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Transcription initiation by the respiratory syncytial virus polymerase

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Dissertation

TRANSCRIPTION INITIATION BY THE RESPIRATORY SYNCYTIAL VIRUS POLYMERASE

by

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EPIGRAPH

“Many of life’s failures are people who did not realize how close they were to success when they gave up.” Thomas Alva Edison

DEDICATION

For Joseph, who always believed in me the most

when I believed in myself the least.

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TRANSCRIPTION INITIATION BY THE

RESPIRATORY SYNCYTIAL VIRUS POLYMERASE

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ABSTRACT

Respiratory syncytial virus (RSV) is the leading cause of respiratory illness in children worldwide. RSV has a negative sense RNA genome, which is the template for viral mRNA transcription and genome replication, and encodes a polymerase to carry out viral RNA synthesis. The promoters for RSV transcription and genome replication are found in a 44-nucleotide (nt), 3´-extragenic region called the leader (Le). Replication is initiated opposite the first nt of the Le, and transcription of the first gene begins at position +45, at a gene start (GS) sequence. However, transcription is also dependent on sequence within Le1-12. Interestingly, Le nucleotides 3-12 bear strong similarity to a GS signal. We hypothesized that this GS-like sequence is the recruitment site for transcribing polymerase. To test this hypothesis, we examined RNA synthesis events at the Le promoter. We identified a previously undescribed RNA initiation

site at Le position +3 (Le+3) that was used frequently during RSV infection. Initiation at Le+3 led to the production of a small ~25 nt RNA. Le+3 initiation was shown to occur independently of replication initiation at +1, indicating it is a *bona fide* initiation site. Mutation of Le1-12 to increase similarity to a GS resulted in elongation of Le+3 RNA and a decrease in transcription initiation at the GS, demonstrating that the Le initiation sequence alters polymerase processivity and impacts downstream transcription events. Preliminary experiments to determine the function of the small RNA showed that it increased levels of viral RNA replication, suggesting it may be involved in influencing a switch from transcription to replication. These studies suggest a model for RSV transcription initiation, whereby the transcribing polymerase enters at the 3´-end of the genome, initiates RNA synthesis from Le+3 and generates a small RNA, and is then positioned to initiate transcription at the first GS. The small RNA that is generated may act as a feedback molecule to promote RNA replication. These findings provide a greater understanding of polymerase behavior at the promoter and may inform rational drug and vaccine design.

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LIST OF ABBREVIATIONS

˚C	degree(s) Celsius
A549	adenocarcinomic human alveolar basal epithelial cells
AcD	actinomycin D
ADP	adenosine diphosphate
(ag)	antigenome
Ago	argonaute
APS	ammonium persulfate
AREs	apical recycling endosomes
Ars	sodium arsenite
AS-ODN	antisense-oligodeoxynucleotide
BHK-21	baby hamster kidney cells
BLAST	basic local alignment search tool
BSR-T7/5	BHK cell clone stably expressing T7 polymerase
$CaCl_2$	calcium chloride
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal phosphatase
CMV	cytomegalovirus
CO_2	carbon dioxide
CPE	cytopathic effect

DAPI	4’,6’-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
dH_2O	distilled H_2O
DIF	deionized formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOC	deoxycholate
dsRNA	double stranded RNA
DTT	dithiothreitol
EboV	Ebola virus
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
ESCRT	endosomal sorting complexes required for transportation
F	RSV fusion glycoprotein
FBS	fetal bovine serum
G	RSV attachment glycoprotein
g	gram
(g)	genome
GAG	glycosaminoglycans

GE	gene end sequence
GMEM	Glasgow Minimal Essential Media
GS	gene start sequence
HEp-2	human epithelial airway cells
hMPV	human metapneumovirus
hpi	hours post infection
HRP	horseradish peroxidase
Hsp-70	heat shock protein 70
ICAM-I	intracellular adhesion molecule 1
IF	indirect immunofluorescence
IFN	interferon
IR	intergenic region
J	Joule
kDa	kiloDalton
L	RSV polymerase large subunit
LB	Luria Broth
Le	leader
LeC	leader complement
LNA	locked nucleic acid
M	Molar
M protein	RSV matrix protein

M2-1	RSV M2 ORF 1 protein, transcription processivity factor
M2-2	RSV M2 ORF 2 protein
mA	milliampere
MarV	Marburg virus
MDA-5	Melanoma Differentiation-Associated protein 5
MeV	measles virus
$MgCl_2$	magesium chloride
miRNA	microRNA
ml	milliliter
mM	milliMolar
MOI	multiplicity of infection
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
MuV	mumps virus
MVA-T7	modified vaccinia Ankara- T7
N	RSV nucleoprotein
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
NB	Northern blot
NDV	Newcastle disease virus

NFκB	nuclear factor κ of B cells
NiV	Nipah virus
nm	nanometer
nM	nanoMolar
NNS	non-segmented negative sense
NS1	RSV nonstructural protein 1
NS2	RSV nonstructural protein 2
nt	nucleotide
NTP	nucleotide triphosphate
ORF	open reading frame
P	RSV phosphoprotein
P2	insoluble fraction (pellet)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	PBS-Tween
PCR	polymerase chain reaction
PE	primer extension
PFU	plaque forming units
PKR	protein kinase RNA-activated
pmol	picomole
PNK	polynucleotide kinase
pre-miRNA	miRNA precursor

pri-miRNA	primary miRNA
RIG-I	retinoic acid-inducible gene 1
RISC	RNA-induced silencing complex
RNase	ribonuclease
RNA	ribonucleic acid
RNAP II	RNA polymerase II
RNP	ribonucleoprotein
rRNA	ribosomal RNA
RSB	reticulocyte standard buffer
RSV	respiratory syncytial virus
RT	reverse transcriptase
S1	soluble fraction 1
S2	soluble fraction 2
Scr	scrambled
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SeV	Sendai virus
SH	RSV small hydrophobic protein
siRNA	small interfering RNA
sRNA	small RNA
SSC	saline-sodium citrate

ssRNA	single stranded RNA
svRNA	small viral RNA
Ta	annealing temperature
TAP	tobacco acid pyrophosphatase
TBE	Tris borate EDTA
TBEV	tick-borne encephalitis virus
TPB	tryptose phosphate broth
TE	Tris-EDTA
TEMED	tetramethylethlenediamine
TEV	tobacco etch virus
Th1	T helper cell type 1
Th2	T helper cell type 2
TIA-1	T-cell-restricted intracellular antigen 1
TIAR	T-cell-restricted intracellular antigen related protein
TLR	toll-like receptor
TNF-α	tumor necrosis factor α
Tr	trailer
TrC	trailer complement
tRNA	transfer RNA
UV	ultra violet
V	volts
VSV	vesicular stomatitis virus

W	watts
WNV	West Nile virus
Wt	wildtype
µ	micron
µg	microgram
µl	microliter
µm	micrometer
µM	microMolar

Introduction

Respiratory syncytial virus

History

Respiratory syncytial virus (RSV) was first isolated in 1955 from a chimpanzee at the Walter Reed Army Institute of Research during an outbreak in the chimpanzee colony (14; 27). The virus was initially called chimpanzee coryza agent, as it produced a cluster of symptoms including sneezing, coughing, and mucopurulent nasal discharge, collectively referred to as “coryza”. The virus was later identified in cases of infantile croup, pneumonia and bronchiolitis, and serological evidence for RSV proved the virus was common within the pediatric population (26; 28).

Clinical significance and Epidemiology

RSV is now recognized as the leading cause of severe respiratory illness in infants and children worldwide, and is increasingly recognized as a significant pathogen in the elderly and immunocompromised (52; 58; 93). Yearly epidemics of RSV occur over 4-5 months during winter and spring, and RSV is highly contagious and easily spread by contact. Between 50 and 70 percent of infants become infected with RSV during their first year of life, and almost all children are infected with RSV by two years of age (76; 101; 121). Re-infection is

frequent during the first few years of life (76). Mortality due to RSV is low, however RSV represents a significant public health burden. An estimated 2 to 4 million children receive outpatient treatment, while between 57,000 and 113,000 children are hospitalized for RSV each year in the United States (95; 140). Risk factors for RSV infection include increased exposure in a community setting such as daycare, having siblings younger than 5 years old, low titers of maternal antibodies, premature birth, lung and heart conditions, exposure to cigarette smoke, and asthma (9; 45; 68; 83; 157; 165). In addition, hospital-acquired RSV infection is a significant cause of RSV spread in pediatric units (89).

In adults, the yearly reinfection rate for RSV is 5-10%, and up to 50% for adults with increased exposure, such as healthcare and daycare workers, and parents of small children (56-58). In adults over 65 years of age, RSV causes an estimated 17,358 deaths per year in the U.S. (246). RSV is also a significant cause of morbidity in patients with T-cell deficiencies and cystic fibrosis (3; 92; 102; 176; 268).

Immunopathology

At the beginning of the infection, RSV replicates in the nasopharynx. The virus has a tropism for airway and lung tissues and has been shown to preferentially infect ciliated cells (252; 272; 279). RSV is not thought to cause persistent infection. Primary RSV infection causes a range of symptoms including

rhinorhea, otitis media, fever, pneumonia and bronchiolitis, and in rare and very severe cases, death. Subsequent re-infections tend to be much less severe, suggesting that a protective immune response comes into play. RSV is not as cytopathic or invasive as influenza virus, but does cause increased shedding of airway cells, which leads to obstruction of bronchioles and alveoli (272; 279). CD4+ T cells and CD8+ T cells have both been shown to be important for viral clearance (77). However, the immune response to RSV may be skewed towards a Th2 CD4+ T cell bias leading to airway hyper-reactivity, wheezing, and prolonged effects on lung function (8; 129; 215). The humoral response to infection is characterized by serum and secretory antibodies to the viral fusion (F) and major attachment (G) glycoproteins, which are the only virus neutralization antigens, and titer of RSV-specific antibodies appears to correlate with protection from re-infection (39; 264; 265).

In addition to the cellular and humoral immune response to RSV, surfactants have been shown to be an important early barrier to infection (5; 75; 145). RSV also triggers activation of toll-like receptors (TLRs) and retinoic acid-inducible gene 1 (RIG-I), leading to the production of a wide range of cytokines (123; 134; 153; 177; 217; 218; 277). Neutrophils are recruited to the lung following infection and comprise the largest population of immune cells in the airway. Neutrophils play a role in viral clearance, though probably at the cost of exacerbation of disease symptoms (55; 155; 166). Macrophages are important to viral clearance

and phagocytic uptake of cellular debris and may be the primary producers of type I interferon (IFN) (130; 206). However, RSV is adept at blocking the IFN response, in addition to inhibiting apoptosis, TNF-α and NFκB signaling, PKR activation and stress granule formation (70; 85; 90; 91; 96; 164; 237).

Challenges to mounting an effective immune response to RSV include immunological immaturity in the pediatric population, and immune senescence and waning titers of RSV-neutralizing antibodies in the elderly population (1; 42; 59; 94; 146; 171; 197; 225).

Therapeutics and Vaccines

Currently there are no vaccines or therapeutics approved for use against RSV. Ribavirin has been indicated in severe cases, but despite established antiviral activity against RSV in tissue culture and animal models, clinical efficacy remains unproven (105; 213). Additionally, ribavirin is difficult to administer and produces adverse side effects, and is therefore not recommended for routine use (235). A humanized monoclonal F-specific antibody prophylactic, palivizumab (Synagis^{TM}), exists for use in preterm, and high-risk infants with congenital heart and lung defects. Licensed in 1998, palivizumab is administered intramuscularly, once monthly throughout the duration of RSV season (60). Although effective, palivizumab does not prevent disease, but restricts viral replication to a level that is manageable, and has been shown to reduce hospitalizations between 45- and

55-percent (86; 226). However, the cost is prohibitive and access to monthly doses of palivizumab in developing countries is limited (205).

Vaccine development for RSV has historically encountered many difficulties. A formalin-inactivated vaccine tested in the 1960's resulted in severe exacerbation of disease symptoms in vaccinees due to an unusual phenomenon called immunopotentiation (119; 207). Vaccination resulted in poor induction of a neutralizing antibody response; the antibodies produced were not to antigenic epitopes (180). However antibody-antigen complexes were still made that activated complement and biased a Th2 response. This led to poor stimulation of natural killer cells and CD8+ T cells, which help to down regulate inflammation and the Th2 response upon infection with RSV (108; 191; 230; 233). Additionally, the process of formalin fixation generates carbonyl groups, which have been shown to enhance the Th2 response. After natural infection with RSV, eighty percent of the trial participants were hospitalized for severe bronchiolitis and asthma-like symptoms and two children died (207). Consequently, vaccine development for RSV has proceeded slowly and with much caution. However, there are several candidate vaccines entering clinical trials that are based on live-attenuated strains of RSV, which do not induce immunopotentiation (120; 273; 274). Their efficacy remains to be seen, as none has yet been approved for use.

Several small molecule inhibitors of RSV proteins have undergone clinical trials including chemical compounds targeting the F and G proteins, nasally-administered small interfering RNA (siRNAs) targeting the viral nucleoprotein (N), and many more compounds are being developed (50; 54; 152; 192; 224). Currently, there is a lot of interest in inhibitors of the RSV polymerase complex. The polymerase is responsible for all viral RNA synthesis and is unique from cellular proteins, making it an ideal target for antiviral therapeutics. However, the polymerase is very large and complex, and relatively little is known about its function. A greater understanding of the molecular mechanisms of RSV polymerase activity is important for the development of polymerase inhibitors and may inform rational drug design.

Overview of the RSV replication cycle (Figure 3)

Virion structure

RSV virions are pleomorphic and consist of a mix of spherical particles, 100- 350 nanometers (nm) in diameter, and filaments, 20- 200 nm in diameter, which can be up to 10 μm in length (23; 184). The cell-derived lipid envelope is studded with three viral surface glycoproteins, the G protein, the F protein, and the small hydrophobic protein (SH). The matrix protein (M) forms a layer on the inner side of the virion envelope and mediates assembly of viral components at the plasma membrane. Viral RNA is packaged within the virion as a nucleocapsid. N protein assembly on the genome creates a flexible, filamentous nucleocapsid structure

with a characteristic “herringbone” pattern (12). The polymerase components, L, P and the transcription processivity factor M2-1, are associated with the nucleocapsid-bound RNA (Figure 1).

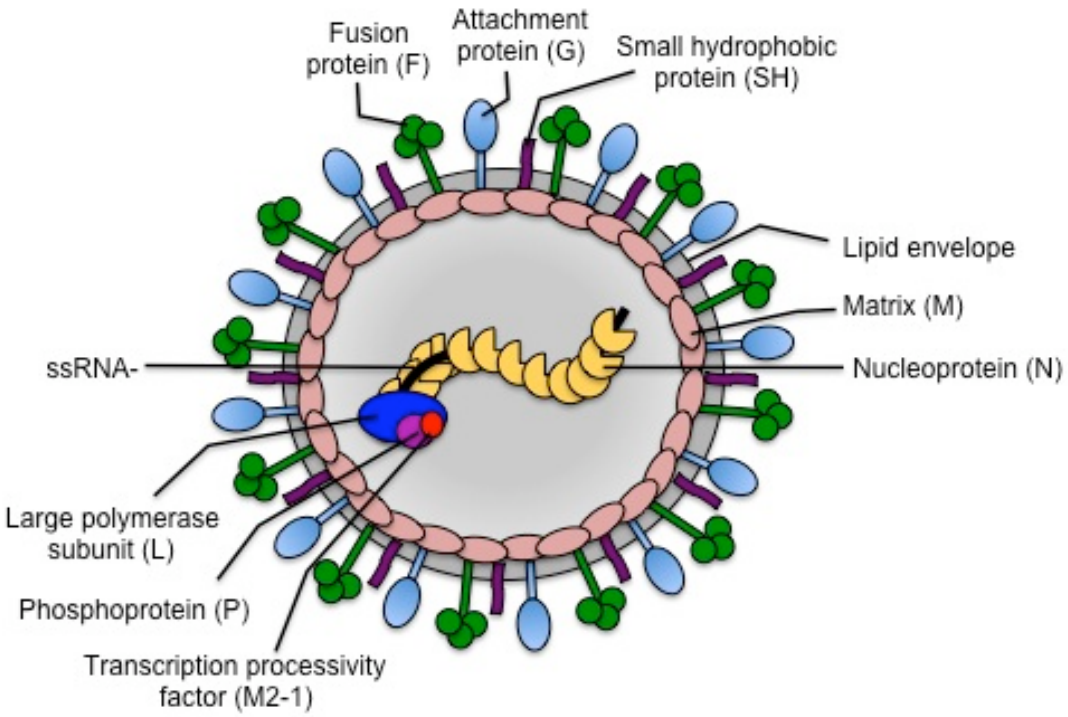


Figure 1; RSV virion structure. Illustration of the RSV proteins that comprise the virion and nucleocapsid structures. Virions have a host-derived lipid envelope, studded with three glycoproteins. The ssRNA genome is encapsidated in N protein assembled as a helical filament, and associated with the RSV polymerase complex.

Cellular entry

The receptor for RSV has not been satisfactorily identified, though several candidates have been suggested including intracellular adhesion molecule (ICAM)-I, RhoA, the CX3CR1 fractalkine receptor, annexin II, and nucleolin (7; 158; 195; 242; 251). Both G and F are also able to bind cellular glycosaminoglycans (GAGs). After attachment, the F protein mediates fusion at the plasma membrane and the viral nucleocapsid is released into the cytoplasm where the replication cycle takes place (115; 231).

RNA production and protein expression

Upon entry into the cytoplasm, RSV forms large, electron-dense inclusion bodies, which are thought to be sites of viral replication, composed largely of viral N protein (24; 73) (Figure 2). RSV encodes its own polymerase that carries out transcription and replication. Transcription results in the synthesis of ten capped and polyadenylated mRNAs, which encode eleven proteins. The viral mRNAs are translated using the host cell machinery. RSV mRNAs and proteins can be detected 4-6 hours post infection (hpi) and reach peak accumulation around 15-20 hpi.

