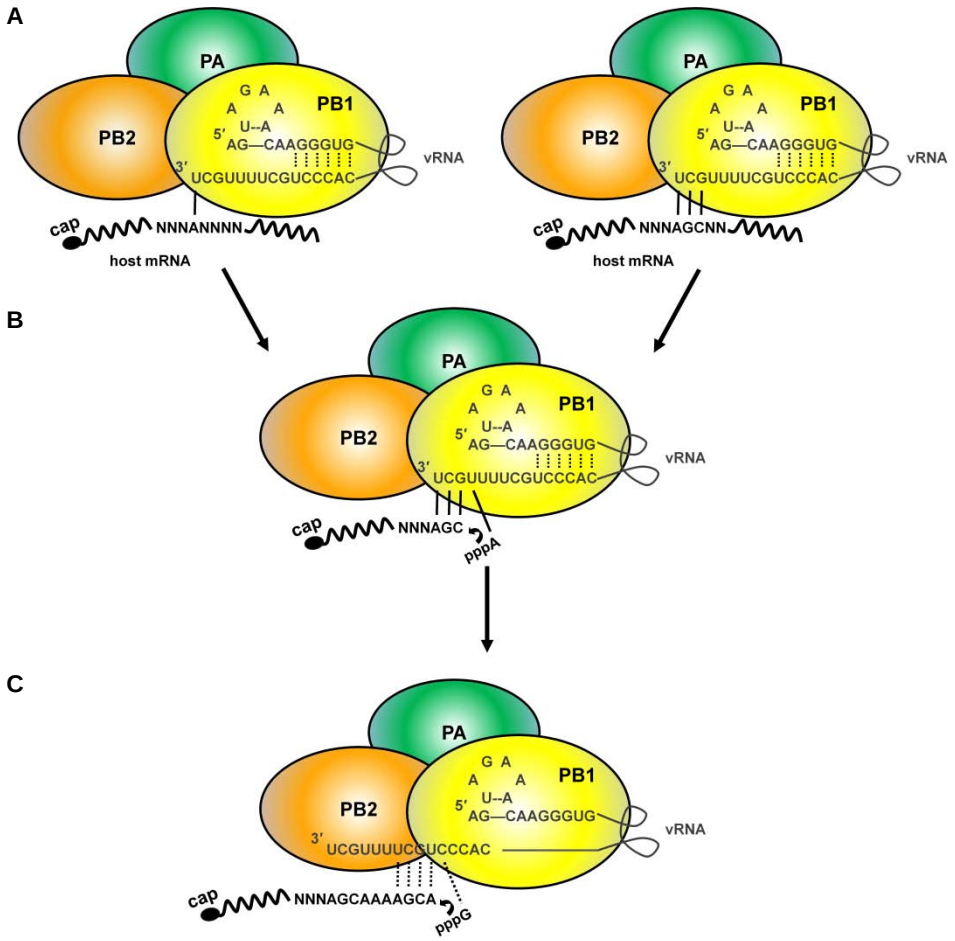


Figure 6-1 Proposed model for influenza virus cap-snatching. (A) Selection between leaders with increasing base-pairing versus a singly residue/-s to the vRNA template. (B) Endonuclease cleavage of the capped RNA leader. (C) Elongation for viral mRNA synthesis.

Transcription initiation of segmented (-) ssRNA viruses: a common mechanism

Segmented (-) ssRNA viruses, both animal- and plant-infecting, share a common mechanism for transcription initiation in which the synthesis of their mRNAs is initiated by cap-snatching. Based on our observations a model is proposed that applies to all segmented (-) ssRNA viruses (**Figure 6-1**). Data from previous transcription initiation studies can now be reinterpreted in the light of our proposed model.

TABLE 6-1

Sequence of the 3' vRNA terminus from members of the segmented (-) ssRNA viruses.

Virus	3' end sequence of vRNA	References
Influenza A	3'-***UCG***UUUU…	Lamb and Horvarth, 1991; Shaw and Lamb, 1984
Influenza B	3'-***UCG***UCUU…	Lamb and Horvarth, 1991; Shaw and Lamb, 1984
Influenza C	3'-***UCG***UCUU…	Lamb and Horvarth, 1991
THOV	3'-***UCG***UUUU…	Albo *et al.*, 1996; Leahy *et al.*, 1997
HTNV	3'-***AUC***AUCU…	Garcin *et al.*, 1995
DUGV	3'-***AGA***GUUU…	Jin and Elliott, 1993b
SSHV	3'-***UCA***UCAC…	Bishop *et al.*, 1983
GERV	3'-***UCA***UCAC…	Bouloy *et al.*, 1990
LACV	3'-***UCA***UCAC…	Dobie *et al.*, 1997
BUNV	3'-***UCA***UCAC…	Jin and Elliott, 1993a
RVFV	3'-***ACA***CAAA…	Collett, 1986
UUKV	3'-***UGU***GUUU…	Simons and Pettersson, 1991
TSWV	3'-***UCU***GCUU…	Duijsings *et al.*, 2001
TACV	3'-***GCG***UGUC…	Garcin and Kolakofsky, 1990
LCMV	3'-***GCG***UG(U/G)C…	Meyer and Southern, 1993

Viral residues potentially base-pairing with the 3' residues of RNA leader are in **bold** and *italics*. Virus abbreviation: BUNV, *Bunyamwera virus*; DUGV, *Dugbe virus*; GERV, *Germiston virus*; HTNV, *Hantaan virus*; LACV, *La Crosse virus*; LCMV, *Lymphocytic choriomeningitis virus*; SSHV, *Snowshoe hare virus*; RVFV, *Rift Valley fever virus*; TACV, *Tacaribe virus*; THOV, *Thogoto virus*; TSWV, *Tomato spotted wilt virus*; UUKV, *Uukuniemi virus*.

In contrast to all other segmented (-) ssRNA viruses, *Thogoto orthomyxovirus* (THOV) mRNAs are thought to lack any host-derived leader sequences, and only the cap structure is used for initiation of viral mRNA synthesis (**Table 6-1 and 6-2**) (***Albo et al., 1996; Leahy et al., 1997***). However, there is no evidence that excludes snatching of a larger capped RNA leader that, by virtue of base-pairing, primes THOV transcription. In light of our model, it is now not only tempting but also very possible that a very small leader

sequence is snatched with 1-3 nt complementary to the first vRNA template residues, like in the case of arenaviruses (**Table 6-1 and 6-2**). The 5' capped RNA leaders of the *Lymphocytic choriomeningitis arenavirus* (LCMV) S mRNAs (***Meyer and Southern, 1993***) and the ones from another arenavirus, *Tacaribe virus* (TACV) (***Garcin and Kolakofsky, 1990, id. 1992***) were found to be heterogeneous in sequence composition. For LCMV the leaders were reported to range from 1 to 7 nt while for TACV they ranged from 1 to 4 nt (**Table 6-2**). Based on our (universal) model, base-pairing now likely occurs between the capped leader and the vRNA template for arenaviruses as well, which implies that the actually snatched leader sequences are most likely 1 to 3 residues larger in size. The snatched RNA leader sequence would then prime transcription by base-pairing at the first G residue at position +1 from the 3' end of the vRNA template (3'-G…), the first two GC residues (3'-GC…) or the first three (3'-GCG…) residues (**Table 6-1 and 6-2**).

TABLE 6-2

Leader sequences at the 5' ends of the indicated mRNA reported as host-derived for *Thogoto orthomyxovirus* and arenaviruses, and postulated to be host-derived according to the current model of cap-snatching.

5' leader sequence of the indicated virus mRNA		Virus	Segment
Reported	Current model		
5'-capAGCAAAA	5'-cap***AGC***AAAA	THOV	3, 4, 5
5'-…(A/U/G)CGCAC(A/C)G	5'-…(A/U/G)***CGC***AC(A/C)G	LCMV	S
5'-…(A/U/G)CGCACAG	5'-…(A/U/G)***CGC***ACAG	TACV	S

Indicated virus sequence is underlined, while residues base-pairing to the viral template and with possible origin from the RNA leader are in **bold** and *italics*; the non-viral 3'-terminal nucleotide at position -1 relative to the first viral residue is shaded. The corresponding RNA segment from which the transcript derived is also indicated. The table is based on data given for arenaviruses (***Garcin and Kolakofsky, 1990; Meyer and Southern, 1993***) and thogotoviruses (***Albo et al., 1996; Leahy et al., 1997***). Virus abbreviation: LCMV, *Lymphocytic choriomeningitis virus*; TACV, *Tacaribe virus*; THOV, *Thogoto virus*.

During our *Influenza A virus* transcription analysis, the phenomena of internal priming, prime-and-realignment, and combination of both were also observed (**Chapters 2, 3 and 4**). For *Influenza B virus*, in contrast to *Influenza A virus*, a preference for leaders with a 3' CA-terminus was anticipated (**Chapter 4**). However, in light of the findings for *Influenza A virus*, and our proposed model for cap-snatching, almost 50% of the published 5' non-viral leader sequences from *Influenza B virus* (**Chapter 4, Table 4-10**), most likely have resulted from internal priming of a G-terminated leader on the 3'-penultimate C residue of the vRNA template. After extension for two nucleotides and subsequent realignment by virtue of base-pairing a repetitive sequence (GCAGC) is obtained, which reflects the other 50% of the leader sequences reported (**Table 6-1 and 6-3**). The

occurrence of internal priming and consecutive re-alignment would not only reject the preference for a 3' CA-terminus but fully match the reported *Influenza B virus* leader sequences to the requirements as determined from our *in vitro* and *in vivo* influenza A studies (**Chapter 3 and 4**).