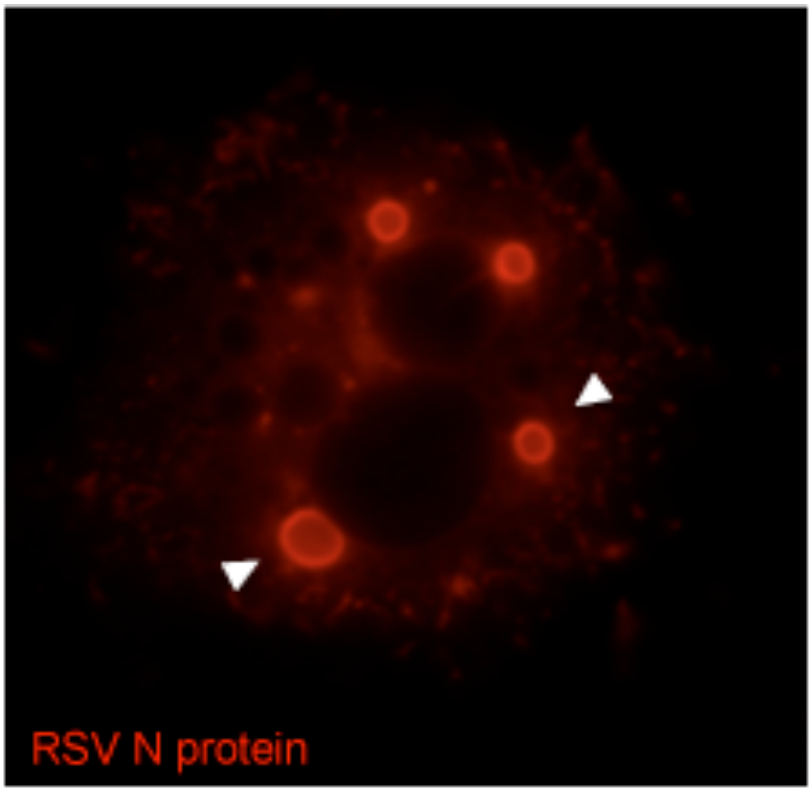


Figure 2; RSV inclusions. RSV forms dense cytoplasmic inclusion bodies, which are thought to be the sites of viral replication. Cells were infected with RSV fixed 24 hpi, permeabilized, stained for RSV N protein (red), and visualized by immunofluorescence microscopy. Image represents a single slice. Two inclusions are indicated with white arrowheads.

Assembly and release

Assembly of viral particles occurs at the plasma membrane associated with lipid rafts (18; 162). In cell culture, progeny virions can be detected 10-12 hpi, and their production peaks around 24 hpi. They are continually released up to 48 hpi or until cell destruction. Unlike many other RNA viruses that hijack the endosomal sorting complexes required for transport (ESCRT) machinery, RSV commandeers cellular apical recycling endosomes (AREs) to accomplish budding (17; 74; 256). Actin and profilin are involved throughout the viral life cycle, and actin appears to be closely involved in the formation of RSV filaments at the plasma membrane (4; 21; 22; 71; 111; 116; 253). Actin may be packaged in the virion along with viral nucleocapsids.

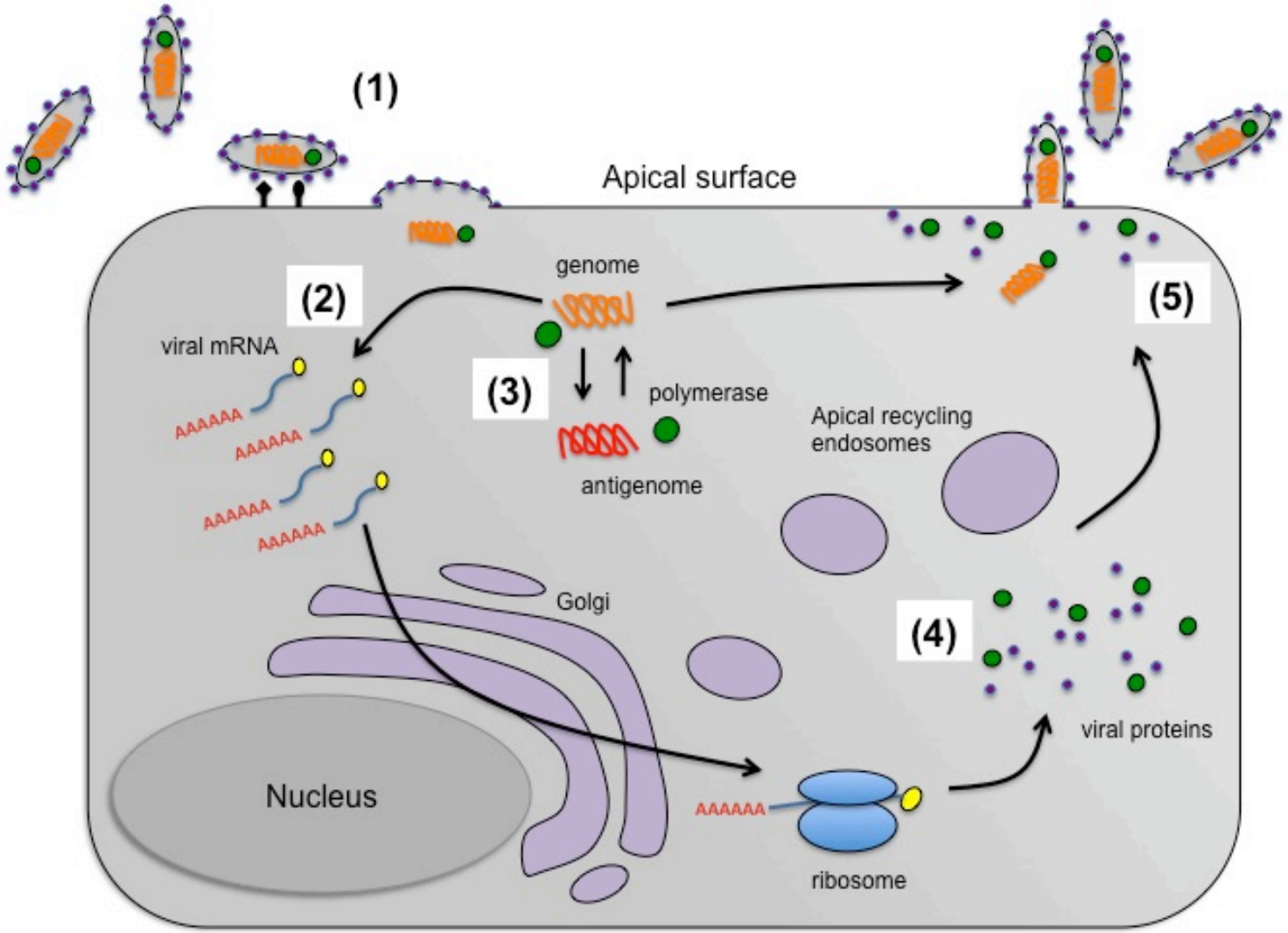

(1)
Apical surface
genome
(2)
viral mRNA
AAAAAA
AAAAAA
AAAAAA
AAAAAA
(3)
polymerase
antigenome
(5)
Apical recycling
endosomes
Golgi
(4)
viral proteins
Nucleus
AAAAAA
ribosome

Figure 3; Overview of the RSV replication cycle. (1) Entry into the cell: RSV attaches to receptors on the apical cell surface via the F and G glycoproteins and enters the cell through fusion at the plasma membrane. Nucleocapsids are released into the cytoplasm. (2) Transcription: the RSV polymerase transcribes the genome into 10 capped and polyadenylated mRNAs. (3) Replication: the polymerase also synthesizes a full-length complement of the genome, the antigenome, from which it synthesizes more copies of genome-sense RNA. (4) Protein synthesis: the mRNAs are translated using the host cell translation machinery into 11 viral proteins. Proteins are trafficked to the plasma membrane utilizing apical recycling endosomes. (5) Assembly and release: nucleocapsids and proteins are assembled at the apical cell surface at sites enriched for lipid rafts. Progeny virions bud from the plasma membrane.

RSV RNA synthesis

Genome structure and organization

RSV possesses a non-segmented, single stranded, negative sense RNA genome, which classifies it in the order *Mononegavirales*. Viruses with a similar genome structure include the human pathogens Ebola virus (EboV), Marburg virus (MarV), Nipah virus (NiV), measles (MeV) and mumps (MuV). RSV is further grouped into the family *Paramyxoviridae*, subfamily *Pneumovirinae*. The RSV A2 strain genome is 15,222 bases long and serves as the template for both transcription and replication. The genome encodes ten genes in the order, NS1, NS2, N, P, M, SH, F, G, M2, and L, which are separated by intergenic regions of varying lengths (38). The genome is bordered by two non-coding extragenic regions, the 3´, 44-nucleotide leader region (Le) and the 5´, 155-nucleotide trailer region (Tr), which contain *cis*-acting sequences involved in transcription and replication (36; 172). (Figure 4)

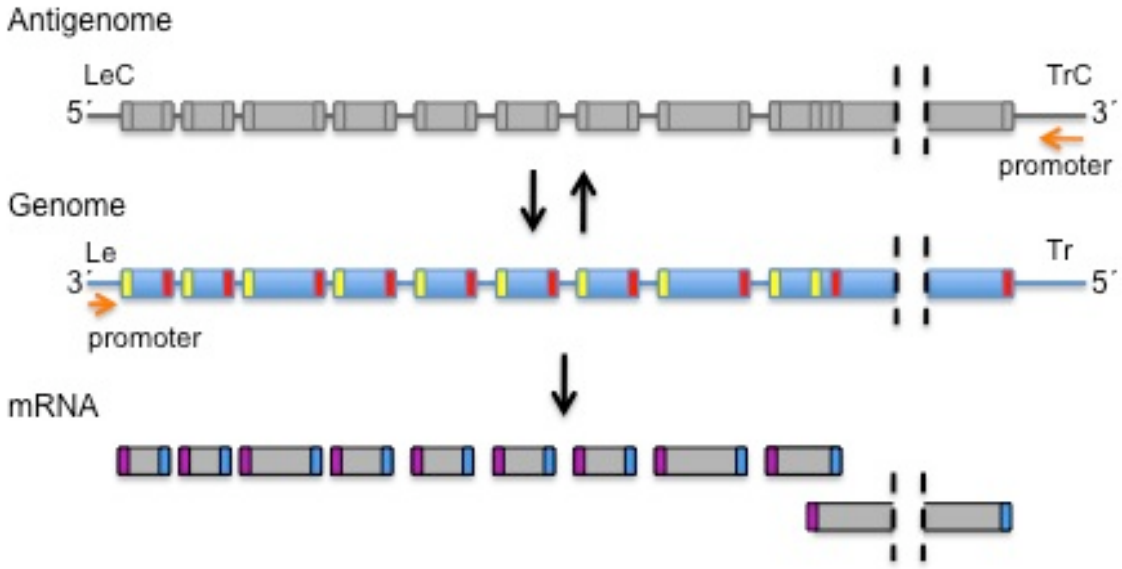


Figure 4; Schematic of the RSV genome. The negative sense RNA genome of RSV is shown at center. The genome is flanked by a 3´, 44- nt Le region, which contains the promoters for transcription and replication (indicated by the orange arrow) and a 5´, 155-nt Tr region. GS signals and GE signals are shaded yellow and red, respectively. The L gene accounts for almost half of the genome coding capacity and is therefore interrupted by dashed lines. The viral mRNAs are shown at bottom. 5´-cap represented in purple, polyA tail represented in cyan. The positive sense antigenome is shown at top. The Trailer complement (TrC) region contains the promoter for replication (orange arrow).

Polymerase components

RSV encodes an RNA-dependent RNA polymerase, which is responsible for carrying out both viral transcription and replication. The polymerase is composed of the large (L) polymerase protein and the phosphoprotein (P) cofactor (161). The stoichiometry of this association is still unknown and a crystal structure has been elusive, however early EM images indicate that the L-P complex is organized as a ring structure with globular appendages, similar to the polymerase of a related virus, vesicular stomatitis virus (VSV) (unpublished data, and (210)). Multiple enzymatic domains have been predicted within the L protein based on homology to polymerase domains from closely related viruses (203). These include domains for RNA polymerization, methyltransferase activity, and polyribonucleotidyltransferase activity (mRNA capping), indicating the L protein is multifunctional (203). This is further supported by the massive size of L, which accounts for close to half of the RSV genome coding capacity (232). The P protein may mediate the interaction between L and the nucleocapsid (169). There is also some evidence that the phosphorylation status of P may influence promoter escape (53). Although L and P represent the minimal polymerase complex necessary for RNA synthesis, the M2 ORF1 protein (M2-1) is required for fully processive mRNA transcription (34).

Transcription

Transcription of the viral genes relies on a single promoter found at the 3´-end of the genome (36; 51). Saturation mutagenesis of the Le region has shown that nucleotides 3, 4, 5, 8, 9, 10, 11, and 12 are critical for transcription, as substitution of these nucleotides ablates downstream transcription (64; 163). Furthermore, the first eleven nucleotides must be located proximal to the 3´-terminus for polymerase recruitment to the template (40). A U-rich region at the end of the Le is also important for transcription, as has been shown for VSV (62; 103; 163).

During transcription, the polymerase is directed by gene start (GS) and gene end (GE) signals, which flank each gene at their 3´- and 5´-ends, respectively. GS signals in RSV are highly conserved, 9-nucleotide sequences that direct the polymerase to initiate transcription (33; 37; 133). In VSV, GS signals also direct the polymerase to cap the nascent transcript (234). GE sequences are semi-conserved 12- or 13- nucleotide sequences, that signal the polymerase to terminate and polyadenylate the transcript (97; 132; 249). Intergenic regions are gene junctions that separate the viral genes and vary in length from 1 to 52 nucleotides (33; 114; 248). When the polymerase reaches a gene junction and terminates the mRNA, it can traverse the intergenic region to find the next GS signal and reinitiate transcription, or it can dissociate from the template. However, termination can be somewhat inefficient and result in read-through

transcription of two or more neighboring genes (38). Because transcription relies on a single promoter in the Le region, transcription is obligatorily sequential. If the polymerase dissociates at a gene junction it must reinitiate transcription at the 3´-end of the genome. This results in a gradient of gene expression such that genes located closer to the 3´-end are transcribed more frequently than genes located nearer the 5´- end (Figure 5) (30; 38; 98; 128; 131). The arrangement of genes has evolved so that genes for proteins needed in large amounts are located at the 3´-end, such as proteins involved in subverting cellular antiviral responses, and genes for proteins needed in smaller amounts are located near the 5´-end, such as the viral polymerase protein, L (38).

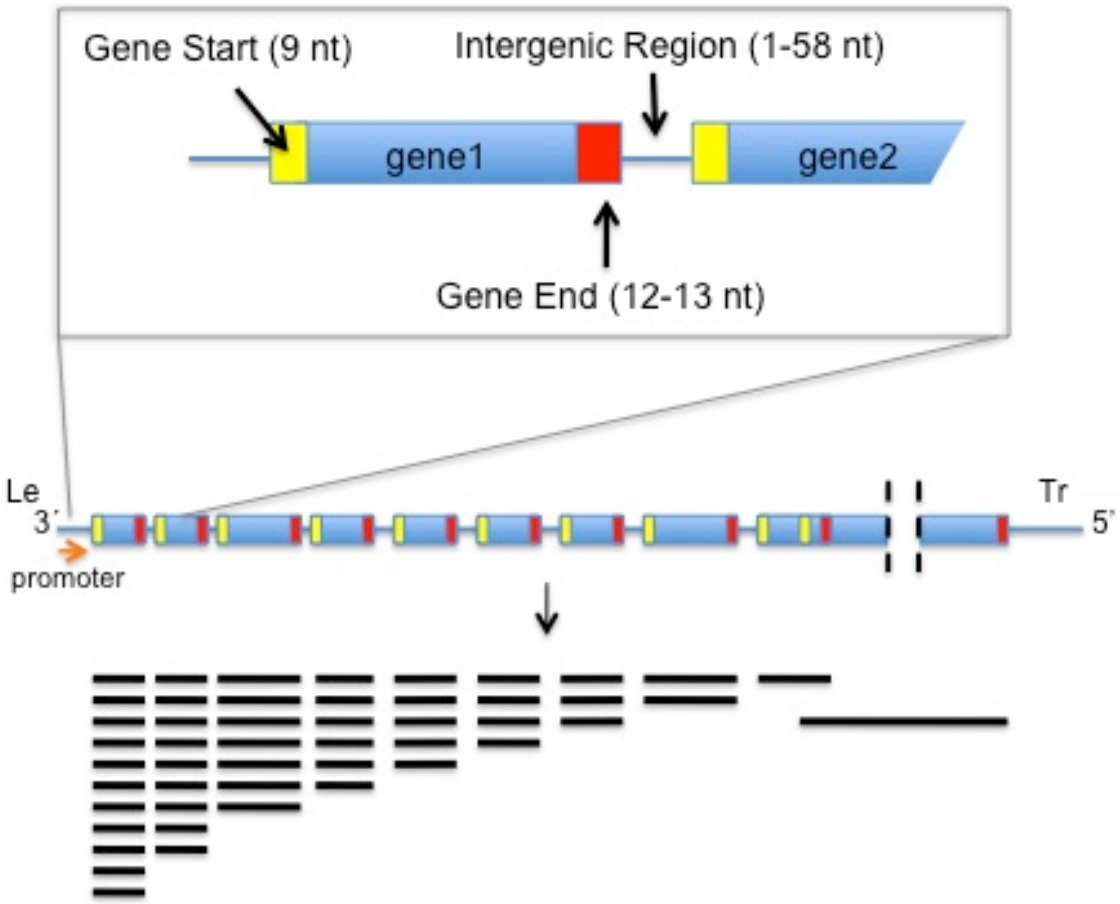


Figure 5; RSV transcription results in a gradient of gene expression. Inset shows a close up of two viral genes separated by a gene junction. Transcription is directed by a single promoter at the 3´-end of the Le (orange arrow) and is therefore obligatorily sequential. Polymerases that dissociate from the template at the gene junction must reinitiate from the 3´-end of the genome. This results in a gradient of gene expression such that genes located near the 3´-end are transcribed more frequently than genes located at the 5´-end.

The last two genes, M2 and L, are not separated by an intergenic region, but instead overlap, such that the L GS signal precedes the M2 GE signal by 68 nucleotides (37). Therefore, it was shown that for the polymerase to initiate at the L GS signal, it must scan backward from the M2 GE signal to locate the L GS signal and initiate transcription of the L gene (38; 61). In order to generate full-length L, the polymerase must readthrough the M2 GE signal (37). The behavior of the polymerase at this unusual overlapping gene junction as well as at the intergenic regions provides evidence that the polymerase is capable of scanning, similarly to cellular proteins (260).

Genome Replication

Genome replication (hereafter referred to as replication) is also dependent on the promoter sequence found at the 3´-end of the Le region, as well as sequence up to the first 34 nucleotides of the Le (36; 163; 182; 183; 239). Saturation mutagenesis of the Le indicated that in addition to the nucleotides shown to be critical for transcription, replication was also significantly impacted by mutations to nucleotides 1, 2, 6 and 7 (64). Further, the first 13 nucleotides are sufficient for recruiting polymerase and initiating RNA synthesis (40; 163). There is evidence that initiation from position +1 occurs in a primer-mediated fashion (185; 187). Minigenome constructs with substitutions or deletions of the first two nucleotides of the Le or trailer complement (TrC) promoters were still able to generate wt products, suggesting the 5´-A-C is non-templated (185; 187). During

replication, the polymerase reads through the GS and GE signals in the genome and generates a full-length complement called the antigenome, which then serves as the template for synthesis of genome sense RNA. The TrC region at the 3′-end of the antigenome is highly similar to the Le region up to the first 26 nucleotides, indicating that the two replication promoters are conserved.

Both genome and antigenome are concurrently encapsidated by the viral N protein as they are synthesized, and do not exist as naked RNA during any part of the viral lifecycle. A single N monomer binds 7 nucleotides, and assembles end-to-end with adjacent N monomers to form the helical nucleocapsid (12; 181; 241). Soluble N protein is associated with P, which may provide specificity for N binding to RSV RNA and bridge the interaction between the polymerase and the nucleocapsid (25; 43; 181). The central region of the Le appears to be required for efficient encapsidation, although this sequence has not been mapped precisely (163). Encapsidation is believed to protect the viral genome from cellular nucleases, as well as to prevent detection by pattern recognition receptors such as RIG-I and melanoma differentiation-associated protein (MDA)-5. Moreover, encapsidation has been shown to increase polymerase processivity during replication of SeV, suggesting that it may allow the polymerase to ignore GS and GE signals during replication (87; 259).

Scientific proposal and hypothesis

As described earlier, both transcription and replication are directed from promoter sequences at the 3´-end of the Le, and carried out by the same polymerase protein. The mechanism for how the polymerase is able to initiate either transcription or replication is poorly understood. Elucidating the mechanism of transcription initiation by the polymerase would not only represent an advancement for molecular biology of the *Mononegavirales*, but may also have implications for therapeutic and vaccine development for RSV. Since the RSV polymerase is responsible for all viral RNA synthesis, a better understanding of the behavior of the polymerase at the promoters may inform the rational design of novel therapeutics or attenuated vaccines. Moreover, uncovering the mechanism of transcription initiation may illuminate how the polymerase is influenced by *cis*-acting sequences, and explain how the polymerase gains access to the encapsidated template and the first GS signal. Thus, the goal of the following studies was to determine the mechanism of transcription initiation by the RSV polymerase. Based on the following lines of evidence, (1) that Le sequence 1-12 is sufficient for recruitment of polymerase, and (2) that nucleotides within this sequence are critical for transcription, **we hypothesize that Le nucleotides 1 through 12 contain the initiation site for transcription.**

This thesis contains two chapters, and an appendix. Chapter 1 describes work in which we identified a previously unrecognized initiation site at position +3 of the promoter, and characterized RNA synthesized from this site. Chapter 2 describes studies performed to test the model that transcription is initiated from position +3 of the Le promoter. Appendix 1 describes preliminary experiments to examine the effect of RNA initiated from the +3 sites of the Le and TrC promoters on RSV replication.

Materials and Methods

Gel recipes

6% urea-acrylamide: Prepared by dissolving 92 g urea (7M) (Sigma Aldrich) in 25 ml 10X Tris/Borate/EDTA (TBE) buffer (American Bioanalytical), 30 ml 40% (19:1 acryl: Bis-acryl) acrylamide (National Diagnostics), and filled to 200 ml in distilled H_2O (dH_2O). Filter sterilized.

15% urea-acrylamide: Prepared by dissolving 16.1 g urea (7M) in 3.5 ml 10X TBE, 13.125 ml 40% acrylamide (19:1), 7.175 ml dH_2O, and syringe-filtered.

20% urea-acrylamide: Prepared by dissolving 96.6 g urea (7M) in 21 ml 10X TBE, 105 ml 40% acrylamide (19:1), 16.8 ml dH_2O, and filter-sterilized.

Gel polymerization reagents: For every 100 ml of gel mix, 1.0 ml 10% ammonium persulfate (APS) (Sigma Aldrich), and 100 µl 1,2-Bis(dimethylamino)ethane; N,N,N',N'-Tetramethyl-1,2-diaminoethane (TEMED) (Sigma-Aldrich), was added and gently swirled, and gels were cast and allowed to polymerize 1-2 hours at room temperature.

1.5% Agarose-formaldehyde gels (Northern blot analysis): Gels were prepared by dissolving 1.5 g agarose (Sigma Aldrich) into 86.4 ml dH_2O, then immediately adding 10 ml 10X 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (American Bioanalytical) and 3.6 ml 36.7% formaldehyde (Sigma Aldrich).

10% SDS gels: Gels were prepared by mixing 2.87 ml dH_2O, 3.75 ml 1 M Tris, pH 8.8 (Sigma Aldrich), 100 µl 10% sodium dodecyl sulfate (SDS) solution (Fisher Scientific), 3.33 ml 30% (w/v) acrylamide (37.5:1) 0.8% bis-acrylamide (National Diagnostics), 50 µl 10% APS, and 10 µl TEMED and cast, ~1 inch from the top of the gel plates, and allowed to polymerize. A 4% stacking gel (7.3 ml dH_2O, 1.25 ml 1 M Tris, pH 6.8, 100 µl 10% SDS, 1.33 ml 30% acrylamide, 50 µl 10% APS, 10 µl TEMED) was cast on top of the 10% SDS gel.

Cell culture

HEp-2 cells (ATCC) were cultured in Opti-MEM Reduced Serum Media (Invitrogen) with 2% fetal bovine serum (FBS) (Gibco-Invitrogen), and 1% GlutaMAX-I Supplement (Invitrogen). BSR-T7/5 cells (generously provided by the Mühlberger lab) were cultured in Glasgow Minimal Essential Media (GMEM) (Invitrogen) with 10% FBS, 1% GlutaMAX-1 Supplement, and 2% 50X MEM-Amino Acids Solution (Gibco), under Geneticin (G418) (Gibco) selection. BHK-21 CCL-10 cells (ATCC) were cultured in GMEM with 8% FBS, 1% GlutaMAX-I Supplement, and 5% tryptose phosphate broth (TBP) (Sigma-Aldrich) and

Minimum Essential Media (MEM) (Invitrogen) with 2.5% FBS and 1% GlutaMAX-I Supplement. For passaging, cell monolayers were disrupted with 0.25% Trypsin-EDTA (Gibco-Invitrogen) and split 1:4 (HEp-2), 1:20 (BSR-T7/5), and 1:3 (BHK-21) twice per week. Cells were incubated at 37˚C, in 5% CO_2.

Plasmids

Plasmids expressing minigenomes used in this study have all been described previously (64; 163; 198). Non-replicating minigenome constructs included 46G (wt Le position 4G), C75Δ (Le substitution at position 4G-C), and 49G (Le substitutions at position 4G-C, and 7 U-G). Replicating minigenome constructs used in this study were lacking the first GS sequence, and included DM138 (wt Le position 4G) and DM88 (Le substitution 4G-C). A replicating minigenome construct with an intact GS sequence, DM66, was used as a molecular weight marker for sizing RNA produced from DM138 and DM88. Plasmids required for minigenome expression were pTM-1 vectors that contained open reading frames (ORFs) of the N (HG53), P (HG60), M2-1 (C32.2) and L (pBP31) proteins, expressed under the control of a T7 promoter (generously provided by Peter Collins, NIH, and described previously- (84)). The C75Δ plasmid (198) was used to synthesize the negative sense CAT riboprobe, CAT(-), and was linearized with XbaI (New England Biolabs). The DM28 plasmid, derived from C75Δ (163), was used to synthesize the positive sense CAT riboprobe, CAT (+), and was linearized using PstI (New England Biolabs). The plasmids expressing the small

LeC and Tr RNAs were generated by subcloning small RNA expression cassettes into plasmid MP28 (198), as described below.

Cloning of small RNA vectors

Plasmids were constructed to express 25-nt small RNAs corresponding to the LeC and Tr small RNAs initiated from position +3, that are natively expressed during RSV infection (Table 1). A scrambled control was also generated based on scrambled LeC sequence (Table 1). Cassettes containing a HindIII restriction site, T7 promoter sequence, the small RNA sequence, a hammerhead ribozyme, and an RsrII restriction site were generated in two rounds of polymerase chain reaction (PCR) using overlapping primers. PCR reactions contained 5 µl 10X Thermopol buffer (New England Biolabs), 1 µl 10 mM dNTP mix (Promega), 1.5 µl of each primer (100 µM stock, ordered from Invitrogen), 40.5 µl H_2O, and 0.5 µl Vent polymerase (New England Biolabs). Amplification was performed over 30 cycles with the following cycler conditions: initialization for 5 minutes at 95˚C, denaturation for 30 sec., 95˚C, annealing for 40 sec., between 55-65˚C, elongation for 45 sec. at 72˚C, and a final elongation step for 10 minutes, 72˚C. Round one PCR products were purified using the QIAQuick Gel Extraction Kit (Qiagen), and round two PCR products were purified using the MinElute PCR Purification Kit (Qiagen), according to manufacturer's instructions. Purified PCR products, were double-digested with HindIII (New England Biolabs) and RsrII (New England Biolabs) in Buffer 2 (New England Biolabs) for ~12-hours at 37˚C,

heat-inactivated for 10 minutes at 65˚C, and PCR purified. PCR products were subcloned into the MP28 vector containing corresponding restriction sites, which was also double-digested and gel extracted. PCR products were ligated with digested vector using the Quick Ligation Kit (New England Biolabs), according to manufacturer's instructions. Controls for specific ligation included ligation reactions without PCR product inserts, without the vector, and without ligase. Ligation reactions were transformed in MAX Efficiency® DH10B™ Competent Cells (Invitrogen) by heat shock at 42˚C, and selected on Lennox LB Broth with Agar (Sigma Aldrich) plates containing carbenicillin (100 mg/ml) (Fisher Scientific). Plasmids were extracted and purified from resultant bacterial colonies using the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions, and sequenced to confirm the proper insert.

Table 1; Sequence of the small RNAs expressed by T7

Construct	**Sequence (5´-3´)**
LeC nt 3-27	GCGAAAAAAUGCGUACAACAAACUU
Tr nt 3-27	GAGAAAAAAAGUGUCAAAAACUAAU
Scrambled (based on LeC)	GGAUGACAACGACAACAUAAAUCUA

PAGE purification of DNA and RNA oligonucleotides

15% urea-acrylamide gels were pre-run in 1X TBE running buffer at 10 Watts (W) for 10 minutes. 25 µg of DNA oligonucleotides or 75 µg RNA oligonucleotides were mixed 1:1 with stop buffer (1.2 ml 95% deionized formamide (Fisher Scientific), 20 mM UltraPure™ 0.5 M EDTA (Invitrogen), 0.1% w/v bromophenol blue (Sigma Aldrich), 0.1% w/v xylene cyanol (Sigma Aldrich)), boiled 5 minutes at 95˚C, and loaded in 20 µl aliquots in adjacent gel lanes. Empty lanes were loaded with 10 µl of stop buffer and the gel was run at 10-15 W for 30-45 minutes. After electrophoresis, the gel was placed on saran wrap, on top of a TLC Plastic Sheet Cellulose screen (EMD Bioscience) in relative darkness. DNA/ RNA bands were visualized by UV-shadowing using a HPTLC/TLC Portable UV Light (EMD Bioscience) and bands of the proper size were excised and placed in individual microcentrifuge tubes. Gel slices were crushed into a fine powder using a clean pipette tip, resuspended in 900 µl 0.3M sodium-acetate (NaOAc), pH 5.2 (Sigma Aldrich), and rocked overnight at 4˚C. Samples were centrifuged 16.1 X g for 10 minutes at 4˚C, and split equally between two tubes and mixed with 8 µl glycogen (5 mg/ml) (Applied Biosystems) and 10 µl 4M NaCl (Sigma Aldrich). 1 ml of 100% ethanol was added and samples were vortexed for 30 seconds and incubated overnight at -20˚C. For maximum recovery, the pelleted acrylamide from the first centrifugation was resuspended in fresh 0.3 M NaOAc and the elution was repeated as described. **Note: the next step differs for DNA and RNA oligonucleotides.* DNA samples were centrifuged 16.1 X g for

30 minutes at 4˚C, supernatent was aspirated and the pellet was washed twice in 1 ml of 70% ethanol, centrifuging 16.1 X g for 5 minutes at 4˚C. Pellets were allowed to air dry briefly and were resuspended in 20 µl diethylpyrocarbonate (DEPC)-treated H_2O (American Bioanalytical), combining pellets by transferring the resuspended material to the subsequent pellet. RNA samples were centrifuged 16.1 X g for 30 minutes at 4˚C, supernatent was aspirated and pellet was washed in 70% ethanol, centrifuging 16.1 X g for 5 minutes at 4˚C. For the second wash, two pellets were combined into a single tube (ex. six tubes becomes three tubes) and centrifugation was repeated. For the final wash, all remaining pellets were combined (ex. three tubes becomes one tube), centrifugation was repeated, and supernatent was aspirated. Pellet was resuspended in 400 µl of 2´-Deprotection Buffer (Dharmacon), vortexed for 10 seconds, centrifuged for 10 seconds, and incubated for 30 minutes at 60˚C. RNA was divided into 100 µl aliquots and dried down using an automatic environmental SpeedVac system (Savant, AES1010), 1-hour at 60˚C. Pellets were resuspended in 20 µl DEPC-treated H_2O. DNA and RNA concentrations were determined using a Nanodrop 2000 UV-vis Spectrophotometer (Thermo Fisher Scientific).

Radiolabeling of oligonucleotides

End-labeled oligonucleotides: 2 µM DNA oligonucleotides (2 µl of a 10µM stock) were mixed in a 10 µl total reaction volume with 1 µl 10X T4-polynucleotide

kinase (PNK) buffer (New England Biolabs), 3 µl DEPC-treated H_2O, 1µl T4-polynucleotide kinase (PNK) (New England Biolabs), and 3 µl ^{32}P-γ[ATP] (3000 Ci/mmol) (Perkin Elmer), and incubated for 20 minutes at 37˚C. 90 µl DEPC-treated H_2O was added to oligonucleotides labeled for primer extension analysis (100 µl total volume). 40 µl DEPC-treated H_2O was added to oligonucleotides labeled for Northern blot analysis, and unincorporated ^{32}P-γ[ATP] was removed on a GE Healthcare Illustra MicroSpin G-25 Column (Fisher Scientific) according to manufacturer's instructions. Labeled oligonucleotides were stored at -20˚C.

Riboprobe synthesis: Riboprobes were prepared by mixing 2.5 µl 100 mM dithiothreitol (DTT) (10 mM) (Sigma Aldrich), 5 µl 10X RNA polymerase buffer (New England Biolabs), 1 µl RNase inhibitor (New England Biolabs), 2.5 µl ribonucleotide (NTP) stock (2.5 mM ATP, 2.5 mM GTP, 2.5 mM UTP, 0.5 mM CTP) (Promega), 4 µl of digested plasmid (CAT+, CAT-, as described under "plasmids"), 2 µl T7 RNA polymerase (New England Biolabs), and 10 µl ^{32}P-α[CTP] (3000 Ci/mmol) (Perkin Elmer), and incubated 1-hour at 37˚C. Reactions were spiked with 1 µl RNase inhibitor and 2 µl RQ1 RNase-free DNase (Promega), and incubated 10 minutes at 37˚C. At the end of the incubation, 1 µl 0.5M EDTA and 25 µl DEPC-treated H_2O were added, and probes were extracted once in phenol (Fisher Scientific). Probes were filtered on pre-prepared columns constructed in 1-cc syringes (Fisher Scientific), plugged with Acros Organics glass wool (Fisher Scientific) and loaded up to 1-ml with G-50

Sephadex beads (Fisher Scientific) saturated in Tris-EDTA buffer (TE). Columns were washed twice with TE prior to loading the probes. The probes were added to the columns and fractions were collected in microcentrifuge tubes. 100 µl TE was added to the columns for nine subsequent fraction collections. Fractions five through eight were typically assumed to contain labeled probe and were pooled, and stored at -20˚C.

Labeling and alkaline hydrolysis of RNA ladder: RNA template corresponding to LeC1-14 sequence was ordered from Dharmacon, and PAGE purified (as described in 'PAGE purification of DNA and RNA oligonucleotides'). To end-label the RNA, 5 µl of RNA (100 µM stock) was mixed with 1 µl 10X T4-PNK buffer, 3 µl (30µCi) ^{32}P-γ[ATP], and 1 µl T4 PNK, and incubated 20 minutes at 37˚C. To hydrolyze the ladder, 10 µl of the labeled RNA was mixed with 0.6 µl tRNA (~0.2 µg; 0.4 mg/ml) (Sigma-Aldrich), and 20 µl 1X alkaline hydrolysis buffer (Ambion), and the reaction was distributed into 5 µl aliquots. The aliquots were incubated at 90˚C and were terminated at various time points (1, 2, 3, and 5 minutes) with 10 µl of stop buffer. Ladders were boiled 5 minutes at 95˚C, and 2 µl of each ladder was loaded in a 20% urea-acrylamide gel, and migrated for 30 minutes at 10 W. Non-hydrolyzed, end-labeled ladder was migrated for comparison. Gel was exposed to film 30-60 minutes at room temperature, and the ladder with the most distinct bands was selected for use as a marker in the *in vitro* RNA synthesis assay.