TABLE 6-3

Leader sequences at the 5' ends of *Influenza B virus* mRNA reported as host-derived and postulated to be host-derived according to the current model of cap-snatching.

5' leader sequence of *Influenza B virus* mRNA		IP	PR
Reported	Current model		
5'-…GAGCAGAA	5'-…G***AGC***AGAA		
5'-…UGCAGCAGAA	5'-…U***GC***AGCAGAA	+	+
5'-…UGCAGCAGAA	5'-…U***GC***AGCAGAA	+	+
5'-…UGCAGCAGAA	5'-…U***GC***AGCAGAA	+	+
5'-…UGCAGCAGAA	5'-…U***GC***AGCAGAA	+	+
5'-…GGCAGCAGAA	5'-…G***GC***AGCAGAA	+	+
5'-…GGCAGCAGAA	5'-…G***GC***AGCAGAA	+	+
5'-…CGCAGCAGAA	5'-…C***GC***AGCAGAA	+	+
5'-…CGCAGCAGAA	5'-…C***GC***AGCAGAA	+	+
5'-…CGCAGCAGAA	5'-…C***GC***AGCAGAA	+	+
5'-…GGCAGAA	5'-…G***GC***AGAA	+	
5'-…GGCAGAA	5'-…G***GC***AGAA	+	
5'-…UGCAGAA	5'-…U***GC***AGAA	+	
5'-…UGCAGAA	5'-…U***GC***AGAA	+	
5'-…CGCAGAA	5'-…C***GC***AGAA	+	
5'-…CGCAGAA	5'-…C***GC***AGAA	+	
5'-…CGCAGAA	5'-…C***GC***AGAA	+	
5'-…CGCAGAA	5'-…C***GC***AGAA	+	
5'-…CGCAGAA	5'-…C***GC***AGAA	+	

Influenza sequence is underlined, while residues base-pairing to the viral template and with possible origin from the RNA leader are in **bold** and *italics*; the non-viral 3'-terminal nucleotide at position -1 relative to the first viral residue is shaded. The proteins encoded by the cloned segments are also indicated. Abbreviations: IP, Internal Priming; PR, Prime-and-Realign.

Most of our knowledge on the transcription and replication processes of the influenza virus genome has been derived from studies carried out on type A viruses and some on type B (***Lamb and Krug, 2001***). Based on the properties of the RNA-dependent RNA polymerase activity associated with influenza C virions (***Nagele and Meier-Ewert, 1984; Yamashita et al., 1989***) and our findings on influenza transcription initiation, it is

very likely that transcription and replication of the *Influenza C virus* follow the same rules and criteria as determined for *Influenza A* and *B viruses*. Since *Influenza A, B and C viruses* all share a common highly conserved 3'-terminal sequence (**Table 6-1**), priming of transcription would thus require similar residues at the 3'-terminal end of capped RNA leaders for all these viruses. The proposed formula describing the sequence of preferred capped RNA leaders for *Influenza A virus*, would thereby apply to all influenza (A/B/C) viruses.

Another look at published non-viral leader sequences from the animal-infecting bunyaviruses reveals that these also fit within the cap-snatching model postulated (**Table 6-4 and 6-5)**. Based on the non-viral leader sequences from S RNA-specific transcripts of *Germiston bunyavirus*, the snatched capped RNA leaders were initially proposed to possess a preference for a 3' U residue. This residue appears at position -1, i.e. the base 5' of the first viral A residue (**Table 6-4**), within the viral transcript sequence (***Bouloy et al., 1990***). In light of the proposed model and the possibility for internal priming and prime-and-realign, this virus seems to favor leaders with a G, C or U at position -1 (**Table 6-4**). In addition, the ultimate 3' residue of the capped RNA leader for *Germiston virus* transcription initiation, likely contains (at least) a single nucleotide residue (A) to support base-pairing at the 3'-ultimate viral template residue (**Table 6-4**). In a similar way, a re-evaluation of the cloned non-viral leader sequences from other animal-infecting bunyavirus transcripts reveals that all of these can be explained to result from priming on the ultimate 3' residue of the vRNA template, or by internal priming or a prime-and-realign (**Table 6-5**).

TABLE 6-4

Leader sequences at the 5' ends of *Germiston virus* mRNA reported as host-derived and postulated to be host-derived according to the current model of cap-snatching.

5' leader sequence of *Germiston virus* mRNA		# clones	IP	PR
Reported	Current model			
5'-…GAGUAGUAGUG	5'-…G***AGU***AGUAGUG	2/19		+
5'-…CAGUAGUAGUG	5'-…C***AGU***AGUAGUG	1/19		+
5'-…GGUAGUAGUG	5'-…G***GU***AGUAGUG	4/19	+	+
5'-…UGUAGUAGUG	5'-…U***GU***AGUAGUG	1/19	+	+
5'-…CGUAGUAGUG	5'-…C***GU***AGUAGUG	1/19	+	+
5'-…CUAGUAGUG	5'-…C***U***AGUAGUG	2/19	+	+
5'-…UUAGUAGUG	5'-…U***U***AGUAGUG	1/19	+	+
5'-…CAGUAGUG	5'-…C***AGU***AGUG	3/19		
5'-…GGUAGUG	5'-…G***GU***AGUG	1/19	+	
5'-…CGUAGUG	5'-…C***GU***AGUG	2/19	+	
5'-…UGUAGUG	5'-…U***GU***AGUG	1/19	+	

Germiston virus sequence is underlined, while residues base-pairing to the viral template and with possible origin from the RNA leader are in **bold** and *italics*; the non-viral 3'-terminal nucleotide at position -1 relative to the first viral residue is shaded. Abbreviations: IP, Internal Priming; PR, Prime-and-Realign.

TABLE 6-5

Leader sequences at the 5' ends of the indicated virus mRNA reported as host-derived and postulated to be host-derived according to the current model of cap-snatching.

5' leader sequence of the indicated virus mRNA		Virus	# of clones	IP	PR
Reported	Current model				
5'-…CUCUCUCAAA	5'-…C***UCU***CUCAAA	**DUGV**	11/24		+
5'-…GUCUCUCAAA	5'-…G***UCU***CUCAAA		2/24		+
5'-…UUCUCUCAAA	5'-…U***UCU***CUCAAA		1/24		+
5'-…GCUCUCAAA	5'-…G***CU***CUCAAA		3/24	+	+
5'-…ACUCUCAAA	5'-…A***CU***CUCAAA		2/24	+	+
5'-…CCUCUCAAA	5'-…C***CU***CUCAAA		4/24	+	+
5'-…GUCUCAAA	5'-…G***U***CUCAAA		1/24		
5'-…UGUAGUAGUG	5'-…U***GU***AGUAGUG	**SSHV**	1/3	+	+
5'-…GGUAGUG	5'-…G***GU***AGUG		1/3	+	
5'-…CGUAGUG	5'-…C***GU***AGUG		1/3	+	
5'-…CAGUAGUAGUG	5'-…C***AGU***AGUAGUG	**LACV**	3/37		+
5'-…GGUAGUAGUG	5'-…G***GU***AGUAGUG		4/37	+	+
5'-…UGUAGUAGUG	5'-…U***GU***AGUAGUG		1/37	+	+
5'-…CUAGUAGUG	5'-…C***U***AGUAGUG		1/37	+	+
5'-…GAGUAGUG	5'-…G***AGU***AGUG		3/37		
5'-…CAGUAGUG	5'-…C***AGU***AGUG		1/37		
5'-…UGUAGUG	5'-…U***GU***AGUG		21/37	+	
5'-…CGUAGUG	5'-…C***GU***AGUG		1/37	+	
5'-…GGUAGUG	5'-…G***GU***AGUG		1/37	+	
5'-…GGUAGUG	5'-…G***GU***AGUG		1/37	+	
5'-…CAGUAGUAGUAGUG	5'-…C***AGU***AGUAGUAGUG	**BUNV**	1/21		+
5'-…GGUAGUAGUAGUG	5'-…G***GU***AGUAGUAGUG		1/21	+	+
5'-…CGUAGUAGUAGUG	5'-…C***GU***AGUAGUAGUG		1/21	+	+
5'-…CUAGUAGUAGUG	5'-…C***U***AGUAGUAGUG		2/21	+	+
5'-…CAGUAGUAGUG	5'-…C***AGU***AGUAGUG		5/21		+
5'-…GGUAGUAGUG	5'-…G***GU***AGUAGUG		7/21	+	+
5'-…UGUAGUAGUG	5'-…U***GU***AGUAGUG		1/21	+	+
5'-…AUAGUAGUG	5'-…A***U***AGUAGUG		2/21	+	+
5'-…CGUAGUG	5'-…C***GU***AGUG		1/21	+	