Labeling of 10 bp ladder: A 10 bp DNA ladder (Invitrogen) was used for sizing primer extension analysis products larger than 100 nt. The 10 bp ladder consists of 33 10-bp repeats and a fragment at 1668 bp. The ladder was labeled and denatured according to the manufacturer's instructions. Ladder stock was diluted tenfold in TE to a final concentration of 0.1 µg/µl. On ice, 2-µl ladder was mixed with 1 µl 5X exchange reaction buffer (250 mM imidazole (Sigma-Aldrich), pH 6.4, 60 mM $MgCl_2$, 5 mM β-mercaptoethanol (Sigma-Aldrich), 350 µM adenosine diphosphate (ADP) (Sigma Aldrich)), 1 µl ^{32}P-γ[ATP], and 1 µl T4-PNK, and incubated 10 minutes at 37 ˚C. The reaction was terminated by heating for 5 minutes at 55˚C. An equal volume (5µl) of stop buffer was added to the labeled ladder and incubated for 5 minutes at 70 ˚C. Ladder was stored at -20˚C.

Virus

Wildtype (wt) RSV was strain A2 (prepared by Robin Djang) and LeC virus (prepared by Laura Dickey) was based on this strain. Modified vaccinia Ankara expressing T7 polymerase (MVA-T7, originally provided by Bernard Moss, NIH) is a highly attenuated strain of vaccinia virus which was used to provide T7 polymerase in *trans* to drive expression of plasmids during transfections. MVA-T7 was propagated in BHK-21 cells, split 1:8 into seven Corning T-150 culture flasks (Fisher Scientific). 10 µl MVA-T7 was added to 190 ml MEM, and 30 ml of virus was added to each of six flasks (30 ml media without virus was added to

one flask for mock infection), and flasks were incubated for 48 hours at 37˚C. Cells were harvested 48-hpi by scraping into media, and collected in 50-ml tubes. Tubes were centrifuged in a Beckman Allegra X-15R centrifuge (Beckman Coulter) 1200X g, for 10 minutes at 4˚C, and supernatent was discarded. The pellets were resuspended in 1 ml MEM and pooled, aliquoted into cryovials (Fisher Scientific), and snap-frozen on dry ice and thawed in a 37˚C water bath, four times. Virus was distributed in 150 µl aliquots and stored at -80˚C.

Virus titration and plaque visualization

HEp-2 cells seeded in 24-well plates were infected at 50-60% confluence. Serial dilutions of virus were prepared in Opti-MEM and a 200-µl inoculum was added to the cells. Virus was allowed to adsorb for 2 hours at 37˚C, and the cells were overlaid with 0.8% methylcellulose (4 g methylcellulose (Sigma-Aldrich) autoclaved with stir bar, 500 ml Opti-MEM, 2% FBS, 1X Antibiotic-Antimycotic (Gibco), stirred at 4˚C for 4 days), and incubated at 37˚C, 4-5 days, until syncytia were visible. Cells were fixed twice with pre-chilled 80% methanol (American Bioanalytical) at 4˚C for 1 hour, and overnight. Cells were washed three times in dH_2O by submerging in a water bath, and blocked in 5% milk powder (Shaw's Supermarket) prepared in 1X phosphate-buffered saline (PBS) (Sigma Aldrich) for 1-hour at room temperature, with gentle shaking. Cells were incubated with a monoclonal mouse antibody specific to RSV F protein (Serotec) diluted 1:200 in 5% milk solution, 1-hour at room temperature. Cells were washed three times

and incubated with goat-anti mouse immunoglobulin (Ig) G conjugated with horseradish peroxidase (HRP) (Abcam) diluted 1:500 in 5% milk solution, 1-hour at room temperature. Cells were washed three times and plaques were visualized by the addition of a 1:1 mixture of 4CN (4-chloro-1-napthol) and peroxidase substrate solution B (KPL). Plaques were counted from the highest dilution of virus, and titer was calculated using the formula: plaque forming units (PFU)/ ml = [(number of plaques) X (1/ dilution factor)] / (inoculum volume in ml)

Virus infections

For analysis of total viral RNA, wt RSV stock (titer 2.0 X 10^7 PFU/ml) was used to infect HEp-2 cells 80-90% confluent in T-75 culture flasks, at a multiplicity of infection (MOI) of 5 PFU/ml, in a 5 ml inoculum volume. Virus was allowed to adsorb for 1-hour at 37˚C, and the inoculum was aspirated and replaced with 15 ml fresh media and serum. Cells were harvested at 17-hpi by scraping into the media, cells were centrifuged 3.5 x g for 5 minutes at 4˚C, and the pellet was disrupted in TRIzol Reagent (Invitrogen). TRIzol preparations were stored at -80˚C until ready to harvest viral RNA (as described in 'RNA isolation'). To test the effect of overexpression of the small RNAs on RSV transcription and replication, wt RSV was used to infect 6-well plates transfected with the small RNA expression plasmids as described in 'Minigenome transfections', at an MOI of 3 PFU/ml, in a 500 µl inoculum volume. Virus was allowed to adsorb 1-2 hours at 37˚C, cells were washed with 1X PBS, and 1.5 ml Opti-MEM with 2%

FBS was added to the cells. Cells were harvested 24-hpi and after centrifugation, cells were resuspended in 1 ml 1X PBS, and distributed for protein and RNA analysis. 200 µl of cell suspension was prepared for protein analysis (as described in 'Western blot analysis') and 800 µl of cell suspension was mixed with 1 ml TRIzol for RNA isolation. For immunofluorescence analysis of the effect of the small RNAs on stress granule formation, transfected cells in 12-well plates were infected with LeC virus (titer 3.45 X 10^6 PFU/ml) at an MOI of ~1 PFU/ml, in a 200 µl inoculum volume. Virus was allowed to adsorb 3 hours at 37˚C, cells were washed in 1X PBS, and 1 ml Opti-MEM with 2% FBS was added to the cells. Cells were fixed at 24- and 48-hpi, as described in 'Immunofluorescence analysis.

Fractionation analysis

Viral RNA was fractionated as previously described (160) with modifications. Infections were performed as described in 'Virus infections'. At 17 hpi, cells were treated with actinomycin D (AcD) (provided as a powder from Sigma; 1 mg dissolved in 50 µl DMSO and 950 µl 1X PBS) for 1 hour at 37˚C, followed by an ice-cold 1X PBS wash. Cells were lysed in 5 mL ice-cold 1X PBS and 250 µg L-α-lysophosphatidylcholine (Sigma-Aldrich) by hand rocking, 1 minute on ice. PBS was aspirated and cells were immediately scraped into 400 µl Buffer A (50 mM Tris-acetate, pH 8.0, 100 mM potassium-acetate, 1 mM DTT*, 2 µg/ml AcD*, **DTT, AcD added fresh immediately prior to the start of the experiment*) and

collected in a microcentrifuge tube. The solution was disrupted by passaging 20 times in a 20-gauge needle, and incubated on ice for 10 minutes. The solution was centrifuged 2400X g for 10 minutes at 4˚C to generate fraction one (S1) and pellet one (P1). P1 was resuspended in 200 µl Buffer B (10 mM Tris-acetate, 10 mM potassium-acetate, 1% Triton™-X100 (Sigma-Aldrich), 1.5 mM $MgCl_2$) and disrupted with a 20-gauge needle. The solution was incubated and centrifuged as above to generate S2 and P2. P2 was resuspended in 300 µl Buffer C (10 mM Tris-acetate, 10 mM potassium-acetate, 1% Triton™-X100, 1.5 mM $MgCl_2$, 0.1% Tween-40 (Sigma Aldrich), 0.5% sodium deoxycholate (DOC) (Sigma-Aldrich)). 100 µl of each fraction was mixed with 1 ml TRIzol and RNA was isolated as described in 'RNA isolation'.

Minigenome transfections

Lipofectin transfection: Lipofectin Reagent (Invitrogen) was used to transfect HEp-2 cells according to the manufacturer's instructions. Cells were seeded in 6-well polystyrene plates and transfected at 60-70% confluence. Lipofectin (10 µl/ well) was prepared in Opti-MEM (90 µl/ well, no additives), vortexed, and incubated for 30 minutes at room temperature. Helper plasmid master mix was prepared by combining 0.4 µg N, 0.2 µg P, 0.1 µg M2-1, and 0.1 µg codon-optimized L plasmids, and 8 µl of this master mix was added to 92.5 µl Opti-MEM (per well) in a polystyrene tube. Master mix without L plasmid was also prepared for a –L control transfection. 0.2 µg of the appropriate minigenome (per well)

was added to each tube containing plasmid master mix. At the end of the 30-minute incubation, 100 µl of the lipofectin mix was added to each tube, and tubes were vortexed and incubated 15 minutes at room temperature. MVA-T7 was prepared for coinfection to provide T7 polymerase in *trans* for plasmid RNA expression. MVA-T7 (10 µl/ well) was mixed with Opti-MEM (900 µl/ well), vortexed and sonicated for 30 seconds (twice), to disrupt viral aggregates. 900 µl MVA-T7 was added to each tube containing lipofectin and plasmids. Tubes were vortexed and 1 ml of the final mixture was added to each well. Plates were incubated 6 hours to overnight at 37˚C, and media was replaced with Opti-MEM containing 2% FBS and 1% L-glutamine. Transfections were harvested 48-hours post transfection.

Lipofectamine 2000 transfection: Lipofectamine 2000 (Invitrogen) was used to transfect BSR-T7/5 cells according to the manufacturer's instructions. For small RNA overexpression experiments, BSR-T7/5 cells (300,000 cells/ well) were seeded in 6-well plates and transfected at 50-60% confluence. For each well, 0.5 µg of the plasmid was added to 150 µl Opti-MEM, and mixed with 108 µl Lipofectamine in Opti-MEM (8 µl Lipofectamine/ well, and 100 µl Opti-MEM/ well, vortexed and incubated for 5 minutes at room temperature). DNA/ Lipofectamine mix was incubated 20 minutes at room temperature, and 263 µl was added dropwise to each well (washed with 1X PBS, and containing 750 µl Opti-MEM). For immunofluorescence assays, BSR-T7/5 cells were seeded very low (~20,000

cells/ well) in 12-well plates containing glass coverslips, and transfected at 30-40% confluence. For each well, 0.5µg of the plasmid was added to 50 µl Opti-MEM, and mixed with 54 µl Lipofectamine in Opti-MEM (4 µl Lipofectamine/ well, and 50µl Opti-MEM/ well, vortexed and incubated for 5 minutes at room temperature). DNA/ Lipofectamine mix was incubated for 20 minutes at room temperature, and 108 µl was added dropwise to each well (washed with 1X PBS, and containing 500 µl Opti-MEM). All plates were incubated for a minimum of 6-hours at 37˚C, and Opti-MEM with 6% FBS was added to all wells (500 µl/ well).

Nuclease protection assay

Cells were harvested by scraping into media, collected in a 1.5 ml microcentrifuge tubes and pelleted 3.5 X g for 5 minutes at 4˚C. 50 ml reticulocyte standard buffer (RSB)-100 (10 mM NaCl, 10 mM Tris, pH 7.5 (Sigma-Aldrich), 1.5 mM $MgCl_2$, 1% Triton™ X-100, 0.5% DOC) was prepared. Cell pellets were resuspended in 100 µl total RNA buffer (998 µl RSB-100, 1 µl aprotinin (Fisher Scientific), 1 mM $CaCl_2$) or 100 µl nuclease buffer (988 µl RSB-100, 1 µl aprotinin, 1 mM $CaCl_2$, 1 µg Micrococcal/S1 nuclease (Thermo-Fisher)). Total RNA samples were mixed with 1 ml TRIzol, incubated for 5 minutes at room temperature, and RNA was extracted. Nuclease-treated samples were incubated in a 30˚C waterbath for 1 hour, vortexing twice to resuspend the pellet. Following incubation, 1 ml TRIzol was added to nuclease-treated samples and RNA was extracted.

RNA isolation

Cells were harvested by scraping into media, collected in a 1.5 ml microcentrifuge tube and pelleted 3.5 X g for 5 minutes at 4˚C. Supernatent was aspirated and cell pellets were resuspended in 1 mL TRIzol, and incubated 5 minutes at room temperature. 200 µl Chloroform:Isoamyl alcohol (24:1) (Sigma-Aldrich) was added, tubes were shaken vigorously for 15 seconds and incubated 3 minutes at room temperature, and centrifuged 12,000 X g for 15 minutes at 4˚C. The aqueous phase was transferred to new tube with 500 µl of isopropanol (American Bioanalytical), mixed by pipetting, and incubated 10 minutes at room temperature. Tubes were centrifuged 12,000 X g for 10 minutes at 4˚C, and supernatent was carefully aspirated. Pellet was dried briefly, resuspended in 300 µl DEPC-treated H_2O, and incubated 10 minutes at room temperature, with frequent vortexing to resuspend the pellet. 33 µl of 2M NaCl-Tris-EDTA (NTE) buffer (2M NaCl, 40 mM Tris, pH 7.4, 1 mM EDTA) was added, and tubes were vortexed. 330 µl phenol was added and tubes were shaken 15 seconds, and centrifuged 7,000 rpm for 3 minutes at 4˚C. The aqueous phase was transferred to a new tube with 330 µl chloroform, shaken 15 seconds, and centrifuged 7,000-rpm for 3 minutes at room temperature. The aqueous phase was transferred to a new tube with 700 µL 100% ethanol, mixed by inverting five to six times, and stored at -80˚C overnight. To precipitate RNA, tubes were centrifuged 16.1 X g for 20 minutes at 4˚C, and supernatent was carefully aspirated. The pellet was

washed in 200 µL 70% ethanol, centrifuged 16.1 X g for 10 minutes at room temperature, and supernatent was carefully aspirated. The pellet was resuspended in 30-50 µl DEPC-treated H_2O, and RNA was snap-frozen on dry ice and stored at -80˚C.

Exonuclease digestion

RNA was digested with Terminator™ 5´-Phosphate-Dependent Exonuclease (Epicentre Biotechnologies) according to the manufacturer's instructions. Terminator Exonuclease is a 5´ to 3´exonuclease that specifically digests RNA containing a 5´-monophosphate. 5 µl of RNA was incubated with 11.5 µl DEPC-treated H_2O, 0.5 µl RNase Inhibitor, 2µl 10X Reaction buffer A (Epicentre Biotechnologies), and 1 µl (1 unit) Terminator Exonuclease, in a 30 µl total reaction volume, for 1 hour at 30˚C. The reaction was terminated by adding 180 µl DEPC-treated H_2O (to bring the reaction volume up to 200 µl), and RNA was extracted once in phenol. The aqueous phase was placed in a new tube and mixed with 0.1 volume of 3M NaOAc and 2.5 volumes of 100% ethanol, and incubated for 30 minutes at -20˚C. RNA was pelleted by centrifuging 16.1 X g for 30 minutes at 4˚C. Supernatent was aspirated, and the pellet was washed once with 70% ethanol by centrifuging 16.1 X g for 10 minutes at 4˚C. RNA was resuspended in 30 µl of DEPC-treated H_2O and stored at -80˚C.

Tobacco acid pyrophosphatase digestion

Tobacco acid pyrophosphatase (TAP) (Epicentre Biotechnologies) hydrolyzes the phosphoric acid anhydride bonds in the triphosphate bridge of 5´-cap structures, leaving a 5´-monophosphate end on the decapped RNA. RNA was treated with TAP according to the manufacturer's instructions. ~25 pmol (5 µl) of RNA was incubated with 5 µl 10X TAP buffer (0.5 M NaOAc, pH 6.0, 10 mM EDTA, 1% β-mercaptoethanol, 0.1% Triton® X-100, provided by Epicentre Biotechnologies), 35 µl DEPC-treated H_2O, and 2.5 µl TAP (25 units), in a 50 µl total reaction volume, and incubated 2-hours at 37˚C. The reaction was terminated by adding 150µl of DEPC-treated H_2O (to bring the reaction volume up to 200 µl) and RNA was extracted once in phenol. The aqueous phase was placed in a new tube and mixed with 0.1 volume of 3M NaOAc and 2.5 volumes of 100% ethanol, and incubated for 30 minutes at -20˚C. RNA was pelleted 16.1 X g for 30 minutes at 4˚C. Supernatent was aspirated and the pellet was washed with 70% ethanol and centrifuged 16.1 X g for 10 minutes at 4˚C. RNA was resuspended in 30 µl of DEPC-treated H_2O and stored at -80˚C.

Primer extension analysis

Primer extension analysis, utilizing end-labeled oligonucleotides (table 2) and the Sensiscript RT Kit (Qiagen), was used to map the 5´-ends of RNA. 5 µl (typically one-fifth to one-tenth of a well or flask) of RNA was mixed with 7 µl DEPC-treated H_2O, 2 µl 10X RT Buffer (Qiagen), 2 µl 5 mM dNTP mix (Qiagen), 2 µl 10 µM ^{32}P-

γ[ATP]-labeled oligonucleotide, 1 µl RNase inhibitor, and 1 µl Sensiscript Reverse Transcriptase (Qiagen), and incubated 90 minutes at 37˚C. Reactions were terminated by adding 20 µl stop buffer. 6% urea-acrylamide gels were pre-run in 1X TBE for 30-60 minutes at 35 milliampere (mA). Samples were boiled 5 minutes at 95˚C, 20 µl of samples were loaded into the gel, and the gel was run 90-120 minutes at 35 mA. Gels were dried down onto Whatman 3MM Chromatography Paper (Fisher Scientific) for 2-hours at 80˚C and exposed to Kodak BioMax MR 8 X 10 inch film (Fisher Scientific) at -80 ˚C for various lengths of time (typically 24 hours to two weeks).

Table 2; Oligonucleotides used for primer extension analysis.

Oligonucleotides were named for the sense and nucleotide positions the primer corresponded to. 'g'= 'genome' sense (to detect RNA containing antigenome sequence) , 'ag'= 'antigenome' sense (to detect RNA containing genome sequence).

Oligo	Sequence (5´-3´)
g15-39	TTTGGTTTATGCAAGTTTGTTGTAC
g45-68	ACTTATCAAATTCTTATTTGCCCC
g24-50	TTCTCCATTCTAGAGGTTTATGCAAGT
g56-75	AAGTGGTACTTATCAAATTC
g91-113	TGAATTGCTGCCCATCTCTAACC
ag13-35	TACGAGATATTAGTTTTTGAC
ag32-55	TATATGTGTATTAACTAAATTACG

Northern blot analysis

This protocol was used for analysis of RNA in the range of ~100 to ~20,000 nt. RNA samples were prepared (35 µl total volume) by mixing 5µl RNA (one-fifth to one-tenth of a well or flask) with 6 µl DEPC-treated H_2O, 2 µl 10X MOPS buffer, 7 µl 36.7% formaldehyde, and 20 µl 95% DIF, and incubated 10 minutes at 65˚C. Samples were mixed with 6.6 µl 6X Loading Dye Solution (Fisher Scientific) and migrated in 1.5% agarose-formaldehyde gels. Gels were run at 110 Voltz (V) in 1X MOPS buffer, approximately 2-hours, and transferred to Whatman Protran Nitrocellulose Blotting Membranes (Fisher Scientific) using the Whatmann TurboBlotter Downward Capillary Transfer System (Sigma-Aldrich) in transfer buffer (3M NaCl, 8 mM NaOH), overnight. Membranes were neutralized in 6X SSC for 15 minutes at room temperature, dried briefly, and UV-crosslinked in a CL 1000 Ultra Violet Crosslinker (UVP) at 1200 Joules (J). For hybridization with end-labeled probes, membranes were pre-hybridized for 1 hour at an appropriate hybridization temperature (see table 3) in hybridization buffer (5X Denhardt's Solution (Sigma-Aldrich), 6X SSC, 0.1% SDS, 0.01% sodium pyrophosphate decahydrate (NaPPi) (Sigma-Aldrich)). For hybridization with riboprobes, membranes were pre-hybridized for 1 hour at 65˚C in hybridization buffer (6X SSC, 2X Denhardt's Soln., 0.5% SDS, and 100 µg salmon sperm DNA (Invitrogen) boiled for 5 minutes at 95˚C to shear DNA). 25-50 µl of radiolabeled probe was added to the hybridization buffer and membranes were incubated 12-18 hours. Blots probed with end-labeled oligonucleotides were washed twice in

6X SSC for 15 minutes at room temperature, and twice in 6X SSC for 10 minutes at the hybridization temperature. Blots probed with riboprobes were washed in 2X SSC and 0.1% SDS for 2 hours at 65˚C, and in 0.1X SSC and 0.1% SDS for 15 minutes at 65˚C. Blots were dried briefly, wrapped in saran wrap, and exposed to film in a cassette at -80˚C for various lengths of time (generally 24-hours up to 2 weeks).

Northern blot analysis of small RNA

This protocol was used for the detection of small RNA in the range of ~17 to ~1,000 nt (adapted from (263)). 6% urea-acrylamide gels were pre-run in 1X TBE for 30 minutes, at 200 V. RNA samples were prepared by mixing RNA 1:1 with stop buffer, and incubated for 20 minutes at 65˚C. A low-range ssRNA ladder (New England Biolabs) was prepared by mixing 2 µl ladder (1µg) with 3 µl DEPC-treated H_2O and 5 µl 2X RNA ladder loading buffer (New England Biolabs), incubated for 5 minutes at 65˚C, and chilled on ice. A microRNA Marker (New England Biolabs) in ready-to-load denaturing solution was incubated 5 minutes at 65˚C, 5 minutes at 95˚C, and chilled on ice. Samples were loaded in 6% urea-acrylamide gels and run in 1X TBE, for 20-30 minutes at 150 V. The ladders were cut off the gels prior to transfer and stained with ethidium bromide (Sigma-Aldrich) for UV-visualization. Gels were transferred to Whatman Nytran Nylon positively charged 0.45 µ membranes (Fisher Scientific)

as described above. Blots were neutralized in 6X SSC, UV-crosslinked, and hybridized as described above.

Table 3; Oligonucleotides used for Northern blot analysis. Oligonucleotides were named for the sense and nucleotide positions the probe corresponded to. 'g'= 'genome' sense (to detect antigenome-containing RNA) , 'ag'= 'antigenome' sense (to detect genome-containing RNA). Probes indicated with an asterisk were locked nucleic acid (LNA) probes, modified with an LNA base every third nucleotide. Probes indicated with a diamond were CAT riboprobes. Riboprobe sequences are not shown here because they represent long (up to 1000 nt) T7 transcribed run-off products. The annealing temperatures (Ta˚C) used for hybridization were determined by subtracting 3-5˚C from the oligonucleotide melting temperatures.

Oligo	Sequence (5´-3´)	Ta˚C
g7-34 *	TTTATGCAAGTTTGTTGTACGCATTTTT	62
g56-75	AAGTGGTACTTATCAAATTC	41
g504-540	GCTAGTTGATATTAATTATAATTTATGGATTAAGATC	56
ag5-32 *	GAGATATTAGTTTTTGACACTTTTTTTC	62
CAT (-) ◆	CAT-specific probe to detect anti-minigenome/ mRNA	65
CAT (+) ◆	CAT-specific probe to detect input minigenome	65

Protein expression

A codon-optimized version of the RSV (strain A2) L protein ORF was chemically synthesized (GeneArt), and the mutant polymerase, N812A, was generated by QuickChange site-directed mutagenesis (Aligent Technologies). Wt and mutant polymerase proteins were cloned in the pFastBac Dual vector (Invitrogen) together with the P protein ORF. The P protein was tagged with a hexahistidine sequence, with a tobacco etch virus (TEV) protease cleavage site between the ORF and the histidine tag. Baculoviruses were recovered using the Bac-To-Bac system (Invitrogen) and used to infect Sf21 cells. RSV L-P polymerase complexes were isolated and purified from cell lysates by affinity chromatography and size-exclusion, cleaved with AcTEV Protease (Invitrogen), and analyzed by SDS-PAGE and PageBlue protein staining as described below.

SDS-PAGE and PageBlue protein staining

Wt L-P polymerase complex and the N812A mutant L-P polymerase complex were mixed 1:1 with Laemmli's Sample Buffer 2X Concentrate (BioRad) with 250 mM DTT added, boiled 5 minutes at 95˚C, and migrated in a 10% SDS-polyacrylamide gel with a 4% stack, 100 V for 1 hour. The BenchMark Prestained Protein Ladder (Invitrogen) and the MagicMark XP Western Protein Standard (Invitrogen) were used to size the protein bands. The gel was fixed in 100 ml 40% ethanol and 10% acetic acid (Sigma-Aldrich) for 10 minutes, then rocked in 100 ml PageBlue Protein Staining Solution (Thermo-Fisher) for 1-2

hours. Gel was washed in dH_2O five times, until wash poured off clear. Gel was dried down on Whatman 3MM chromatography paper at 80˚C for 2 hours.

Western blot analysis

Protein samples were prepared from cell pellets resuspended in 1 ml 1X PBS. 200 µl of lysate was mixed 1:1 with Laemmli's buffer 2X concentrate and vortexed. Samples were boiled 5 minutes at 95˚C, and migrated in 10% SDS-polyacrylamide gels poured with a 4% stack, 125 V in 1X SDS-PAGE running buffer (10X SDS-PAGE running buffer prepared by dissolving 60.6 g tris base (Sigma-Aldrich), 288 g glycine (Sigma-Aldrich), 20 g SDS in 2 L dH_2O), for 1-2 hours. The PageRuler Plus Prestained Protein Ladder (Thermo-Fisher) was used as a molecular weight marker. 1X Western transfer buffer was prepared by mixing 40 ml 25X tris-glycine buffer (prepared by dissolving 18.2 g tris base (12 mM) and 90.0 g glycine (96 mM) in 500 ml dH_2O) with 200 ml methanol and 760 ml dH_2O. Gels were transferred to nitrocellulose membranes in 1X transfer buffer for 2 hours at 25 V. Membranes were blocked in 5% milk solution in PBS-Tween (PBS-T) (1X PBS and 0.1% Tween-20 (Sigma-Aldrich)) overnight at 4˚C, with gentle rocking. Membranes were washed 5 minutes in PBS-T, three times, and incubated with primary antibody 1-hour at room temperature. Membranes were washed three times, and incubated with secondary antibody 1-hour at room temperature. Membranes were washed three times and exposed to 3 ml Western Lightning Chemiluminescence Reagent (Perkin Elmer) for 1 minute.

Membranes were exposed to X-Omat Blue film (Fisher Scientific) for 1, 3, and 5 minutes.

Immunofluorescence microscopy

To examine the effect of the RSV small RNAs on stress granule formation, cells grown on coverslips were transfected with small RNA overexpression plasmids and infected with LeC virus, as described. As a positive control for stress granule formation, mock-transfected/ mock-infected cells were treated with 0.5 mM sodium arsenite (Sigma-Aldrich) for 2-hours at 37˚C, immediately prior to fixation. At 48- and 72-hours post transfection, cells were washed in 1X PBS and 2 ml of pre-chilled fixative (5% formaldehyde, 2% sucrose, in 1X PBS) was added to each well. After fixing, cells were washed in 1X PBS and 1 ml permeabilization buffer (0.5% Igepal (Sigma-Aldrich), 10% sucrose, in 1X PBS) was added for 20 minutes at room temperature, with gentle shaking. Coverslips were blocked in 1X PBS with 5% FBS for 1-hour at room temperature, and primary antibody cocktail (anti-RSV N protein (Serotec) diluted 1:500, and anti-eIF3 (Santa Cruz) diluted 1:100) was prepared in 1X PBS. Coverslips were incubated with primary antibodies for 1-hour at room temperature in a humid chamber, and washed 5 minutes in 1X PBS with 5% FBS, three times. Isotype-specific secondary antibodies labeled with AlexaFluor 488 and AlexaFluor 568 (Invitrogen, diluted 1:1,000) were mixed with 0.1 µg 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) in 1X PBS, and cells were incubated 1-hour at room

temperature in the humid chamber, in darkness. Cells were washed 5 minutes in 1X PBS with 5% FBS, three times, mounted on slides with VectaShield Mounting Medium for Fluorescence (Fisher Scientific), and visualized by fluorescence microscopy.

In vitro RNA synthesis assay

RNA oligonucleotides corresponding to nt 1-14 of the Le promoter, the LeC, and a mutant Le (position 2G-A) (Dharmacon) were PAGE purified as described. For radiolabel incorporation reactions, 10 µl of 10 µM RNA template was combined with 5µl 10X RNA synthesis buffer (500 mM Tris HCl pH 7.4, 500 mM NaCl, 50 mM $MgCl_2$, 50 mM DTT), 20 µl 1 mM NTP mix (containing all four NTPs, 10mM stock concentrations, New England Biolabs), 12 µl DEPC-treated H_2O, and 1µl (10µCi) ^{32}P-α[GTP] (3000 Ci/mmol) (Perkin Elmer). End-labeling reactions were identical to incorporation reactions, except that the cold GTP or ATP concentration was reduced to 10 µM in the NTP mix for reactions labeled with ^{32}P-γ[GTP] or ^{32}P-γ[ATP], respectively. For –UTP experiments, DEPC-treated H_2O was added to the NTP mix in place of UTP. Reactions were mixed by pipetting and 1 µl of the purified L/P polymerase complex (containing ~100 ng of L protein) was added (without mixing), in a final volume of 50 µl. Reactions were incubated for 3-hours at 30˚C, followed by incubation for 3 minutes at 90˚C to inactivate the polymerase, and cooled on ice. For reactions containing ^{32}P-α[GTP], 1 µl calf intestinal phosphatase (CIP) (New England Biolabs) was added

and reactions were incubated for 1-hour at 37˚C. For reactions containing ^{32}P-γ[GTP] or ^{32}P-γ[ATP], 7.5 µl 10% SDS, 0.5 µl 100 mM Tris, pH 7.4, 0.5 µl 500 mM EDTA and 0.5 µl Proteinase K (New England Biolabs) was added, and reactions were incubated for 45 minutes at 45˚C. Reactions were extracted once by adding 200 µl H_2O and 5 µl 10% SDS, with 250 µl UltraPure Phenol:Chloroform:Isoamyl alcohol (25:24:1) (Invitrogen) and vortexing for 30 seconds. Reactions were incubated 5 minutes on ice and centrifuged full speed for 5 minutes at room temperature. The aqueous phase was transferred to a new tube containing 10 µl 4M NaCl and 1 µl GlycoBlue Coprecipitant (Applied Biosystems). 500 µl 100% ethanol was added and tubes were shaken to mix, and incubated overnight at -20˚C. The RNA was precipitated by centrifuging full speed for 30 minutes at 4˚C, and the pellet was washed in 800 µl 70% ethanol, briefly air-dried, and resuspended in 30 µl DEPC-treated H_2O. The RNA was combined 1:1 with stop buffer, boiled 5 minutes at 95˚C, and analyzed by electrophoresis on a 20% urea-acrylamide gel. Gels were exposed to film and the nucleotide lengths of the RNA products were determined by comparison with a LeC molecular weight ladder generated by alkaline hydrolysis as described.

Quantitation and statistical analysis

All experiments were performed with consistent results three times, unless otherwise indicated. For quantitation, phosphorimager analysis was performed on a Personal Molecular Imager (PMI) System (BioRad) using Quantity One 1-D

Analysis Software (BioRad). Data are presented as the mean ± standard error pooled from three replicates.

Chapter 1: Additional initiation sites in the RSV promoters

Introduction

Cis-acting sequences involved in transcription.

Since transcription and replication are both initiated from a promoter sequence at the 3´-end of the Le, this raises the question of how two distinct RNA synthesis processes are initiated from the same or overlapping promoters. Sequence from positions 1 to 13 has been shown to be sufficient for recruitment of both transcription- and replication-competent polymerases (40). Furthermore, saturation mutagenesis of the Le implicated many of the same nucleotides (nt 3, 5, 8, 9, 10 and 11) as being critical for both transcription and replication (64). However, mutations to nucleotides 4 and 12 appeared to specifically affect transcription (64). Additionally, transcription required a uridine-rich region at the end of the Le, as well as the first GS signal (40; 163). A schematic of the *cis*-acting elements involved in transcription is shown in Figure 6.

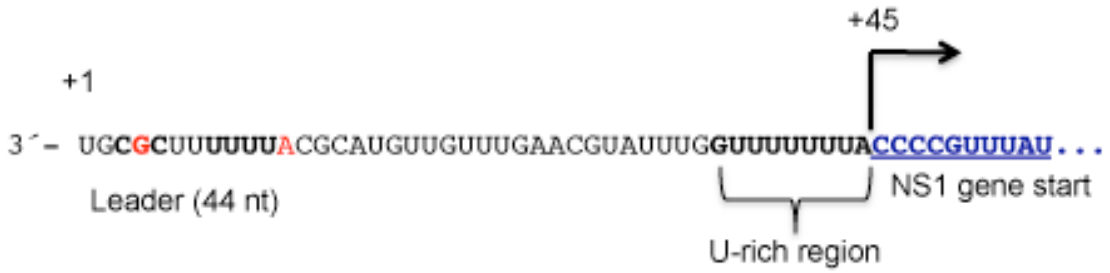


Figure 6; Schematic of the Le sequence elements involved in transcription. The sequence of the 44-nt Le region and NS1 GS (blue) is shown. Nucleotides critical for transcription indicated in bold. Note that positions 4 and 12 (red) can be substituted with little effect on replication, while having a great effect on transcription, indicating these positions are optimized for transcription. U-rich region at the end of the Le indicated in bold. Transcription initiates from position +45 at the first nucleotide of the NS1 GS sequence. *Adapted from Cowton* et al*, 2006, J Gen Virol* (41)

Sequence comparison of the viral promoters and the L GS signal.

Upon closer inspection of the sequences at the 3´- end of the Le and Tr complement promoters, we noticed that the sequences are highly similar to a GS sequence, specifically the L GS sequence. RSV GS sequences are highly conserved; 9 are identical, and the L GS signal differs from the others by two nucleotides (33; 37). Regardless of the sequence differences, the L GS signal functions like the other GS signals (37). The sequences from position 3 to 11 of the Le and TrC promoters can be aligned with the L GS sequence, and correspond in 8 out of 10 nucleotides, and 7 out of 10 nucleotides, respectively (Figure 7). This intriguing observation led us to ask if RNA synthesis initiation could occur at this site, as it does at an authentic GS signal.

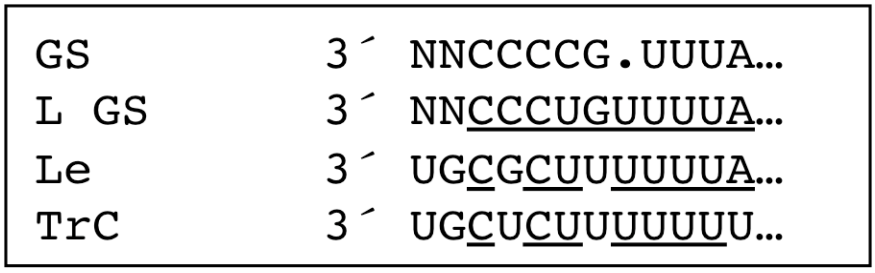


Figure 7; Nucleotides 3-12 of the Le and TrC promoters contain a gene start-like sequence. The sequences of the canonical RSV GS signal (GS), the L gene start signal (L GS), the leader promoter (Le) and the TrC promoter (TrC) are written from 3´to 5´, and aligned from the first 'C' residue of the GS and position +3 of the Le and TrC. The sequences in the Le and TrC promoters and the L GS that align are underlined. The Le shares 8 out of 10 nucleotides with the L GS, while the TrC shares 7 out of 10 nucleotides.

Evidence for an additional initiation site at position +3 of the Le and TrC promoters in the minigenome system.