TABLE 6-5 – *Continued*

5' leader sequence of the indicated virus mRNA		Virus	# of	IP	PR
Reported	Current model		clones		
5'-…CACACACAAA	5'-…C***ACA***CACAAA	**UUKV**	8/25		+
5'-…UACACACAAA	5'-…U***ACA***CACAAA		1/25		+
5'-…GCACACAAA	5'-…G***CA***CACAAA		3/25	+	+
5'-…UCACACAAA	5'-…U***CA***CACAAA		1/25	+	+
5'-…GACACAAA	5'-…G***ACA***CAAA		1/25		
5'-…AACACAAA	5'-…A***ACA***CAAA		1/25		
5'-…GCACAAA	5'-…G***CA***CAAA		9/25	+	
5'-…UCACAAA	5'-…U***CA***CAAA		1/25	+	
5'-…UGUAGUAGA	5'-…U***G***UAGUAGA	**HTNV**	1/35	+	+
5'-…CGUAGUAGA	5'-…C***G***UAGUAGA		6/35	+	+
5'-…GGUAGUAGA	5'-…G***G***UAGUAGA		3/35	+	+
5'-…CAGUAGUAGA	5'-…C***AG***UAGUAGA		1/35	+	+
5'-…AAGUAGUAGA	5'-…A***AG***UAGUAGA		1/35	+	+
5'-…GGUAGUAGUAGA	5'-…G***G***UAGUAGUAGA		3/35	+	+
5'-…UGUAGUAGUAGA	5'-…U***G***UAGUAGUAGA		6/35	+	+
5'-…AAGUAGUAGUAGA	5'-…A***AG***UAGUAGUAGA		1/35	+	+
5'-…GAGUAGUAGUAGA	5'-…G***AG***UAGUAGUAGA		1/35	+	+
5'-…UUAGUAGUAGUAGA	5'-…U***UAG***UAGUAGUAGA		1/35		+
5'-..GGUAGUAGUAGUAGA	5'-..G***G***UAGUAGUAGUAGA		1/35	+	+
...GAGUAGUAGUAGUAGA	...G***AG***UAGUAGUAGUAGA		2/35	+	+
5'-…CGUAGUAGUAGA	5'-…C***G***UAGUAGUAGA		2/35	+	+
5'-…AUAGUAGUAGA	5'-…A***UAG***UAGUAGA		1/35		+
5'-…CUAGUAGUAGA	5'-…C***UAG***UAGUAGA		1/35		+
5'-…UUAGUAGUAGA	5'-…U***UAG***UAGUAGA		1/35		+
5'-UAGUAGUAGA	5'-***UAG***UAGUAGA		3/35		+

Indicated virus sequence is underlined, while residues base-pairing to the viral template and with possible origin from the RNA leader are in **bold** and *italics*; the non-viral 3'-terminal nucleotide at position -1 relative to the first viral residue is shaded. The table is based on data given for hantaviruses (***Garcin et al., 1995***), nairoviruses (***Jin and Elliott, 1993b***), orthobunyaviruses (***Bishop et al., 1983; Dobie et al., 1997; Jin and Elliott, 1993a***), phleboviruses (***Simons and Pettersson, 1991***). Abbreviations: IP, Internal Priming; PR, Prime-and-Realign. Virus abbreviation: BUNV, *Bunyamwera virus*; DUGV, *Dugbe virus*; HTNV, *Hantaan virus*; LACV, *La Crosse virus*; SSHV, *Snowshoe hare virus*; UUKV, *Uukuniemi virus*.

Dysfunctional capped RNA leaders with increasing base complementarity: possibilities for new antiviral drug design

Viruses, despite their simplicity as pathogens, have evolved highly efficient mechanisms for replication and expression of their genomes to generate progeny virus. In the case of pathogenic avian influenza, its widespread geographical distribution and continuous evolution (genetic drift and shift) requires permanent attention in light of annual vaccine strain selection (WHO) and virus production investments. Also reason for serious concern is the observation that influenza viruses, via mutations, may obtain the capacity to cross species barriers, as observed for the H5N1 virus (poultry and human) (***van Kerkhove et al., 2011***). Although its spread among the human population is still limited, H5N1 may turn into a strain capable of efficient human-to-human transmission in near future. The constant danger for newly emerging variants (Mexican flu 2010) underscores the increasing need and interest for general antiviral compounds.

Current options to limit the clinical impact of influenza virus infections include vaccination and the use of antiviral drugs. Since the current manufacturing capacity for vaccines is insufficient to cope with the demand during a pandemic, the availability of antiviral drugs may help to contain the emerging pandemic virus at its emergence. The efficacy of the two currently available classes of antiviral drugs, neuraminidase inhibitors (e.g. Tamiflu) and ion channel blockers, is limited and heavily dependent of a very early start of treatment. In addition, more and more cases are reported on development of resistance, e.g. against Tamiflu (***Orozovic et al., 2011***).

The observation that a viral capped RNA leader sequence with two to three bases complementary to the 3'-ultimate residues of a (-) ssRNA virus template sequence is preferred over a leader sequence with less base complementarity offers possibilities for development of new antiviral compounds. In **Chapter 3**, a preferred capped RNA leader for *Influenza A virus* was proposed to have the consensus sequence ^{7m}G-(N)$_{7-8}$-(A/U/G)-(A/U)-*AGC*-3' based on the *in vitro* analysis, while based on the additional *in vivo* data (**Chapter 4**) could be expanded to ^{7m}G-(N)$_{7-8}$-(A/U/G)-(A/U/G)-*AGC*-3'. Based on the additional available *Influenza B virus* data, a consensus sequence of this leader and applicable to all influenza viruses, could be represented by the general formula ^{7m}G-(N)$_{7-8}$-(A/U/G)-(U/G)-*AGC*-3'.

In vitro influenza transcription experiments have shown a stronger *in vitro* reduction of α-globin RNA leader initiated transcription by cap donors that fitted the aforementioned formula most close (triple base-pairing) compared to those with a less preferred sequence context (**Chapter 3**). This clearly suggests that such molecules may exhibit a strong competitive advantage over host cellular mRNA leader sequences during transcription initiation in a natural infection.

Chemical modification of capped leader sequences to prevent i) cleavage by the viral endonuclease (**Figure 6-1A to B**) or ii) elongation by the viral polymerase (**Figure 6-1B to C**) could make these molecules dysfunctional and after application inhibit and treat a viral infection. Modifications to achieve this comprise i) a 3'-terminal phosphate group, ii) one or more phosphorothioate or phosphorodiamidate bonds, or iii) one or more morpholino rings. In the first case, the 3'-OH terminus of a capped RNA leader is modified into a 3'-phosphate, giving the following consensus ^{7m}G-(N)$_{7\text{-}8}$-(A/U/G)-(U/G)-*AGC*p-3' where p is a 3'-phosphate group. RNA polymerases need a 3'-OH terminus for elongation, thus the presence of a 3'-phosphate terminus instead of a 3'-OH will block elongation by the viral RNA polymerase. In contrast, a modification of the phosphodiester bonds within the capped leader sequence will introduce nuclease resistance and thereby prevent cleavage or further trimming of a leader sequence by the viral endonuclease (and other host nucleases) into a functional cap donor that can readily prime and become elongated. Modification of the phosphodiester bonds could involve replacement by phosphorothioate or phosphorodiamidate bonds, preferably at least the phosphodiester bonds between the A, G, or C at the 3' terminus. But it could also involve the entire cap donor molecule (***Tado et al., 2001***). An additional advantage of the nuclease resistance of such molecules is their stability during application as antiviral drug. A last possible modification, i.e. a replacement of one or more ribose rings (or when a DNA consensus sequence is used of the dideoxyribose rings) within the capped RNA leader with one or more morpholino rings, will also provide resistance to endonucleases (***Summerton and Weller, 1997; Summerton, 1979***). In morpholino modified molecules the phosphate groups are additionally replaced by phosphorodiamidate groups.

Based on all our data it is not unlikely that capped RNA leaders when made dysfunctional may have a down regulating effect, due to their preferential usage, on the overall transcription level. They thereby decrease virus titres and detrimental effects of a viral infection providing the host's immune system with more time to generate an adequate immune response. In this way a reduced illness can be achieved.

According to our model and data from reported studies, a few examples of capped-RNA leader sequences that, once made dysfunctional, should inhibit influenza transcription initiation are listed in **Table 6-6**. It is expected that inhibitors with more than one base complementarity to the vRNA (AG or AGC) will be more effective in the reduction of viral transcription compared to those with only a single (A) base-pairing residue. A capped RNA leader with more than three base-pairing residues and containing modified phosphate bonds may also function as inhibitor as this molecule would still bind the viral polymerase and align on the viral template. However, due to its modification it will not be endonuclease cleaved/trimmed to render a proper 3' end required for transcriptional elongation and from that moment onwards block transcription. In a similar way to influenza virus, dysfunctional capped RNA leaders could be designed and synthesized against many other segmented (-) ssRNA viruses, or even groups of viruses which share a homologous 3' viral RNA terminus

such as influenza viruses and GERV (**Table 6-1**). As an example, dysfunctional molecule (DM) 1 could be applied against *Influenza A/B/C virus*, while DM2 against influenza viruses and/or GERV bunyavirus (**Table 6-7**). Multifunctional inhibitors, such as DM2, not only could extend their applicability against different type of viruses but also reduce production costs.

TABLE 6-6

Dysfunctional capped leaders for influenza virus inhibition.

Dysfunctional capped leader	5' end sequence of capped leader
	11
DMp 1bp	5'-GUAUUAAUGU***A***p
DMp 2bp	5'-GUAUUAAUGU***AG***p
DMp 3bp	5'-GUAUUAAUGU***AGC***p

Residues potentially base-pairing (bp) to the viral template (3'-UCG…) are in **bold** and *italics*. Abbreviations: DM, dysfunctional molecule; p, phosphate group.

TABLE 6-7

Dysfunctional capped RNA leaders against different viruses.

Dysfunctional capped leader	5' end sequence of capped leader	Virus
DM1	5'-GUAUUAAUGU***AGC***p	*Influenza A/B/C virus*
DM2	5'-GUAUUAAUGU***AG***p	*Influenza A/B/C virus* and/or GERV

Residues potentially base-pairing to the indicated viral template are in **bold** and *italics*. Abbreviations: DM, dysfunctional molecules; GERV, *Germiston virus*; p, phosphate group THOV, *Thogoto virus*.