It is generally accepted that initiation at position +1 of the Le promoter corresponds to synthesis of full-length antigenome, and from the TrC promoter, genome sense RNA. During the course of experiments studying the mechanism of RSV replication using the minigenome system, primer extension analysis using primers that hybridize close to the 5´- end of RNA synthesis products revealed that in addition to the position +1 initiation site in the Le and TrC promoters, there was an additional site for RNA synthesis initiation in both promoters at nucleotide position 3 (Figure 8, panel A) (185; 187). No other initiation sites were identified. Identification of RNA initiated from position +3 was a surprising finding; until recently (220; 280), additional initiation sites had not been reported for RSV or related viruses.

In the minigenome system, initiation at position +3 of either promoter in a wildtype (wt) template appears to be a minor event. However, a 1U to A substitution in the TrC template results in nearly 9-fold more initiation from position +3 than from position +1 (Figure 8, panel A (ii), compare lanes 5 and 6). This result likely reflects the polymerase preference to initiate at a pyrimidine. To determine whether RNA initiated from position +3 was elongated to the end of the template, we performed primer extension analysis on the RNA initiated from position +3 of wt and 1U-A minigenomes using two primers that hybridized

further downstream of the initiation site. Analysis with these primers showed that RNA initiated from position +1 was elongated, as expected. But no products were detected that migrated faster than +1 initiated products, suggesting that the RNA initiated from position +3 was not efficiently extended beyond 50 and 100 nucleotides (Figure 8, panel B (i) and (ii), lanes 2 and 3. Note that the faint band detected in lane 2 migrates at the same size as the RNA initiated from position +1 in lane 3). Thus, these data show that the polymerase is capable of initiating RNA synthesis at the gene start-like sequence in the viral promoters, and RNA initiated from this site is not efficiently extended.

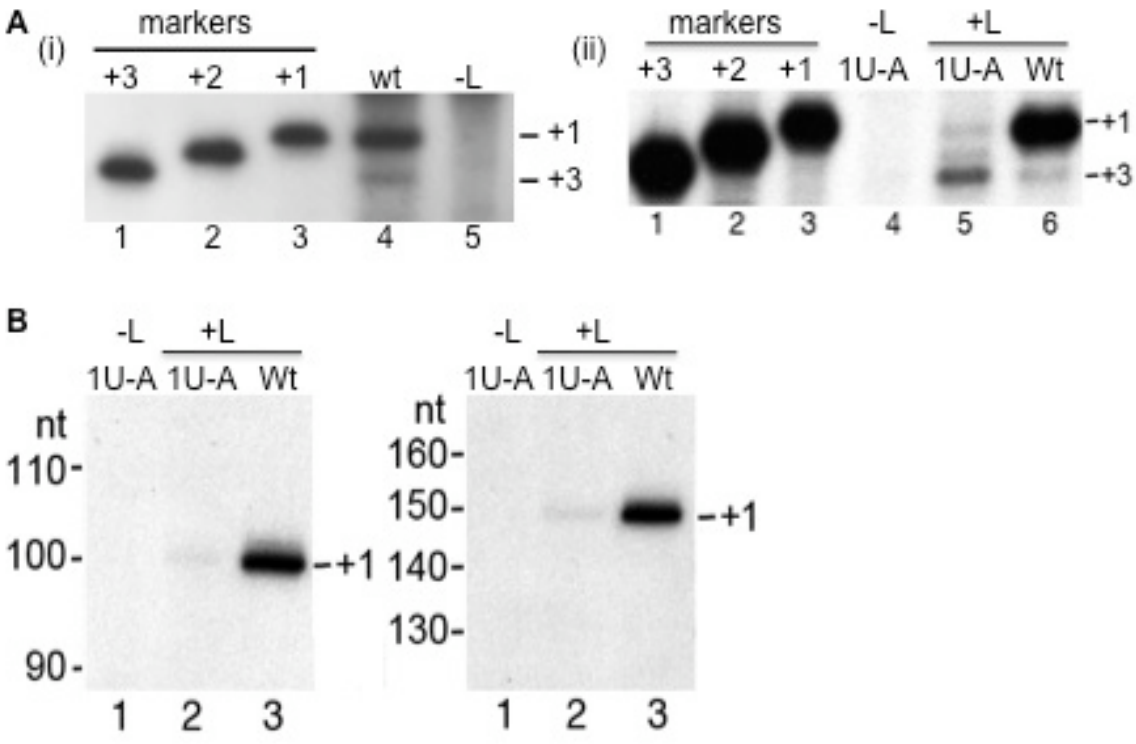


Figure 8; Evidence for an additional initiation site at position +3 of the Le and TrC promoters in the minigenome system. (A) (i) Primer extension analysis of RNA synthesized from the Le promoter using a primer hybridizing to LeC positions 24-50, (ii) and from the TrC promoter using a primer hybridizing to Tr positions 24-48. Molecular weight markers representing initiation at position +3, +2 and +1, present in lanes 1, 2, and 3, respectively. Panel (i): Wt Le-containing minigenome (lane 4) compared to –L control (lane 5). Panel (ii): Wt TrC promoter (lane 6) compared to 1U-A substitution mutant (lane 5). –L control (lane 4). +1 and +3 initiations are indicated. (B) Primer extension analysis of the same RNA samples in panel A (ii), using primers that hybridized from positions

74-100 (i) or 124-148 (ii) of Tr, to examine extension of the RNA initiated at position +3. *Panel A (i) adapted from Noton and Fearns, 2011* (187)*. Panel A (ii) and Panel B adapted from Noton* et al, *2010* (185)*. Sarah Noton performed experiments shown in Panel A. I performed the experiments in Panel B.*

In the following studies, we examined RNA isolated from wt RSV infections with the goal of determining whether the polymerase uses this additional initiation site during viral infection. We also characterized the length and properties of the RNA initiated from position +3.

Results

1.1 The +3 initiation site is used during RSV infection, and the RNA initiated from position +3 is not efficiently extended.

To determine if RNA synthesis initiation from position +3 also occurs during viral infection, intracellular RNA from RSV-infected cells was examined by primer extension analysis using a genome sense primer that corresponded to nucleotides 15 to 39 of the Le sequence (genome (g)15-39), and an antigenome sense primer that corresponded to nucleotides 13 to 35 (antigenome (ag)13-35) of the TrC sequence. By using primers that hybridized so close the 5´ends of the LeC and Tr RNAs, the initiation sites of RNA products from the 3´ends of the Le and TrC promoters could be determined precisely. A schematic of the positions that the primers correspond to on the genome is shown in Figure 9 (panel A).

Primer extension analysis using the g15-39 primer indicated that RNA was initiated from two sites within the Le promoters, the +1 initiation site, which is the expected initiation site for antigenome synthesis, and position +3 (Figure 9, panel

B, (i)). Surprisingly, significantly more RNA initiated at position +3 could be detected from the Le than RNA initiated from position +1. It is not possible to determine the absolute frequency of initiation at +1 versus +3 based on levels of intracellular RNA because of the potential differences in RNA stability in the cellular environment. However, antigenome initiated at +1 would be expected to be very stable due to encapsidation, therefore, this finding indicates that the RSV polymerase could initiate at the +3 site relatively frequently during infection.

Primer extension analysis with the ag13-35 primer also detected RNA initiated from both position +1 and +3 of the TrC promoter (Figure 9, Panel B, (iii)). However, in contrast to the results for the Le promoter, the levels of +3 initiation from the TrC promoter were lower than the levels of +1 initiation. It is important to point out however, that if the RNA initiated from position +3 is not encapsidated it may be underrepresented in this assay, and may actually be much more abundant. These analyses showed that RNA synthesis initiation occurs relatively frequently at position +3 from both the Le and TrC promoters during RSV infection.

To determine whether the RNA initiated from position +3 was extended, primers that hybridized further downstream of the Le and TrC promoters were used for analysis. Using a primer that corresponds to genome positions 45-68 of the Le, and antigenome positions 32-55 of the TrC, RNA initiated from position +1 was

still readily detected, as expected (panel B, ii and iv). However, normalizing to RNA initiated from position +1, there was a 7.8-fold decrease in the level of RNA initiated at position +3 of the Le promoter, and RNA initiated from position +3 of the TrC promoter was no longer detectable, demonstrating that these RNAs were not efficiently elongated (Panel B, (ii) and (iv), quantitation in Panel C). Thus, this result shows that RNA synthesis can be initiated from position +3 of the Le and TrC promoters, and the bulk of this RNA is terminated within a short distance of the initiation site.

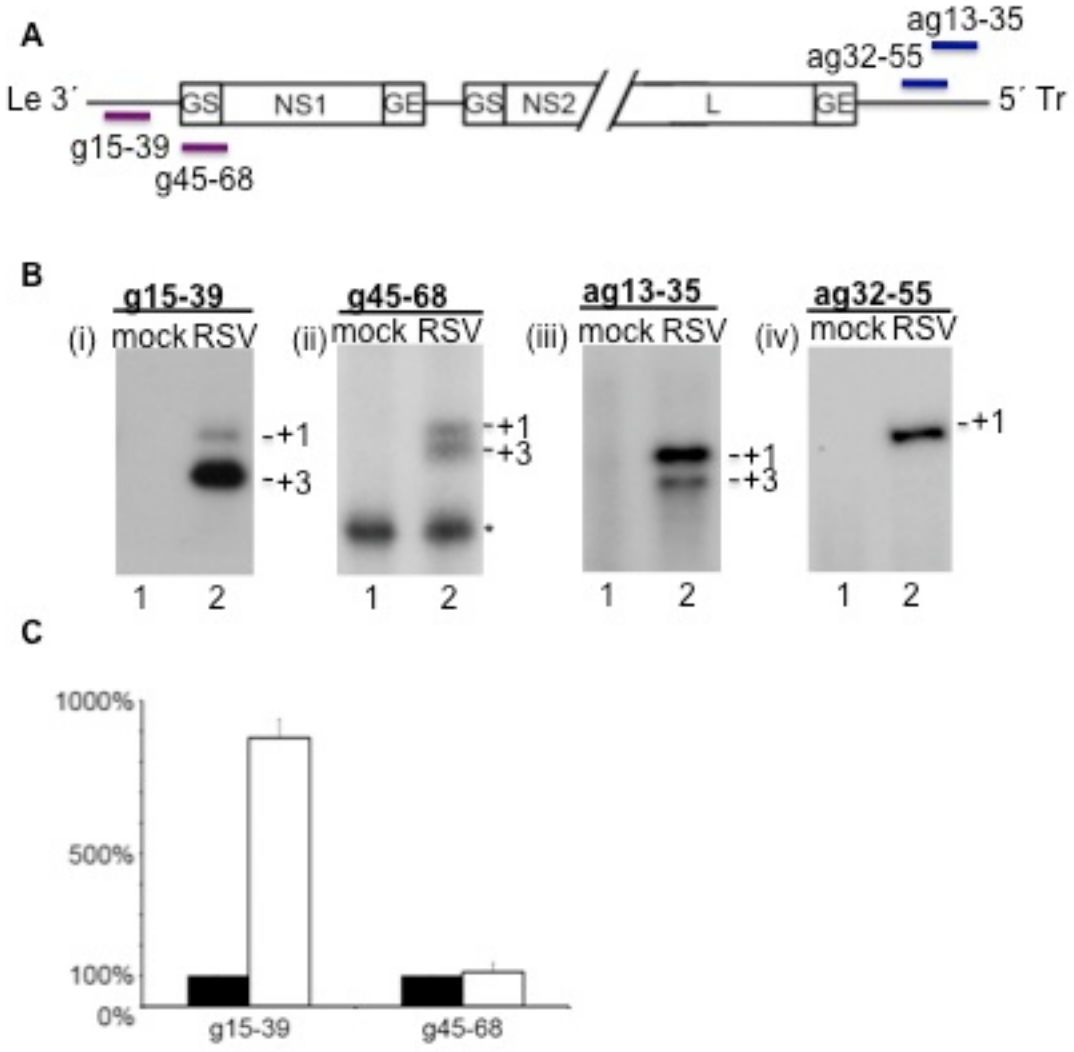


Figure 9; Evidence for initiation at position +3 of the Le and TrC promoters in RSV-infected cells. (A) Schematic diagram showing the hybridization positions of the primers relative to genome (purple bars, below) and antigenome (blue bars, above). (B) Primer extension analysis of viral RNA using negative sense primers corresponding to genome positions 15-39 (i) and antigenome

positions 13-35 (iii). Primer extension analysis of the same RNA using primers that hybridized further downstream at genome positions 45-68 (ii) and antigenome positions 32-55 (iv). RNA isolated from mock-infected cells was used as a negative control (lane 1). End-labeled oligonucleotides corresponding to initiations at positions 1, 2 and 3 were used as markers (not shown). Asterisk indicates background band (ii). (C) Quantification of the RNA detected from the position +3 initiation site (white bars), as determined by quantification of replicates of the experiment shown in panel B, (i) and (ii). For each primer the data were normalized to the RNA initiated from position +1 at 100% (black bars). The bars show the means of three experiments, with standard errors indicated.

1.2 RNAs initiated from position +3 do not contain 5´-monophosphate ends.

One possible explanation for the observance of RNA corresponding to initiation from position +3 could be that RNA initiated from position +1 is cleaved or processed to generate this RNA. There is precedence for cleavage of viral genomic RNA, as Borna disease virus is known to cleave its 5´- triphosphate ends in order to avoid detection by RIG-I (88). If RNA initiated from position +1 was cleaved to generate the RNA detected from +3, it would be expected to contain a 5´-monophosphate end. To determine if this was the case, RSV RNA was treated with an exonuclease that digests RNA containing a 5´-monophosphate, but is unable to digest 5´-tri- and di-phosphorylated RNA, RNA containing a 5´-cap, or RNA containing a 5´-hydroxyl group. As an internal control for exonuclease digestion, ribosomal RNA (rRNA), which possesses a 5´-monophosphate, was visualized by 0.8% agarose gel with ethidium bromide. The rRNA was largely digested by treatment with exonuclease, although the rRNA from the mock-infected cell samples was not digested as completely as the rRNA from RSV-infected cell sample (Figure 10, panel A, lanes 2 and 3). Next, primer extension analysis was performed to determine the effect of exonuclease treatment on the RNA initiated from position +1 and +3 of the Le and TrC promoters. Analysis with the g15-39 primer showed no change in the levels of +3 initiation from the Le, suggesting that this RNA does not contain a 5´-monophosphate end (panel B, i, compare lanes 2 and 4). The RNA initiated from

position +3 of the TrC promoter was also examined. Analysis with primer ag13-35 showed no change in the levels of +3 initiations from the TrC promoter, indicating that this RNAs also does not contain a 5´-monophosphate end (panel B, ii, compare lanes 2 and 4). While these results do not determine the structure of the 5´-ends of the RNA initiated from position +3, they do rule out the possibility that these RNAs contain a 5´-monophosphate, and therefore strongly suggests that these RNAs are initiated from position +3.

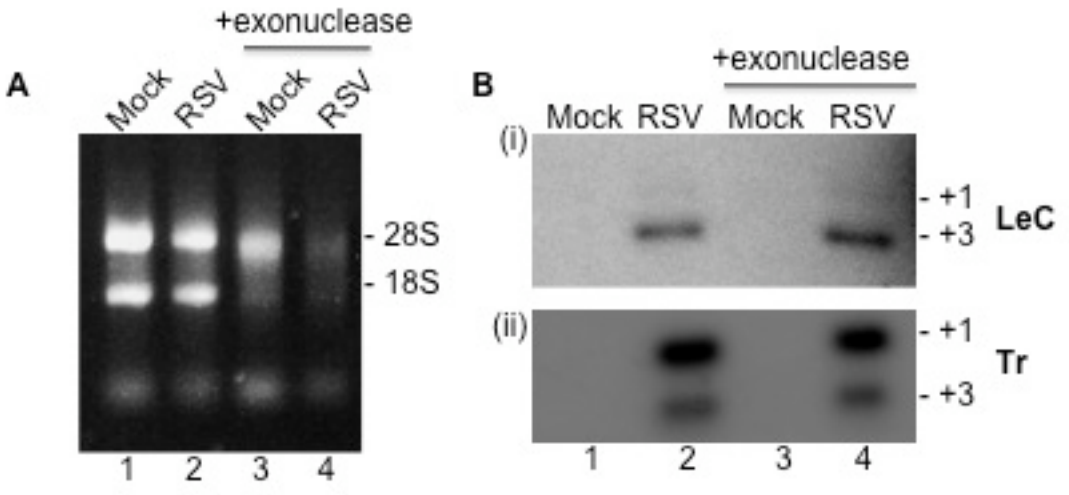


Figure 10; RNA initiated from +3 does not have a 5´- monophosphate. (A) 0.8% agarose gel stained with ethidium bromide to visualize ribosomal RNA. Exonuclease-treated RNA is migrated in lanes 3 and 4. 28S and 18S rRNA bands are indicated. (B) Primer extension analysis of exonuclease-treated RNA to determine if there is an effect on +1 and +3 initiations. RNA was analyzed using a primer that corresponded to genome positions 15-39 (i) and antigenome positions 13-35 (ii). Initiations from +1 and +3 are indicated.

1.3 Small RNAs are generated from the Le and TrC promoters.

To further characterize the RNAs generated from position +3 of the Le and TrC promoters, RNA isolated from RSV-infected cells was analyzed by Northern blot. A negative sense probe specific to genome positions 7-34 was used to detect LeC-containing RNA (antigenome), while a positive sense probe specific to antigenome positions 5-32 was used to detect Tr-containing RNA (genome). A negative sense probe specific to genome positions 504-540 was also used to detect NS1 mRNA (for sequences, refer to table 3). The Northern blot analysis was performed using two different electrophoresis conditions; formaldehyde-agarose gel electrophoresis, designed to detect RNAs in the range of ~100-~20,000 nucleotides in length (Figure 11, panel B), and polyacrylamide gel electrophoresis, to allow resolution of small RNAs in the range of 17 to 500 nucleotides in length (panel C). Northern blot analysis of larger RNAs using agarose gels showed that the probes bound to the expected RNA species. The g7-34 probe bound to a high-molecular-weight band corresponding to antigenome RNA, and the NS1 probe identified bands of appropriate sizes to be NS1 and NS1-NS2 readthrough mRNAs (panel B, i and iii). Long exposures were required to detect antigenome RNA with this probe reflecting the relative scarcity of antigenome RNA compare to NS1 mRNA (panel B, iv). The ag5-32 probe bound to a high-molecular-weight band corresponding to genome RNA (Panel B, ii).