Translation initiation of segmented (-) ssRNA viruses

Eukaryotic mRNAs commonly contain a 5' cap structure and a 3' poly(A) tail required for translation initiation (***Gallie, 1998, id. 2002; Millevoi and Vagner, 2010; Cowling, 2009; Dreyfus and Régnier, 2002; Gu and Lima, 2005***), in which both elements act synergistically to stimulate protein synthesis (***Gallie, 1991; Michel et al., 2001***). Translation initiation is a stepwise process by which the 40S ribosomal subunit is recruited to the mRNA and then starts scanning in a 5' to 3' direction until the first initiation codon is encountered in a favorable sequence context. At that moment, the 80S ribosome is assembled (***Jackson et al., 2010; Sonenberg and Hinnebusch, 2009***). Critical to the

translation process is the eIF4F cap-binding complex which mediates the initiation of cellular mRNA translation. This complex is composed of eIF4E (eukaryotic translation initiation factor) which binds to the mRNA cap, eIF4A which is a helicase necessary for initiation, and eIF4G which indirectly links the mRNA cap with the 43S pre-initiation complex (***Jackson et al., 2010; Sonenberg and Hinnebusch, 2009***). eIF4G also binds poly(A) tail-bound PABP [poly(A)-binding protein], leading to mRNA circularization by bringing its 5' and 3' termini into close contact [cap-eIF4E/eIF4G/PABP-poly(A)] (**Figure 6-2**) (***Kahvejian et al., 2001; Mangus et al., 2003***). It is in this context that the poly(A) tail interacts with the 5' cap and stimulates translation initiation (***Gallie, 1991; Michel et al., 2001***).

In contrast to eukaryotic mRNAs, viral mRNAs of segmented (-) RNA viruses contain a 5' cap structure but lack a common poly(A) tail at their 3' end. To compensate the lack of this tail, these viruses have evolved alternative mechanisms to support translational enhancement of their mRNAs. In general, specific sequences within their 3' UTR act as a functional analog of a poly(A) tail.

For influenza virus, all eight (-) strand RNA segments contain a stretch of 5-7 U residues about 17-22 nt from the 5' end. This U stretch sequence has been mapped as the viral polyadenylation signal (***Robertson et al., 1981***). It is iteratively copied by the viral polymerase during transcription to generate a poly(A) tail at the end of the mRNA. This process of polyadenylation, also described as stuttering of the polymerase, is favored by the binding of the viral trimeric RNA polymerase complex to the 5' end of the vRNA template. Viral mRNA translation in influenza virus was found to be enhanced by binding of the viral NS1 protein to both PABP and eIF4G (***Marión et al., 1997; Salvatore et al., 2002; Qiu and Krug, 1994; Aragón et al., 2000; Burgui et al., 2003***). Something similar has been postulated for the mRNA from the M segment of *Sin Nombre hantavirus* (***Hutchinson et al., 1996***).

In contrast to influenza virus, less is known on the mechanism of translation for bunyaviruses, which generally lack a common poly(A) tail at the 3' end of their mRNAs. Recently, the *Bunyamwera bunyavirus* (BUNV) and *Andes hantavirus* (ANDV) were shown to translate their own transcripts in a PABP-independent manner (***Blakqori et al., 2009; Vera-Otarola et al., 2010***), which is not surprising due to the lack of a common poly(A)-tail. Instead, a translation-enhancing element was reported for the 3' UTR of the BUNV S RNA segment encoded transcripts (***Blakqori et al., 2009***). Similarly, observations have been reported for the 3' UTR of the ANDV S mRNA where translation was additionally found to be independent of viral proteins (***Vera-Otarola et al., 2010***). For the plant-infecting bunyavirus TSWV, transcripts from the ambisense S and M RNAs segments have been shown to contain a potential 3' hairpin structure which is proposed to act as a translation-enhancing element, functionally equivalent to a poly(A) tail (**Chapter 5; *van Knippenberg et al., 2005b***). In contrast, the TSWV L RNA segment is of entire (-) polarity and does not contain sequences with similarity to the intergenic region of the S and M RNA

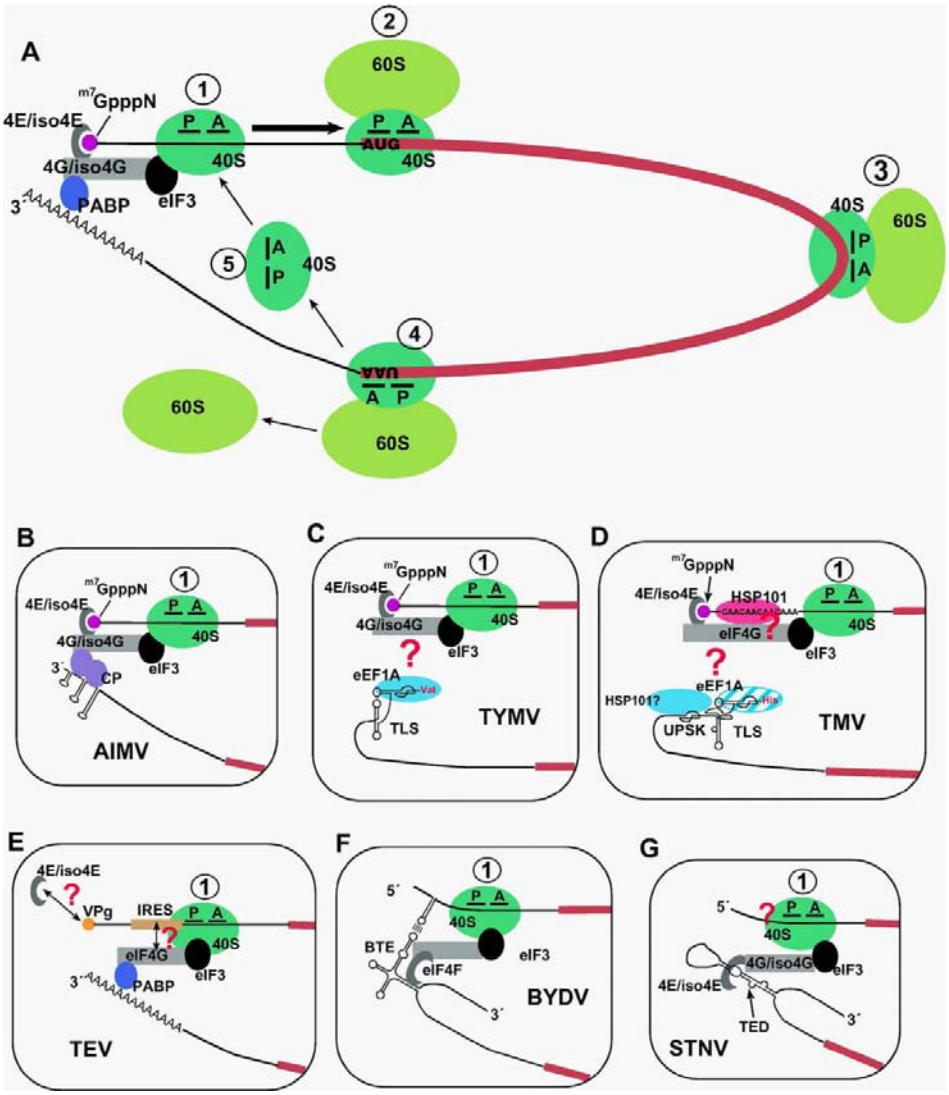


Figure 6-2 **The closed loop scheme for translation initiation of plant positive strand RNA viruses.** Panel A shows in simplified terms the circularized format in which efficiently translated cellular mRNAs are believed to exist. The key responsible *cis*-acting features are the 5' ^{m7}GpppN cap (purple dot) and 3'-poly(A) tail, which synergistically enhance expression. Bridging interactions through eIF4E or eIF-iso4E (the cap-binding proteins), eIF4G or eIF-iso4G, and the poly(A) binding protein (PABP) bring the 5'- and 3'-termini into close proximity, and the interactions are mutually stabilizing. The first stage of translation initiation, the recruitment of the 40S small ribosome subunit to the 5'-end, depends on simultaneous interaction of eIF3 (a complex of multiple proteins) with eIF4G or eIF-iso4G and the 40S subunit. This step is depicted in step (1) and is followed by ribosome scanning (arrow) along the 5'-UTR (black line) in search of the AUG initiation codon. In response to

segments, that provides these hairpin structures. A closer look, though, reveals a sequence downstream the stop codon that is also predicted to fold into a secondary folding structure (**Figures 6-3; *van Knippenberg et al., 2005b***). This suggests that translation of all TSWV viral mRNAs might involve a similar mechanism, i.e. in which a 3'-terminal RNA folding structure is involved in the translational enhancement and recruitment of viral/host factors to assist in translation, although this remains to be elucidated in particular for the L mRNA.

The presence of a hairpin structure sequence is not restricted to the tospovirus ambisense M and S RNA segments, but also appears within the ambisense S RNA segments from the related phleboviruses (***Schmaljohn and Hooper, 2001***), within plant viruses from the floating genus *Tenuivirus* (***Ramirez and Haenni, 1994***), and within members of the *Arenaviridae* (***Buchmeier et al., 2001***). In some of these, the predicted hairpin structure also involves stretches of A- and U-rich sequences [e.g. *Punta toro virus* (***Emery and Bishop, 1987***)], in a few others involves GC-rich sequences [e.g. *Tacaribe virus* (***Iapalucci et al., 1991; Lopez and Franze-Fernandez, 2007***)], while also examples exist in which the IR does not fold into a stable hairpin structure [e.g. *Rift Valley fever virus*

base-pairing with the initiator tRNA (not shown) located in the ribosomal P site, the 60S large ribosome subunit joins (step 2) to initiate the elongation phase of translation. During peptide elongation (step 3), the codons in the ORF (thick red line) are read by tRNAs entering the ribosomal A site, until a termination codon, such as UAA, is reached (step 4), triggering termination of protein synthesis and subunit dissociation and release (step 5). Because of the closed loop format, ribosomes are near the 5'-end upon termination, facilitating new initiation. The boxed diagrams (B-G) illustrate variations of the initial 40S subunit recruitment step for viral mRNAs that lack a cap, poly(A) tail, or both. Red question marks indicate unknown or uncertain details. AlMV, TYMV, and TMV are examples of viruses whose RNAs have a cap but no poly(A) tail. For AlMV, a coat protein (CP) dimer binds to the 3'-terminal region and to eIF4G/iso4G. For TYMV RNA, aminoacylation (indicated by Val in the diagram) of the 3'-terminal tRNA-like structure (TLS) is needed for full 3'-translational enhancement, and it has been postulated that eEF1A binding is involved in closed loop formation. TMV RNA also has a 3' TLS capable of aminoacylation (His) and eEF1A binding, but 3'-translational enhancement relies on an upstream pseudoknot (UPSK). Intriguingly, this feature can also bind eEF1A, which may be involved in closed loop formation, apparently in a way that predominates over a TYMV-like interaction involving the TLS. TEV RNA has a poly(A) tail, but no 5'-cap. The 5'-end is covalently linked to VPg, which is not needed for translation but does interact with eIF4E and eIFiso4E; it is not known whether this interaction influences translation. 40S ribosome subunits are recruited to the 5'-UTR through an IRES element whose function requires eIF4G and that may involve direct base-pairing to 18S ribosomal RNA. BYDV and STNV RNAs lack both canonical terminal elements and possess translational enhancer elements (BTE, TED) in an internal position (not at the 3'-terminus) of the 3'-UTR. These elements recruit translation initiation factors that are normally recruited to the 5'-end by the cap. In BYDV RNA, ribosome delivery to the 5'-end is accomplished through direct RNA base-pairing between elements in the 5'- and 3'-UTRs, while in STNV RNA, base-pairing between the 5'-UTR and rRNA may be involved.
Taken from Dreher and Miller (*2006*).

(***Giorgi et al., 1991***)]. It is postulated that transcription termination of ambisense mRNAs is dictated by the formation of a hairpin structure in nascent transcripts (***Emery and Bishop, 1987; Iapalucci et al., 1991***), in the same manner as transcription termination occurs in prokaryotes and in some eukaryotes. The presence of hairpin structure encoding sequences in many ambisense RNA segments of bunyaviruses would support this idea. In the case where a hairpin structure is not present or RNA segments are involved of entire negative polarity, transcription termination is suggested to involve G- or C-rich regions present near or at the transcription termination sites (***Collett, 1986; Giorgi et al., 1991; Gro et al., 1997; Schmaljohn and Hooper, 2001***). Some of these cases involve inverted complementary repeat sequences that could fold into a small stem-loop structure (***Bouloy et al., 1990***). In a more recent publication by Ikegami *et al.* (***2007***), a 13 nt-long inverted complementary sequence, with the ability to fold into a RNA secondary structure, was shown to be required for RVFV L RNA transcription termination. Although transcription termination of the RVFV M and (ambisense) S RNA segment are suggested to occur differently (***Albarino et al., 2007***), the 3' UTR sequence involved in L RNA transcription termination was able to terminate transcription of the (ambisense) S RNA encoded genes and thus supports the idea of a common transcription termination strategy. For orthobunyaviruses, ongoing transcription has been shown to require translation (***Barr, 2007; Bellocq et al., 1987; Vialat and Bouloy, 1992***), and it is explained to prevent nascent mRNAs from (premature) transcription termination. This tight coupling not only indicates a resemblance with prokaryotic systems, as proposed by Barr (***2007***), but additionally indicates that for orthobunyaviruses mRNA circularization is not mandatory for translation initiation.

While the information on transcription termination of bunyaviruses is still limited, and different strategies have been proposed to account for transcription termination of bunyavirus genomic RNA elements, it is possible that RNA folding structures play a major role in all these RNA segments, whether of ambisense or of negative polarity. Even though the information on translation (initiation) of bunyavirus transcripts is also limited, with the appearance of more publications on this topic it becomes tempting to hypothesize that segmented, (-) ssRNA viruses share a common transcription termination/translation initiation mechanism that applies to all their genomic RNA segments, irrespective of their host. In such model, transcription termination occurs due to stalling of the RNA polymerase on a 3' RNA folding structure in nascent transcripts; this folding structure would next act as a poly(A) tail analog to enable mRNA circularization and translational enhancement. The latter might involve viral proteins to functionally act as PABP-analog. Functional replacement of the poly(A) tail by a highly structured 3' UTR has been also demonstrated for the 3' UTR of flaviviruses, such as *Dengue virus*, rotavirus and many plant-infecting positive-strand RNA viruses (***Dreher and Miller, 2006; Edgil et al., 2003; Ito et al., 1998; Vende et al., 2000***). Rotavirus NSP3A and *Alfalfa mosaic virus* (AlMV) coat protein associate with both the 3' end of their respective genomes and with eIF4G (**Figure 6-2; *Piron et al., 1998; Bol, 2005***), thereby enabling genome circularization.

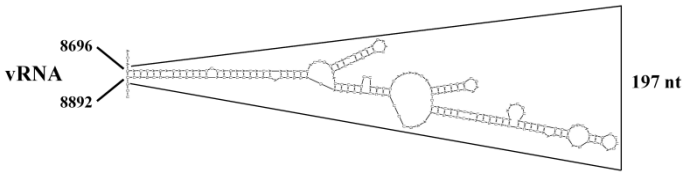


Figure 6-3 RNA folding predictions of TSWV L RNA segment. Mfold predictions of the 3' end of the L ORF. Abbreviation: vRNA, viral sense RNA; vcRNA, viral complementary RNA.

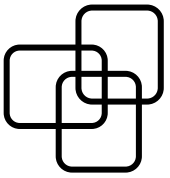
Outlook

During the research presented in this thesis, the requirements for alignment of capped RNA leader sequences along the viral genome during influenza cap-snatching have been disclosed (**Chapter 2, 3 and 4**). The approaches used have shown to be successful for the identification of the RNA leader origin and to subsequently support the design of capped RNA leaders that would become preferentially used during the priming of viral mRNA synthesis. In the light of the observation that *Influenza A virus* prefers leaders with increasing base complementarity to its vRNA template (**Chapter 2, 3 and 4**), similar to the plant-infecting bunyavirus (TSWV), supports the idea of a conserved mechanism applicable to all segmented (-) ssRNA viruses. To prove this hypothesis, experiments as described in this thesis, will need to be performed using viruses other than influenza and TSWV. For the near future, it will become most interesting to test the efficacy of proposed dysfunctional capped RNA leaders as an antiviral drug against these viruses.

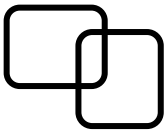
Our translation studies have indicates an important role for the 3' UTR of TSWV mRNAs in translation (**Chapter 5**), in which a 3' hairpin structure is postulated as a functional analog of a poly(A) tail. Due to many commonalities with the animal-infecting bunyaviruses, the question rises whether a similar strategy applies to their viral transcripts as well, and possibly to all segmented (-) ssRNA viral transcripts. In this respect, it will be interesting to see whether 3' UTR sequences from distinct (animal-infecting) bunyavirus RNA segments or even arenavirus RNA segments, and predicted to fold into a secondary structure or not, are able to functionally replace the existing TSWV hairpin structure sequence. One of the first candidate UTR sequences to analyze in such functional replacement test is the 3' UTR from the TSWV L RNA segment. Once these analyses demonstrate the requirement of RNA folding structures in transcription termination and translation initiation it will be interesting to find out whether or not a viral protein (complex) is involved in translation enhancement. The comparative analysis of plant- and animal-infecting bunyaviruses may even be a very useful and interesting tool to investigate the functional similarities between mechanisms within eukaryotic organisms belonging to different kingdoms.

“Once you have eaten the donkey, you should not leave the tail.”

Greek proverb



Appendix



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Abbreviation list

(-)	negative
(+)	positive
A	Adenine
AlMV	*Alfalfa mosaic virus*
AlMV3	*Alfalfa mosaic virus* RNA3
BHK-21	Baby Hamster Kidney (cells)
bp	base-pairing (residue)

BUNV	*Bunyamwera virus*
c	complementary (strand)
C	Cytosine
CCHFV	*Crimean Congo hemorhagic fever virus*
CTP	Cytidine triphosphate
DM	dysfunctional molecule
dsRNA	double-stranded RNA
DUGV	*Dugbe virus*
e.g.	*exempli gratia* [Latin phrase with meaning “for example”]
ead.	*eadem* [Latin word with meaning “the same (woman)”]
ER	endoplasmic reticulum
et al.	*et alii* [Latin phrase with meaning “and others”]
G	Guanine
Gc	glycoprotein (carboxy-terminal position within the precursor)
Gn	glycoprotein (amino-terminal position within the precursor)
Hep δ	*Hepatitis delta virus* (ribozyme)
HFV	*Hemorrhagic fever virus*
HTNV	*Hantaan virus*
i.e.	*id est* [Latin phrase with meaning “that is”]
id.	*idem* [Latin word with meaning “the same (man)”]
IR	intergenic region
IRES	internal ribosome entry site
L	large (RNA segment)
LACV	*La Crosse virus*
LCMV	*Lymphocytic choriomeningitis virus*
LFV	*Lassa fever virus*
M	medium (RNA segment)
MBP	Maltose binding protein
MDCK	Madin-Darby canine kidney (cells)
mRNA	messenger RNA
m.o.i.	multiplicity of infection
N	nucleoprotein
nt	nucleotide
NP	nucleocapsid protein
NS1	non-structural protein 1
NSm	cell-to-cell movement protein
NSs	non-structural protein
NTP	nucleoside triphosphate
ORF	open reading frame
p	phosphate