Analysis of the same RNA samples using polyacrylamide gel electrophoresis showed that the g7-34 probe was also able to detect a band of small RNAs that were somewhat heterogeneous in size but predominantly ~25 nucleotides, with some transcripts detectable as a faint smear, extending up to ~50 nucleotides in length (Panel C, i). The ag5-32 probe also detected heterogeneous RNAs of similar size, as well as a much less abundant RNA ~60 nucleotides in length (Panel C, ii). These are the only small RNAs detectable with these probes. Under these conditions the NS1 probe detected full-length NS1 mRNA (532 nt excluding the poly(A) tail) near the top of the gel, as well as some prematurely terminated NS1 RNAs that could be detected with a probe that hybridizes closer to the 5´end of NS1 mRNA (Panel C, iii and iv). However, no ~25 nt RNA species were detected with the NS1 probes, indicating that the small RNAs detected with the Le and Tr probes were not antigenome and genome that had become degraded during the RNA purification procedure. These results show that small transcripts predominantly 25 nucleotides in length are generated from the Le and TrC promoters during RSV infection.

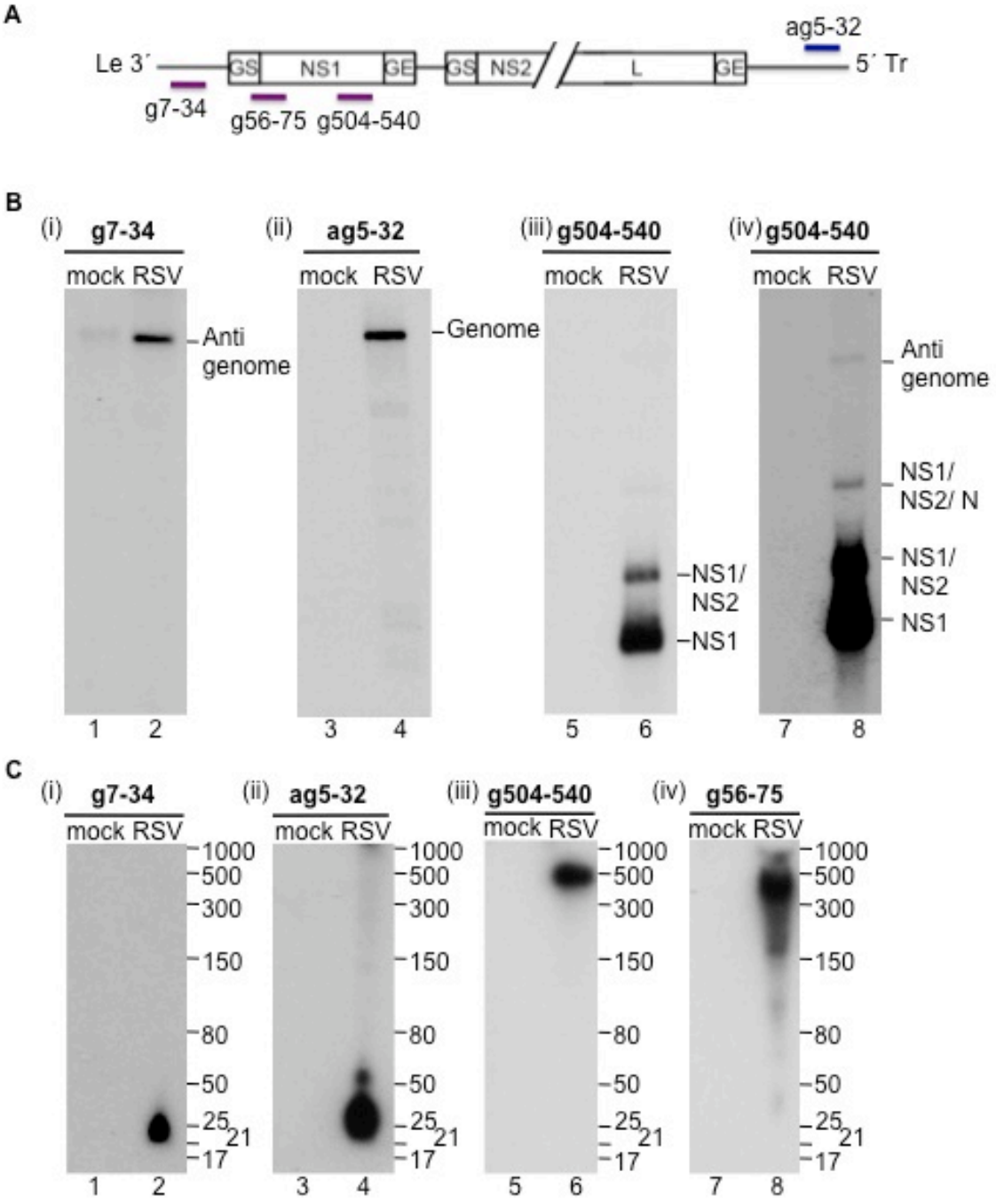


Figure 11; Small RNAs are generated from the Le and TrC promoters. (A) Schematic diagram showing the positions primers correspond to on the genome

(below, purple) and antigenome (above, blue). (B) RNA migrated on 1.5% agarose-formaldehyde gels was probed with oligonucleotides corresponding to genome positions 7-34, to detect antigenome and LeC-containing RNA (panel i), antigenome positions 5-32, to detect genome and Tr-containing RNA (panel ii), and genome positions 504-540, to detect NS1 mRNA (panel iii). Panel (iv) is a longer exposure of panel (iii) showing antigenome levels compared to NS1 mRNA. Antigenome, genome, NS1, NS1/ NS2 and NS1/ NS2/ N readthrough mRNA are indicated. (C) RNA migrated on 6% urea-acrylamide gels was probed as above to detect small RNA in the range 17- 1000 nts (i, ii and iii). Probe corresponding to genome positions 56-75 was also used to detect small RNA generated from the NS1 GS (iv). RNA from mock-infected cells was used as a negative control (lanes 1, 3, 5, and 7 for both panels).

1.4 RNA initiated from position +3 of the Le fractionates with subgenomic mRNAs during RSV infection.

Taken together, the primer extension and Northern blot data described above are consistent with a model in which RNA initiated at the +1 site is elongated into antigenome RNA, whereas RNA initiated at +3 is truncated at ~25 nt. However, the data do not exclude the possibility that some RNA initiated at +1 is also truncated, or that some RNA initiated at position +3 is elongated to the end of the genome, similarly to antigenome RNA. Therefore, to further investigate the relationship between +1 and +3 initiated RNA, it was of interest to separate the products of the +1 and +3 initiation sites and determine their lengths. To accomplish this, RSV-infected cell extracts were fractionated using a modified version of the method described by Mason and colleagues (160), which we had found is able to separate nucleocapsids from cytoplasmic mRNAs (Noton and Fearns, unpublished data). Briefly, mock- or RSV-infected cells were harvested and treated with a series of increasingly stringent buffers to generate two supernatant fractions (S1 and S2) and one pellet fraction (P2) (Figure 12).

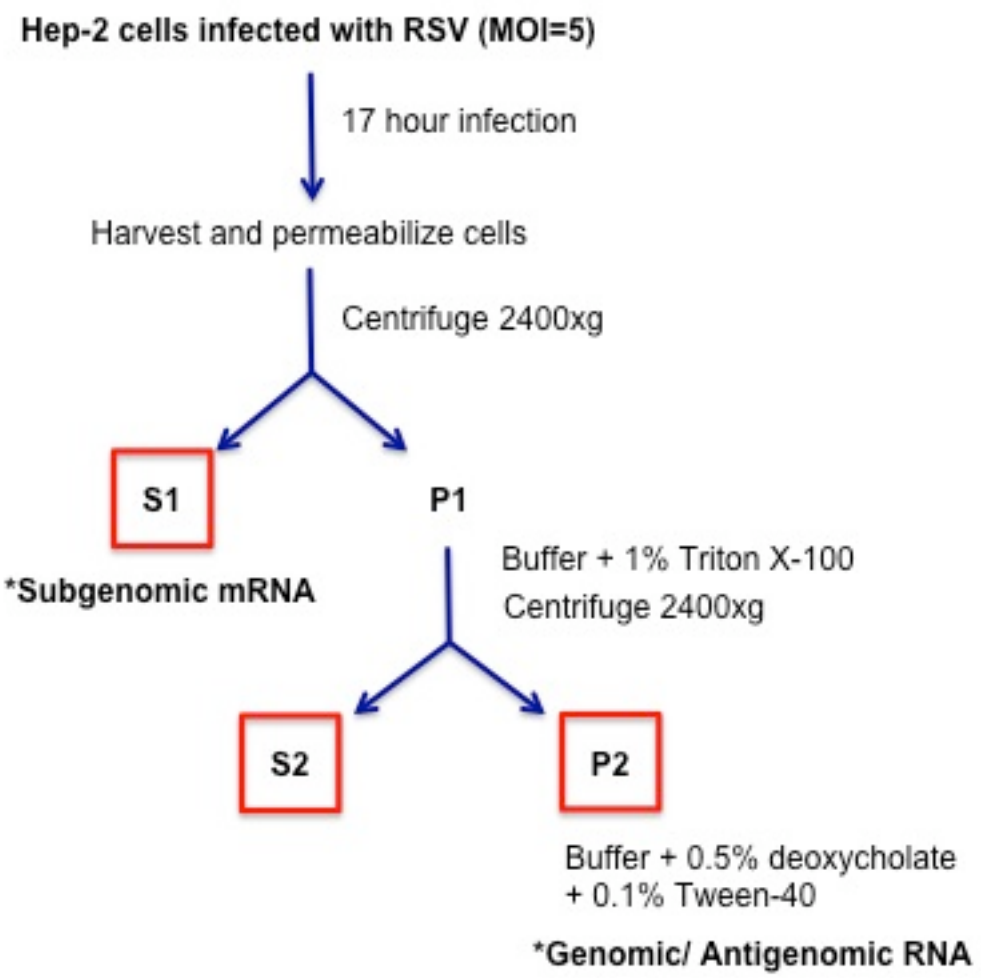


Figure 12; Schematic of modified fractionation analysis. The original protocol involved centrifugation of resuspended P2 to generate fractions S3 and P3 (160), however, we combined the final two fractions into resuspended P2 to concentrate genome and antigenome RNA.

RNA was isolated from each fraction and was analyzed by formaldehyde-agarose gel electrophoresis and Northern blotting to determine if genome and antigenome RNAs had been separated from mRNA. Analysis with probe ag5-32 to detect genome RNA revealed that genome fractionated predominantly in the P2 fraction, with small amounts detectable in S1 and S2 (Figure 13, panel i), and analysis with the g7-34 probe to detect antigenome RNA showed antigenome fractionated exclusively in P2 (panel ii). In contrast, an NS1 specific probe, detected NS1 and NS1-NS2 read through mRNAs primarily in the S1 fraction, with some RNA detected in fraction S2 (panel iii). No RNAs were detected in mock-infected fractionated controls (lanes 1, 2 and 3). These data demonstrate that this method can be used to largely separate subgenomic mRNA from genome and antigenome.

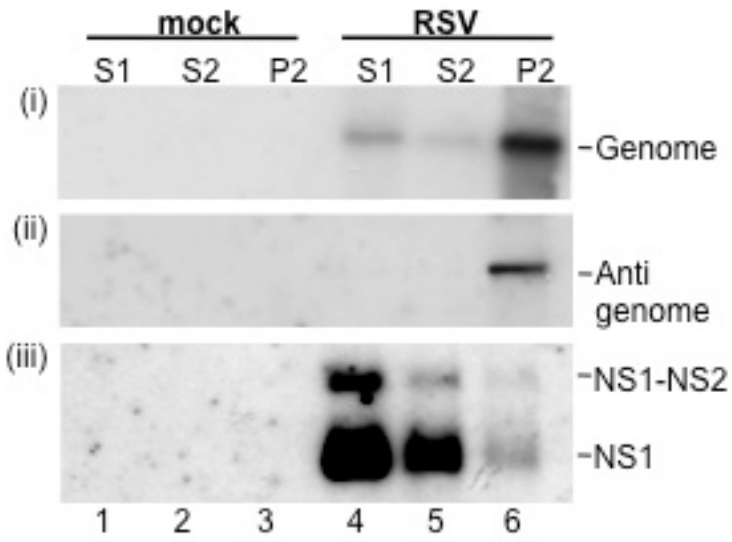


Figure 13; RSV mRNA can be separated from genome and antigenome by fractionation analysis. RNA was isolated from fractionated mock- and RSV-infected cells, and was migrated on 1.5% agarose-formaldehyde gels and Northern blotted, and probed with ag5-32 to detect genome (i), g7-34 to detect antigenome (ii), and g504-540 to detect NS1 mRNA (iii). Fractions from mock infections were used as negative controls (lanes 1, 2, and 3). Genome, antigenome, NS1 and NS1-NS2 readthrough mRNA are indicated. Note that each fraction is not equally concentrated; the S1 fraction is resuspended in 300 µl of buffer, S2 is resuspended in 100 µl of buffer, and P2 is resuspended in 200 µl of buffer, but an equal volume of each fraction was taken for RNA extraction. Thus, the RNA in fraction P2 is 1.5 times more concentrated than RNA in fraction S1. This means that RNA in fraction P2 is overrepresented compared to RNA in fraction S1. Therefore, it is not appropriate to compare quantities between lanes.

The same RNA fractions were then subjected to primer extension analysis to examine which fractions contained RNA initiated at +1 or +3. Primer extension analysis was performed using primer g45-68. Analysis with this primer revealed RNA initiated from position +3 fractionated predominantly in the S1 fraction, with only small amounts detectable in fractions S2 and P2. Conversely, RNA initiated from position +1 fractionated primarily into fraction P2, with very little in fractions S1 and S2 (Figure 14, panel A, i).

Small RNA Northern blot analysis of the RNA fractions showed that the ~25 nt RNA fractionated predominantly in the S1 fraction, but a significant amount was also detectable in P2 (panel A, ii). As a control, small RNA Northern blot analysis of NS1 supports the finding that mRNA fractionates into S1 (panel iii). Based on these results it can be concluded that initiation from position +3 of the Le region usually results in synthesis of a ~25 nt RNA that could be detected in fraction S1, consistent with the primer extension results shown in panel (i). In contrast, initiation at +1 can result in synthesis of full-length antigenome RNA that could be detected in P2. However, the data also show a relatively high level of ~25 nt length RNA in P2, despite a relatively low level of RNA initiated at +3 in this fraction. This result indicates that at least some of the ~25 nt RNA that can be detected is initiated at +1. In summary, these data show that the dominant product of the Le +3 initiation site is ~25 nt in length whereas initiation at Le +1 can yield either full-length antigenome or small RNAs ~25 nt in length.

Fractionation analysis of the RNA generated from the TrC promoter was somewhat less conclusive. Primer extension was performed using the primer that hybridized from positions 13-35 to examine RNA initiated from position +1 and +3 of the TrC promoter. Despite detection of genome RNA in the insoluble fraction by Northern blot (Figure 13, panel i) it was difficult to detect RNA initiated from position +1 of the TrC promoter in this fraction by primer extension (Figure 14, panel B, i; see lane 6). Indeed, RNAs initiated at +1 and +3 are both detected in the S1 fraction predominantly (compare lanes 4 and 6). Primer extension analysis of the Tr containing RNA with alternative primers was not performed due to time constraints.

Small RNA Northern blot analysis of the small Tr RNAs showed they were detected largely in the S1 fraction, in good agreement with the primer extension analysis (Figure 14, panel B, ii). Very low levels of the ~25 nucleotide RNA were also detected in the S2 and P2 fractions. No RNA was detected from mock-infected controls (lanes 1, 2, and 3).

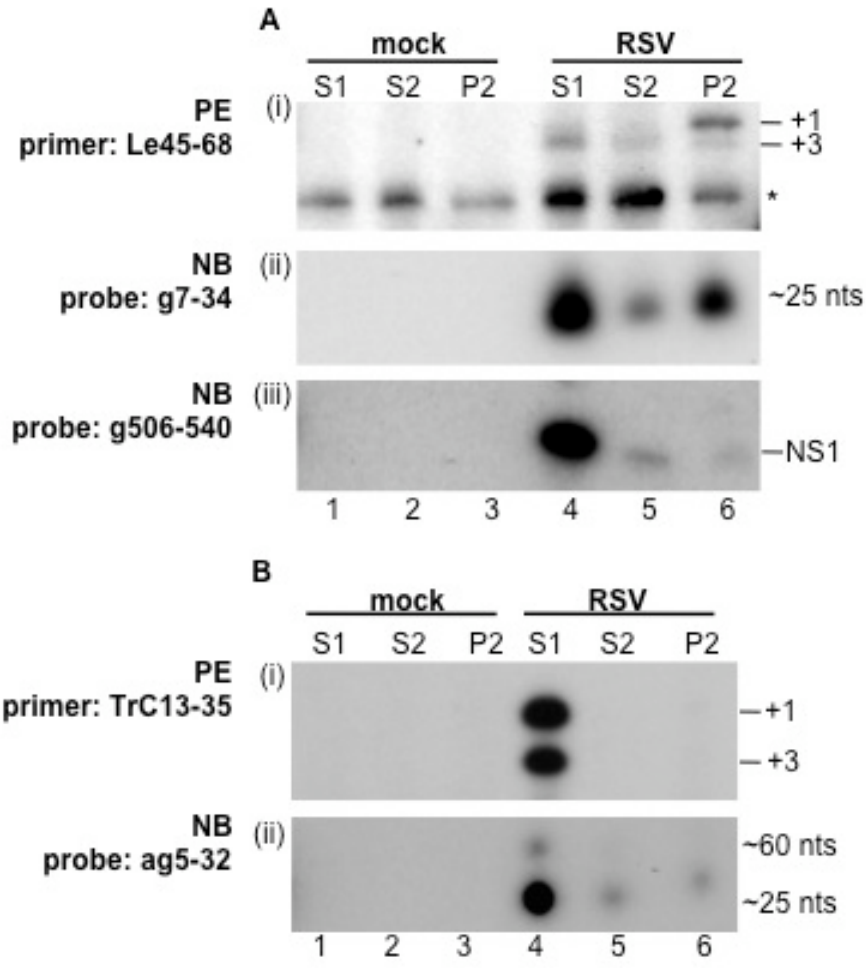


Figure 14; Fractionation analysis of the small LeC and Tr RNA. (A) Primer extension analysis using a primer hybridizing to genome positions 45-68 to detect initiation from positions +1 and +3 from the Le promoter (i). Asterisk indicates a background band. Panels (ii) and (iii) are urea-acrylamide Northern blots probed with g7-34 (ii) and g504-540 (iii) to detect the small LeC RNA and NS1, as indicated. (B) Primer extension analysis using a primer hybridizing to antigenome positions 32-55 to detect initiation from positions +1 and +3 from the

TrC promoter (i). Panel (ii) is a urea-acrylamide Northern blot probed with ag5-32 for the detection of small Tr RNA, as indicated. RNA isolated from mock-infected fractionated cells was used as negative controls in lanes 1-3. *Note that the type of analysis, primer extension (PE) or Northern blot (NB), and the probe/ primer used is indicated in bold on the left side of each panel.*

In summary, these data show that the bulk of RSV mRNA can be separated from genome and antigenome by fractionation. RNA initiated from position +3 of the Le fractionates predominantly into fraction S1 with subgenomic mRNAs, while RNA initiated from position +1 fractionates almost exclusively into fraction P2, as expected, with genome and antigenome. The small LeC RNA fractionates primarily in the S1 fraction, indicating that initiation from position +3 is responsible for the generation of at least some of the small RNA, since the only initiation event detected is from position +3. However, some of the small LeC RNA is also detected in P1. Therefore, this result does not rule out the possibility that some of the small LeC RNA is also generated from position +1. In contrast, RNA initiated from positions +1 and +3 of the TrC promoter were largely detected in fraction S1, which corresponds with detection of the small Tr-containing RNAs predominantly in fraction S1 as well.