PA	polymerase acidic protein
PABP	poly(A) tail binding protein
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pol I	polymerase I
Pol II	polymerase II
RdRp	RNA-dependent RNA polymerase
RNP	ribonucleoprotein or ribonucleocapsid
Rv	reverse
Fr	forward
rNTP	ribonucleoside triphosphate
REN	*Renilla*
FF	Firefly
RRL	Rabbit reticulocyte lysate
RSV	*Rice stripe virus*
RT-PCR	reverse transcriptase PCR
RVFV	*Rift Valley fever virus*
S	small (RNA segment)
SFSV	*Sandfly fever Sicilian virus*
ssRNA	single-stranded RNA
siRNA	small interfering RNA
miRNA	micro RNA
T	Thymine
THOV	*Thogoto virus*
TOSV	*Toscana virus*
TS-oligo	template-switch oligonucleotide
TSWV	*Tomato spotted wilt virus*
TV	*Tacaribe virus*
TYRV	*Tomato yellow ring virus*
U	Uracil
UTR	untranslated region
UUKV	*Uukuniemi virus*
vc	viral-complementary (strand)
vRNA	viral RNA
vv-T7	T7-recombinant (or modified) *Vaccinia virus*
WT	wild type
vs.	versus
resp	respectively

Summary

The research described in this thesis focuses at the mechanisms of transcription (initiation/termination) and translation of segmented (-) ssRNA viruses. Although segmented (-) ssRNA viruses have already been studied in detail for many years, several research issues still have remained unsolved. One major question that still puzzles is how transcription initiation (cap-snatching) in detail takes place. In this thesis, the requirements for alignment of capped RNA leader sequences along the viral genome during influenza transcription initiation have been analyzed and to this end two systems (*in vitro* and *in cell*) were being developed. Using these systems, various *Alfalfa mosaic virus* RNA 3-derived mutant leaders, differing in i) base complementarity to the viral RNA template, ii) residues 5' of potential base-pairing residues and iii) leader length, have been tested as cap donor either singly or in (pair-wise/multiple) competition during influenza virus transcription initiation.

Sequence analysis of *de novo* synthesized transcripts within both systems, revealed that influenza transcriptase prefers leaders with increasing base complementarity to the 3'-ultimate residues of the viral template, 10 or 11 nt from the 5' cap. For the first time, the occurrence of prime-and-realign during transcription initiation of influenza virus was observed. Additionally, internal priming at the 3'-penultimate residue was observed. Application of capped RNA leaders with enhanced complementarity to the viral RNA template showed stronger reduction of globin leader RNA initiated influenza transcription *in vitro* compared to those with a single base complementarity, demonstrating the competitiveness of such molecules during transcription initiation.

The finding that influenza virus displays a preference for multiple base-pairing cap donors during transcription initiation is similar to recent observations made on this account for the plant-infecting bunyavirus *Tomato spotted wilt virus* (TSWV), and suggests a highly conserved mechanism of transcription initiation that most likely applies to all segmented, (-) ssRNA viruses. The insight obtained offers possibilities for development of new antiviral drugs based on capped RNA leader sequences made dysfunctional for transcription initiation/elongation.

In contrast to transcription initiation, less is known on the signals that trigger transcription termination and support translation initiation. While common eukaryotic messenger RNAs contain a 5' cap structure and a 3' poly(A)-tail, transcription of the TSWV M and S RNA ambisense-encoded genes terminates in an intergenic region (IR) that contains stretches of highly A- and U-rich sequences. The latter are predicted to fold into a

stable hairpin structure. To guarantee enhanced translation of these viral mRNAs, it is postulated that the predicted hairpin structure from this IR sequence acts as an equivalent to a poly(A) tail. To test this hypothesis, various N gene-based constructs were made in which the N gene was swapped for *Renilla* luciferase the 5' and/or 3' UTRs mutated/deleted or swapped for bunyaviral analogs, and tested in BHK-21 cells for translation efficiency in a *Vaccinia*-T7 expression system.

Whereas the *Renilla* luciferase gene, flanked with the 5' and 3' untranslated region (UTR) of the N gene and including the sequence for the hairpin structure, showed good expression levels, no luciferase expression was observed when the hairpin structure sequence was deleted from this *Renilla* sensor construct. Exchanging the TSWV hairpin structure sequence by the one from *Tomato yellow ring virus* (TYRV), another distinct tospovirus, did not affect luciferase expression levels. While the 3' UTR of the N gene was sufficient, and did not require the additional presence of the 5' UTR sequence for translation, the 3' UTR from the NSs gene only supported good translation in the additional presence of the homologous 5' UTR and indicating a possible interaction. Finally, the presence of the viral N and NSs proteins enhanced translation of luciferase constructs containing the hairpin structure sequence, suggesting a concerted action for translational enhancement.

Nederlanse Samenvatting

Het onderzoek beschreven in dit proefschrift richt zich op transcriptie- (initiatie/terminatie) en translatie-mechanismen van gesegmenteerde, negatief (-) strengs RNA virussen en dan in het bijzonder van het *Tomatenbronsvlekken virus* [Engels "*Tomato spotted wilt virus*" (TSWV)] en het griepvirus [Engels "*Influenza A virus*"]. Alhoewel deze virussen al voor lange tijd in detail bestudeerd worden, zijn tal van vragen nog steeds onbeantwoord gebleven. Dit gaat onder andere op voor transcriptie-initiatie (ook wel "cap-snatching" genoemd) van het virale RNA genoom. In dit proefschrift zijn de vereisten voor ge'cap'te RNA leadersequenties, om transcriptie op het influenza A RNA genoom te kunnen initiëren, onderzocht en daartoe werden twee systemen (*in vitro* en *in vivo*) ontwikkeld. Gebruik makend van beide systemen zijn vervolgens verschillende *Luzerne mozaïek virus* [Engels "*Alfalfa mosaic virus*" (AlMV)] RNA-3 mutanten die verschilden in i) basecomplementariteit tot het virale RNA genoom, ii) nucleotideresiduen 5' van de potentiële base-parende residuen, en iii) RNA leader lengte, getest als capdonor in een individuele of simultane, competitie-achtige setting tijdens influenzagenoom transcriptie-initiatie.

Nucleotidensequentie-analyse van nieuw gemaakte boodschapper RNA transcripten in beide systemen liet een voorkeur zien van *Influenza A virus* voor RNA leaders met toegenomen basecomplementariteit tot het 3'-uiteinde van het RNA genoom, op een positie rond 10-11 nucleotiden vanaf de 5' cap structuur. Voor het eerst werd een zogenaamd "prime-and-realign" tijdens influenza transcriptie initiatie waargenomen. Tevens werd interne initiatie op nucleotidepositie +2 van het virale RNA genoom aangetoond. Toediening van ge'cap'te RNA moleculen met toegenomen basecomplementariteit tijdens *in vitro* influenza virus transcriptie initiatie leidde tot een grotere reductie in globine RNA geïnitiëerde genoomtranscriptie dan wanneer ge'cap'te RNA moleculen met lagere basecomplementariteit werden toegediend. Hiermee werd het belang van base-parende en het competitie karakter van dergelijke moleculen gedurende influenza virus transcriptie initiatie onderstreept.

De constatering dat *Influenza A virus*, net als het TSWV, een voorkeur heeft voor cap donoren met verhoogde basecomplementariteit gedurende transcriptie initiatie suggereert een zeer geconserveerd mechanisme van transcriptie initiatie, dat hoogstwaarschijnlijk van toepassing is op alle gesegmenteerde (-) ssRNA virussen. Het inzicht dat is verkregen biedt mogelijkheden voor de ontwikkeling van nieuwe antivirale

middelen gebaseerd op ge'cap'te RNA leadersequenties, welke disfunctioneel gemaakt zijn voor transcriptie initiatie/elongatie.

In tegenstelling tot het proces van transcriptie initiatie bij gesegmenteerde (-) ssRNA virussen is veel minder bekend over de nucleotiden sequenties die uiteindelijk leiden tot transcriptie terminatie en die daarnaast vereist zijn voor, en eventueel stimulerend zijn tot translatie (initiatie). In tegenstelling tot eukaryote boodschapper RNA's, bezitten de dier- en plant-infecterende bunyavirussen, waaronder TSWV, aan het 3' uiteinde geen poly(A) staart. Voor TSWV vindt transcriptie terminatie van de M en S RNA ambisense gecodeerde genen plaats in de zogenaamde intergene sequentie regio (IR). Deze laatste is rijk in A- en U- nucleotide residuen en in staat tot vouwing in een zogenaamde haarspeld of "hairpin" structuur. Deze structuur wordt verondersteld een belangrijke rol te spelen in het bevorderen van een goede translatie van de TSWV M en S RNA transcripten, op een manier die functioneel analoog wordt geacht aan een poly(A)-staart. Om dit te toetsen, zijn verschillende constructen gemaakt van het N gentranscript waarin het open leesraam voor het N eiwit was vervangen door dat van het gevoelige marker enzym *Renilla* luciferase, en de flankerende 5' en 3' "untranslated regions" (UTR) of zijn verwijderd, òf gemutageniseerd òf vervangen voor de analoge UTR's van andere (plant- en dier-infecterende) bunyavirussen. De gemaakte constructen zijn vervolgens op translatieefficiëntie getoetst via het *Vaccinia virus*-T7 expressie systeem.

Terwijl het *Renilla* luciferase gen geflankeerd met de 5' en 3' UTR sequenties van het N gen, inclusief de "hairpin" coderende sequentie, goed tot expressie kwam werd er geen *Renilla* luciferase activiteit gemeten indien de "hairpin" coderende sequentie verwijderd was. Daarentegen bleek een uitwisseling van de "hairpin" coderende sequentie voor die van het *Tomato yellow ring virus* (TYRV), een ander afwijkend tospovirus, geen invloed te hebben op het luciferase expressieniveau. Uit de analyse van mutanten bleek tevens dat de 3' UTR sequentie voor expressie van het N gen voldoende was en geen aanwezigheid van de 5' UTR sequentie vereiste, terwijl voor expressie van het NSs gen zowel de 5' en 3' UTR vereist waren. Dit laatste wijst op een mogelijke interactie tussen beide UTR sequenties. Translatie studies in aanwezigheid van de virale N en NSs eiwitten bleek te leiden tot verhoogde expressie niveau's, hetgeen wijst op een gezamelijke (inter)actie van deze eiwitten met de "hairpin" stuctuur tijdens translatie.

Acknowledgments

Look for the right people
Look for the right people...
You only have to look carefully...
And if you make a wrong choice…
Do not give up…continue looking…
Somewhere there…outside…exist some good ones…
(like cloning…within a pool of clones - although sometimes
difficult to find - there always exist some good ones…
in most cases, you only need one...)

Christina Geerts-Dimitriadou

After a long journey to the PhD title, I finally (almost) arrived to the end. Herewith, I would like to thank some people for helping this dream come true *or* for just being pleasant co-travelers…

Dear Dr. ***Richard Kormelink***, I could only begin with you. I would like to thank you for offering me the opportunity to start this research in Virology and for being my co-promotor. Thank you for all the discussions we had and especially for your comments (which although were not always so easy to *digest*… however, I definitely learned a lot from them). Thank you for the thesis corrections, the time you invested in this thesis and your support with the "influenza" manuscripts. Thank you for everything!!!

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I can't fully express my gratitude to Prof. ***Just Vlak*** for taking over the role of promotor after Prof. Goldbach's fatal accident. THANK you for the time you invested correcting my thesis during the last few months and for making sure to be submitted before my contract ends, as I kindly requested. I think an advantage to our collaboration was that we (both) share the *attitude* to keep to deadlines and to try to avoid delays.

I would also like to thank *in advance* the ***Thesis Committee*** for taking the time to critically read the thesis concept and prepare critical questions in order to make an interesting Thesis defense. Special thanks to Prof. ***Richard Elliott*** for coming from the United Kingdom for my PhD defense. Thank you (all) very much!!!

For his kind interest in my research and for the critical reading of the "influenza" manuscripts I would like to acknowledge Dr. ***Gorben Pijlman***. I would also like to thank Dr. ***Marcel Prins*** here. Your critical questions during the lunch meetings were very helpful and contribute to my further development as a researcher. In addition, thank you for bringing me in contact with Richard for this PhD project and for the nice talks during your "PhD and Master student's outdoor activities". Many thanks to my Master thesis (daily) supervisor Dr. ***Etienne Bucher*** for sharing his enthusiasm about research, and for teaching me all these small (but very important) technical tricks in order to obtain good results. Dear Etienne, to be your paranymph during your PhD defense was an honor to me, to have you as my paranymph now and for coming from Switzerland *especially* for my PhD defense that is a GREAT honor, THANK YOU!!!

I owe a great deal to Dr. ***Mark Zwart*** who has helped me with setting up the real-time PCR and performing the statistical analyses. Dear Mark, thank you so much for all your suggestions, critical questions during my presentations and mostly for your kind interest and advice in personal matters. Dear ***Esther***, we (both) started our PhDs the same day… from how many *waves* we had to *swim away* or to *survive* together… Thank you for all the nice talks and the good times (your wedding, Istanbul and all other places and meetings). Dear ***Corinne***, firstly thank you for taking over the experimental steps with the *Vaccinia*-infected cells during my pregnancy. This great favor allowed me to continue my experiments without putting Eleni in danger. And secondly, thank you (and ***Simon***) for taking care of our cat and parrots during our holidays, and for having nice dinners and chats. Then, I would like to thank my Master students ***Jan***, ***Lu*** and ***Bram*** for their contribution to the TSWV part. A special "thanks" to ***Lu*** (***Yun-Yueh***) who worked very hard and with great pleasure to the topic. Thank you Lu for your enthusiasm in *our* project, all the nice discussions we had and for performing not only a Major Thesis but also a Minor with me. THANKS and good luck with your PhD.

In the Laboratory of Virology, but also within the Wageningen University and Institutes, I met many people who contributed to my scientific and social life. Hereby, I want to thank: ***Afshin***, ***Alex***, ***Bjarne***, ***Carien***, ***Dick Peters***, ***Dieu*** (***Bui***), ***Douwe***, ***Dowty***, ***Dryas***, ***Elena***, ***Els***, ***Geja***, ***Hanke***, ***Hans***, ***Harry***, ***Ingeborg, Jac***, ***Jacky***, ***Jan***, ***Janneke***, ***Jorg***, ***Ke***, ***Kelli***, ***Kelly***, ***Lara***, ***Luisa***, ***Marcel Westenberg***, ***Marcio***, ***Marianna***, ***Marjolein***, ***Marleen***, ***Miguel***, ***Monique***, ***Patrick***, ***Paulus***, ***Peter***, ***René***, ***Seyda***, ***Stefan***, ***Stineke***, ***Thea***, ***Thomas***, ***Vera***, ***Willem***, ***Wim*** *et al*. Special thanks to ***Dick Lohuis***, the "Father of the Virology lab", for always offering his assistance, for his kind daily interest and for his amazing personal humor, which made my long days enjoyable.

In your work (or educational) environment, you do not only meet colleagues, but you can also find friends… I have been lucky because I have found a lot of friends everywhere… MANY thanks to: ***Agah*** and ***Asli***, ***Christina***, ***Daniela*** and ***Sjors***, ***Dionyssia*** and ***Nikos***, ***Èrico*** and ***Emanuela***, ***Etienne*** and ***Danijela***, ***Giorgia*** and ***Sotiris***, ***Ioannis***, ***Kiki***, ***Maartje***, ***Maëlle***, ***Maria*** and ***Giorgos***, ***Muriel*** and ***David***, ***Nikos***, ***Ntina*** and ***Nikos***, ***Rania***, ***Rika***, ***Simone*** and ***Cristiano***, ***Vaggelis*** *et al.* for the unforgettable moments, dinners and discussions!!! To my former mathematician Mr. ***Jannis Xatzis***: thank you for being my number one source information on one of my biggest passions (Mathematics), thank you for all the discussions we had and your support (and for believing in me…). *I met few people in my life that I feel like knowing them forever…* Dear ***Magda***, thank you for accepting being my paranymph, for your friendship, for all the good moments and conversations. I wish you all the best in your life and career. *My lovely BEST friends* ***Vana*** (*my longest in time friend*), ***Jana*** (*my sister in spirit*) and ***Miwa*** (*my longest in distance friend*) thank you for fighting together with me against the *time* and *distance* in order to save this friendship!!! I would like to thank ***Marjo*** and ***Carine*** for the great (only) ladies-dinners, and ***Julia*** for the nice talks in my own language. Dear ***Andrea,*** you were one from the few people standing by me in one of the most difficult moments in my life. Your support helped me to move on and take my life from where I left it before this incident (and to my surprise even quicker than what I ever thought). I will never forget this… a big THANKS for all your friendship, for the nice times we had all together (with ***Ken***, ***Timmy*** and my family), and for always listening to what I have to say!

To the best family a kid but also a grown-up could hope for…

My parents [***Argiris*** and ***Helen*** (***Eleni***)], my sisters [***Natassa*** and ***Dimitra*** (my ***Mimi***)] and their families, I would like to thank for all their love and support all these years. I would also like to thank all my foster brothers and sisters (***Anna-Maria***, ***Jannis***, ***Christina***, ***Maria***, and ***Lefteris***) for the "*challenging years*", I learned a lot from having you at home. *I grew up in* ***Athens*** *and* ***Salamina*** *(known also as* ***Salamis****) while I spent all my summers at my family's summer house in* ***Aegina****…* ***Aegina*** *has always been my second home which provided me with great memories…* For these memories, I would like to thank my grandparents (***Christos*** and ***Elfriede***), my Godfather (uncle ***Achilles***), my late uncle ***Aristotelis***, my aunt ***Antonia***, and their families, and all the ***Aeginetans friends***! To my lovely grandfather ***Christos*** (from who I have the honor to carry his name): though, you are the step-father of my mother, you are the *best* grandfather that I could ever have! Special thanks to my lovely aunt ***Kalliopi***, uncle ***Paulos***, cousins ***Natasa*** and ***Eleni*** for all their support during my studies in the Peloponnese but also during the years of "*far away from home...*".

To lose someone that you know is hard…to lose someone that you love is terrible… I will never forget Boxing Day of 2010, the day that my late uncle ***Aristotelis*** (*our lovely* ***Telis***) passed away three days after his 55th birthday. His sudden and tragic death "*broke a*

big piece of my heart" and has been a *huge loss* for my family. He was a man with a "*pure*" heart! I am very grateful to him for all his support, the wonderful memories and for the fact that he *never* forgot me, even here in The Netherlands.

Special thanks to my parents-in-law (***Jan*** and ***Wilma***) and brothers-in-law (***Niels*** and ***Tom***) for their amazing help and support all this time and for the warm welcome to their family…I would also like to thank the late oma ***Mina Teunissen***, the late opa ***Geerts***, all the aunts and uncles, and their families, and the ***Ulft friends*** for welcoming me to their homes and for the nice moments!

There are definitely not enough words…… and not enough space here……. in order to *THANK* my husband ***Roy*** and children (***Jannis*** and ***Eleni***) for their support, their love and for making my life so colorful … Roy "*my love*", thank you for your support all these years, for always being next to me (in the good days and *especially* in the bad days) and for believing in me (and in my *sometimes* crazy ideas) for 100%!!! My lovely children, "*my treasures*", your smiles can take away any tiredness, disappointment, sadness. You are my motivation and inspiration to everything… *Thank You My Lovely Ones!!!!!!!!* and my apologies for so many late meals…

In these last lines, I would like to thank all of you whose name did not appear here (and my apologies for this) but I REALLY had to stop now. THANK YOU ALL!!!

Biography

Christina Geerts-Dimitriadou was born on the 18th of August 1977 in Athens, Greece. In 1988 she was awarded the 1st prize in the "Youth Chess Tournament" of Attica, where she was the first and only *female* student attending. In 1992 she attended the General High School of Salamina where she completed her secondary education. In addition to her secondary education, at the age of 17, she got her diploma in music with "Excellent" from the Kenner Music School of West Attica in Athens. In September 1997 she started her Bachelor studies at the Educational Institute of Technology of Kalamata in the Peloponnese after she was admitted by "National Examinations". In September 2001 she was awarded her Bachelor of Science degree (Top 5%) in Plant Sciences. Her Major Bachelor thesis was performed at the Plant Research International Institute in Wageningen in The Netherlands, and it was financed by a "*Leonardo da Vinci*" grant. In parallel with her Bachelor studies, she worked as a music teacher in the Conservatory of Kalamata in the Peloponnese. After her graduation she decided to return to The Netherlands and to perform a Master of Sciences at Wageningen University, which she finished in January 2003. She performed her research for her Master's thesis at the Laboratory of Virology under the supervision of Dr. Marcel Prins and Dr. Etienne Bucher, and from that moment her interest in Molecular Sciences was born. In addition to her Master studies she worked at the former ATO-DLO Institute in Wageningen as a research assistant in a project financed by Vitabric Holland BV. In May 2003 Christina began a PhD thesis work at the Laboratory of Fungal Genomics (Wageningen University). The aim of the research project was to identify and characterize novel pectin degrading enzymes, secreted by the fungus *Rhizopus oryzae*. In March 2006 she transferred to a new PhD project at the Laboratory of Virology within the same University, examining the involvement of *cis*- and *trans*-acting factors in transcription/translation of the segmented (-) ssRNA viruses, which resulted in the work presented in this thesis. The results obtained from the influenza work were patented. During her PhD she worked under the supervision of the late Prof. dr. Rob Goldbach and Dr. Richard Kormelink. After Prof. Goldbach's fatal accident in April 2009 she was further supervised by Prof. dr. Just Vlak. In September 2010 she was awarded the 1st "Bryan Harrison" prize for the "Best Oral Presentation" during the "International Advances in Plant Virology Conference" in Arnhem. In addition to her career, Christina married Roy Geerts in April 2003. They have two children, Jannis Argiris (6 years old) and Eleni Mina (1 and a half years old), and the family currently lives in Wageningen.

Publication list

Geerts-Dimitriadou C., Kormelink R. Transcription Initiation of Segmented, Negative-Strand RNA Viruses re-visited: a new Perspective for Antiviral Drug. *Manuscript in preparation.*

Geerts-Dimitriadou C., Lu Y.Y., Geertsema C., Goldbach R., Kormelink R. Analysis of the *Tomato spotted wilt virus* S RNA-encoded hairpin structure in translation. *Submitted for publication.*

Geerts-Dimitriadou C., Goldbach R., Kormelink R., 2011. Preferential use of RNA leader sequences during influenza A transcription initiation *in vivo*. Virology 409, 27-32.

Geerts-Dimitriadou C., Zwart M.P., Goldbach R., Kormelink R., 2011. Base-pairing promotes leader selection to prime *in vitro* influenza genome transcription. Virology 409, 17-26.

Bucher E., Lohuis D., van Poppel P.M., **Geerts-Dimitriadou C.**, Goldbach R., Prins M., 2006. Multiple virus resistance at a high frequency using a single transgene construct. J. Gen. Virol. 87, 3697-3701.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: **Christina Geerts-Dimitriadou**
Date: **28 June 2011**
Group: **Laboratory of Virology, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
Genome Transcription of Segmented Negative-Strand RNA Viruses	Jun 26, 2006
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Radiation hygiene, level 5B	Sep 23, 2003
Subtotal Start-up Phase	*3,0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
PhD week VLAG	2003
EPS PhD student day, Wageningen	Sep 19, 2006
EPS PhD student day, Wageningen	Sep 13, 2007
EPS PhD student day, Leiden	Feb 26, 2009
► **EPS theme symposia**	
EPS Theme 2: Interactions between Plants and Biotic agents, Amsterdam	Feb 2, 2007
EPS Theme 2 symposium & Willie Commelin Scholten day, Utrecht	Jan 22, 2009
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting, Lunteren	Apr 03-04, 2006
NWO-CW meeting, Lunteren	Dec 04-05, 2006
NWO-ALW meeting, Lunteren	Apr 02-03, 2007
NWO-CW meeting, Lunteren	Dec 10-12, 2007
NKPV meeting, Wageningen	Feb 28, 2008
NWO-ALW meeting, Lunteren	Apr 07-08, 2008
NWO-CW meeting, Veldhoven	Dec 08-10, 2008
NWO-ALW meeting, Lunteren	Apr 06-07, 2009
► **Seminars (series), workshops and symposia**	
Dutch Annual Virology Symposium (DAVS), KNAW, Amsterdam	Mar 10, 2006
Symposium 'Influenza Ecology and Pandemics, NIOO-KNAW, Wageningen	Apr 19, 2006
Symposium 'BioNanotechnology', Wageningen	May 12, 2006
Dutch Annual Virology Symposium (DAVS), KNAW, Amsterdam	Mar 09, 2007
Seminar "SARS, avian influenza and distemper: emerging viruses and host species barriers", Virology, Wageningen	Jan 25, 2008
Dutch Annual Virology Symposium (DAVS), KNAW, Amsterdam	Mar 07, 2008
Seminar "Viruses as vectors for gene therapy", Virology, Wageningen	Jun 09, 2008
Dutch Annual Virology Symposium (DAVS), KNAW, Amsterdam	Mar 06, 2009
► **Seminar plus**	
► **International symposia and congresses**	
XIV. International Congress of Virology, Istanbul, Turkey	Aug 10-15, 2008
Dutch-German Virology binational Plant Virology Symposium, Hamburg, Germany	Apr 08-09, 2009
Negative Strand Virus meeting (Conference), Bruges, Belgium	Jun 21-25, 2010
International Advances in Plant Virology Conference, Arnhem	Sep 05-07, 2010
► **Presentations**	
Oral: Dutch-Japanese meeting, Nagoya University, Japan	Apr 2004
Poster: Ibaraki–Wageningen Young Investigators' Symposium on Gut Microbiology in Japan, Ibaraki University, Japan	Apr 2004
Poster: Summer course 'Glycosciences',Wageningen	Jun 28-Jul 01, 2004
Oral: NWO-CW meeting Veldhoven	Dec 09, 2008
Oral: EPS Theme 2 symposium, Utrecht	Jan 22, 2009
Poster: EPS PhD student day, Leiden	Feb 26, 2009
Oral: Dutch Annual Virology Symposium (DAVS), KNAW, Amsterdam	Mar 06, 2009
Oral: Dutch-German Virology binational Plant Virology Symposium, Germany	Apr 09, 2009
Poster: Dutch-German Virology binational Plant Virology Symposium, Germany	Apr 08, 2009
Oral: Negative Strand Virus meeting (Conference), Bruges, Belgium	Jun 21, 2010
Oral: International Advances in Plant Virology Conference, Arnhem	Sep 05-07, 2010
Poster: International Advances in Plant Virology Conference, Arnhem	Sep 05-07, 2010
► **IAB interview**	Dec 05, 2008
► **Excursions**	
PhD Study Tour; Organizations: Ibaraki University, Osaka University, Kyoto Encyclopedia of Genes and Genomes KEGG, Kyoto University, Nagoya University, Japan Marine Science and Technology Centre, Yakult, KAO Corporation, RIKEN; Tokyo-Kyoto-Nagoya-Yokosuka-Tsukuba-Saitama; Japan	Apr 09-26, 2004
Subtotal Scientific Exposure	*30,3 credits**

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Experimental Plant Sciences

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3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Food Enzymology	Feb 16-18, 2003
	Industrial Proteins	Nov 03-07, 2003
	Bioinformation Technology I	Dec 08-16, 2003
	Summer course 'Glycosciences' + Poster presentation	Jun 28-Jul 01, 2004
	Confocal Light Microscopy: Fundamentals and Biological Applications	Jun 04-08, 2007
►	**Journal club**	
	Literature discussion with virology group	2006-2009
►	**Individual research training**	
	Subtotal In-Depth Studies	*9,6 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Working with EndNote	Feb 19, 2003
	Teaching and Supervising Thesis students	Oct 2004
	Project- and Time Management	Nov-Dec, 2004
	Professional Communication Strategies	Feb 14-15, 2005
	Techniques for Writing and Presenting a Scientific Paper	Mar 28-31, 2006
	Scientific Publishing	Mar 21, 2007
	Making a PhD Thesis	Mar 29, 2007
►	**Organisation of PhD students day, course or conference**	
	Organising "EPS PhD student day 2007"	Sep 13, 2007
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*7,0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**49.9**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study*

The work presented in this Thesis was carried out at the Laboratory of Virology of Wageningen University, The Netherlands. The research was financially supported by The Netherlands Organization of Scientific Research, division of Chemical Sciences (NWO/CW).

The attendance of the “XIV International Conference on Negative Strand Viruses” in Bruges, Belgium, was financially supported by a travel grant of the LEB Foundation.

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