1.5 Small RNA initiated from position +3 of the Le is partially encapsidated.

The small RNA may either be encapsidated in N protein, or unencapsidated. To determine the encapsidation status of the small RNA, mock- and RSV-infected cell lysates were treated with nuclease to digest unencapsidated RNA prior to RNA isolation, and the RNA was analyzed by Northern blot. As expected, genome and antigenome were resistant to degradation by nuclease because they are encapsidated in N protein, while NS1 mRNA, which is not encapsidated, was digested by nuclease treatment (Figure 15, panel A, compare lanes 2 and

4). Nuclease-treated RNAs were then analyzed by urea acrylamide gel electrophoresis to detect the small LeC RNA as well as NS1 mRNA. Analysis with probe g56-75 showed that the NS1 mRNA was efficiently digested (panel B, ii). Analysis with probe g7-34 showed that nuclease treatment resulted in a reduction in the level of the ~25 nucleotide RNA (panel B, i). However, the nuclease treatment did not completely ablate the small LeC RNA, indicating that either the nuclease digestion of this very small RNA was incomplete, or that the ~25 nucleotide RNA that could be detected was comprised of a mixture of encapsidated and unencapsidated RNA. Additionally, the nuclease treated RNA was also analyzed by primer extension with the g45-68 primer to examine the effect of nuclease on the RNA initiated from position +3 of the Le promoter (panel C). RNA initiated from position +1 appeared to be protected. RNA initiated from position +3 was no longer detected, suggesting it was digested by nuclease treatment, although some shorter RNA was detected (lane 4, shorter RNA indicated with an ellipse). It is not possible to determine from this result whether the shorter RNA represents partial digestion of +3-initiated RNA or +1-initiated RNA, since some of the ~25 nt RNA appeared to be initiated from position +1, and may also be unencapsidated.

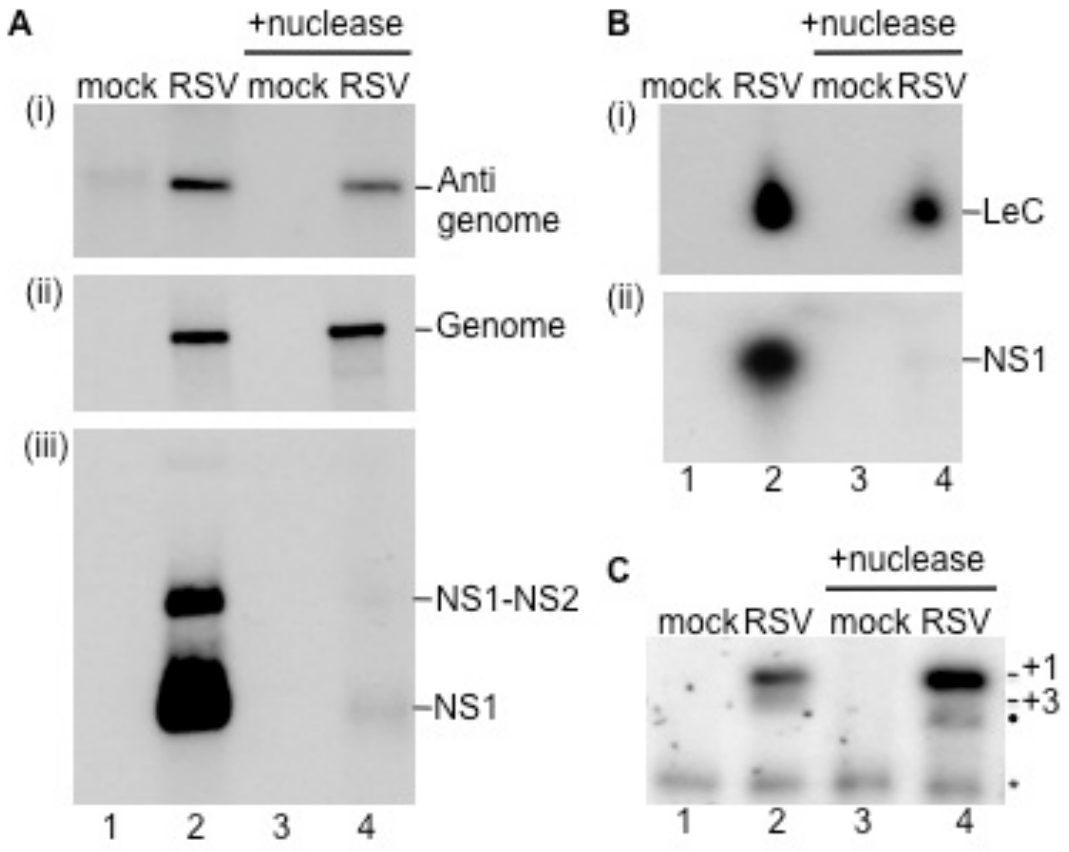


Figure 15; Nuclease treatment of RSV RNA. (A) Agarose-formaldehyde Northern blots probed with g7-34 to detect antigenome (i), ag5-32 to detect genome (ii), and g504-540 to detect NS1 mRNA (iii). Nuclease-treated RNA is migrated in lanes 3 and 4. RNA from mock-infected cells was used as negative controls (lanes 1 and 3). Antigenome, genome, NS1 and NS1-NS2 readthrough mRNA are indicated. (B) Urea-acrylamide Northern blots for the detection of small RNA were probed with g7-34 to detect LeC containing RNA (i) and g504-540 to detect NS1 mRNA (ii). (C) Primer extension analysis of the same RNA using a primer that hybridizes to positions 45-68 of the genome. Initiations at

positions +1 and +3 are indicated. Partially digested RNA (lane 4) is indicated by an ellipse. Background bands are indicated by an asterisk. *Note that the results in panels B and C are representative of two experiments.*

Discussion

Summary of Results

We have shown that the RSV polymerase is able to use an previously undescribed site for RNA synthesis initiation within the Le and TrC promoters during infection. Initiation at position +3 resulted in an RNA that was not efficiently elongated, yielding the small LeC and Tr containing RNAs. The RNA initiated from position +3 of the Le was very abundant, and represented the dominant initiation event in the Le region. The small LeC RNA predominantly fractionated with viral subgenomic mRNA, and was also partially degraded by nuclease indicating some of it is unencapsidated. This is the first demonstration of an additional initiation site within the viral promoters being used during viral infection, and challenges the dogma that RNA synthesis initiation only occurs opposite the first nucleotide of the genome or antigenome.

Elucidating the structure of the 5´-ends of the small RNAs

The exonuclease digestions showed that the small RNAs do not contain 5´-monophosphate ends, suggesting that the RNAs are not cleaved from RNA initiated from position +1. However, these experiments did not exclude the possibilities that the RNAs contain 5´- di- or tri-phosphate moieties, or 5´- cap structures. Since capping is known to stabilize the elongation complex during transcription, it seems unlikely that RNA initiated from +3 would be capped since

it is not efficiently extended. Moreover, in another NNS virus, VSV, mRNAs are not capped until they are 31 nt in length, thus the RNA initiated from position +3 in RSV may not be long enough to be capped (243). It is therefore most likely that the small RNA initiated from position +3 contains a 5´-di- or tri-phosphate. In good agreement with a predicted triphosphate, LeC RNA isolated from RSV-infected cells and stripped of viral N protein, has been shown to be a ligand for RIG-I, leading to the activation of IFN-β in A549 cells (13). Since RIG-I is activated in response to detection of tri-phosphorylated viral RNA, this strongly suggests that the LeC RNA contains a 5´- triphosphate.

Truncation of the small RNAs

Based on the results of the primer extension and Northern blots, RNA initiated from position +1 is efficiently elongated, whereas RNA initiated from position +3 is not and is only ~25 nt in length. Since mRNAs initiated from GS sequences are typically extended many hundreds of nucleotides, this raises the question of why RNA initiated from position +3 at a sequence highly similar to a GS are not extended. Despite the sequence similarity of nucleotides 3-12 of the Le and TrC promoters to a GS signal, there is no sequence downstream that resembles a GE signal. Moreover, the sequence from nucleotides 23-27 of Le and TrC, where the small RNAs are terminated, do not share obvious sequence similarity, suggesting that the sequence of the template is not responsible for the polymerase terminating RNA synthesis. In addition, the small RNAs are

heterogenous in size, which argues against a termination signal. Therefore, termination of the small RNA is likely in response to the polymerase reaching a checkpoint for RNA synthesis. One potential checkpoint may be failure to concurrently encapsidate nascent RNA. In Sendai virus (SeV) it has been shown that concurrent encapsidation results in greater processivity of the polymerase during replication (87). Thus, in this model, RNA that fails to become encapsidated within synthesis of ~25 nucleotides may be aborted. Failure of the RNA initiated at position +3 to become encapsidated may therefore explain why the RNA is prematurely terminated, as at least some of the RNA was digested by nuclease treatment, indicating that it was not protein bound.

Importantly, these assays cannot distinguish if some RNA initiated at +1 was also terminated prematurely; the small LeC RNA may contain RNA initiated from position +1 as well as RNA initiated at +3. Small LeC RNA can be detected in the insoluble fraction by Northern blot with genome and antigenome RNA, suggesting that at least some of the small LeC RNA was generated from position +1. Moreover, the primer extension analysis of fractionated Tr-containing RNA failed to clearly detect RNA initiated from position +1 in the insoluble fraction, but detected a high level of +1 RNA in the S1 fraction. This further suggests there may be a lot of small RNA initiated from position +1. This RNA may represent abortive replication product that did not become encapsidated with N protein, and was therefore released within the Le region.

While the nuclease treatment indicates that some of the small LeC RNA is protected, this experiment does not determine whether or not the RNA is bound by N protein or another cellular protein. Another group has shown that La autoantigen, an ~50 kiloDalton (kDa) cellular protein, binds to RSV LeC-containing RNA to subvert activation of RIG-I (13). Interestingly, they show that the majority of early LeC transcripts are bound by La protein, and that over time as viral N protein accumulates, LeC RNA is predominantly bound by N protein. However, LeC RNA appears to be specifically bound either by La or by N, and not by both proteins, for reasons unclear at this time. La has been shown to act as a chaperone in the biogenesis of small eukaryotic RNAs, and aids in assembly of small RNAs into functional ribonucleoprotein complexes (RNPs) (15; 167; 193; 275). It is exciting to consider the possibility that La is behaving analogously to its cellular function with regards to the LeC small RNA. Thus, La may not only serve to subvert the activation of RIG-I by this RNA, but may also serve to direct the assembly of the small RNA into a higher order viral or cellular complex, where it then influences viral replication or the cellular response to infection.

Future Directions

It would be interesting to determine if the small RNA initiated from position +3 of the Le is bound with cellular La protein. LeC RNA in the form of RNPs could be

affinity purified on Sepharose beads coupled to antibody against La or N protein, and deproteinized to release naked LeC RNA, as described previously (13). Primer extension could then be performed on this RNA to determine whether RNA initiated from position +3 is associated with La-containing RNPs, viral N protein, or both. It would be remarkable if +3 RNA is only associated with La protein, while +1 initiated RNA is only associated with N protein. If this were the case, then it would be important to understand what is responsible for the recruitment of N protein specifically to +1 initiated viral RNA. There is some evidence that the first two initiating nucleotides (5´- AC) may be part of an encapsidation signal required to recruit N to the RNA, which would not be present in an RNA initiated from position +3 (64; 163). The experiments proposed above may provide support for this idea.

In conclusion, this work presents the novel finding of an previously undescribed RNA synthesis initiation site at position +3 of the viral promoters, leading to the generation of small RNAs. This of course raises the question as to why the RSV polymerase generates these small RNAs, and how the polymerase engages with two closely spaced sites during infection. The role of the +3 initiation site within the Le promoter is explored in more detail in Chapter 2.

Chapter 2: Transcription can initiate from position +3 of the Le promoter

Introduction

Prevailing models of transcription initiation in the non-segmented negative sense RNA viruses.

For more than two decades, the mechanisms of viral replication and transcription employed by non-segmented negative sense (NNS) RNA viruses have been in contention. Two models that are well supported by data derived from studies on VSV and SeV have been proposed and are generally accepted. The first model, based on studies with VSV, posits that there are two pools of polymerase, a transcriptase and a replicase, which consist of a basic L-P complex but are associated with different viral and cellular proteins, and initiate at two different sites on the genome (Figure 16). Two distinct polymerases have been purified and shown to be functional *in vitro,* in support of this model (209). The transcriptase is bound with cellular cofactors and is capable of initiating transcription directly at the first GS signal without first transcribing the Le region. In contrast, the replicase is not bound by cellular cofactors but instead is

complexed with viral N protein, and initiates replication opposite the first nucleotide of the genome. The idea that prior Le synthesis is not required for transcription is consistent with the results of experiments on the VSV polR1 mutant, which produces a molar excess of N mRNA over Le RNA (31; 32). Additionally, evidence that the transcriptase enters directly at the first GS signal is supported by experiments in which UV treatment of VSV genomes to introduce lesions randomly throughout the Le, which would block the movement of the polymerase, showed that mRNA synthesis was independent of the number of UV cross linkages in the Le (267). However, the inverse was true for UV-treated VSV templates tested *in vitro,* and transcription *was* dependent on prior Le RNA synthesis (267). This disparity between *in vivo* and *in vitro* data has not been satisfactorily explained.

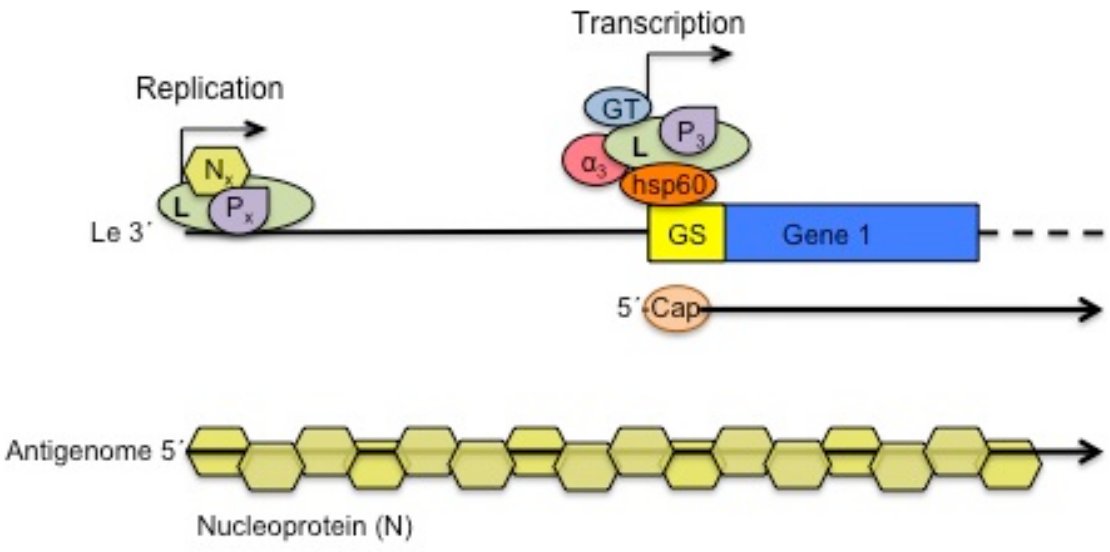


Figure 16; Model of transcription initiation for VSV. Two distinct polymerase complexes carry out transcription and replication. The transcriptase is associated with cellular cofactors and enters the template directly at the first GS sequence, where transcription is then initiated and the transcriptase caps the mRNA. There is no requirement for prior Le RNA synthesis for transcription to occur. The replicase is associated with viral N protein and enters at the 3´-end of the template, and generates antigenome that is concurrently encapsidated.

The second model has been proposed based on studies done with SeV. This model suggests that there is a single polymerase composed of L and P, which is responsible for carrying out both replication and transcription, always enters the template at the 3´-end of the genome. When the polymerase is in transcription mode, it initiates opposite the first nucleotide of the Le, and generates a positive sense Le transcript (Le+), releases the nascent Le+ RNA, and reinitiates transcription at the first GS signal. Upon sufficient accumulation of N protein, replication can be initiated opposite position +1 and the RNA is concurrently encapsidated (Figure 17). This model is supported by substitution analyses of SeV demonstrating that promoter sequence can impact initiation at the GS signal, and that there is competition between these sites for polymerase (138; 261; 262). This model hinges on the idea that transcription is dependent on prior initiation from the Le.

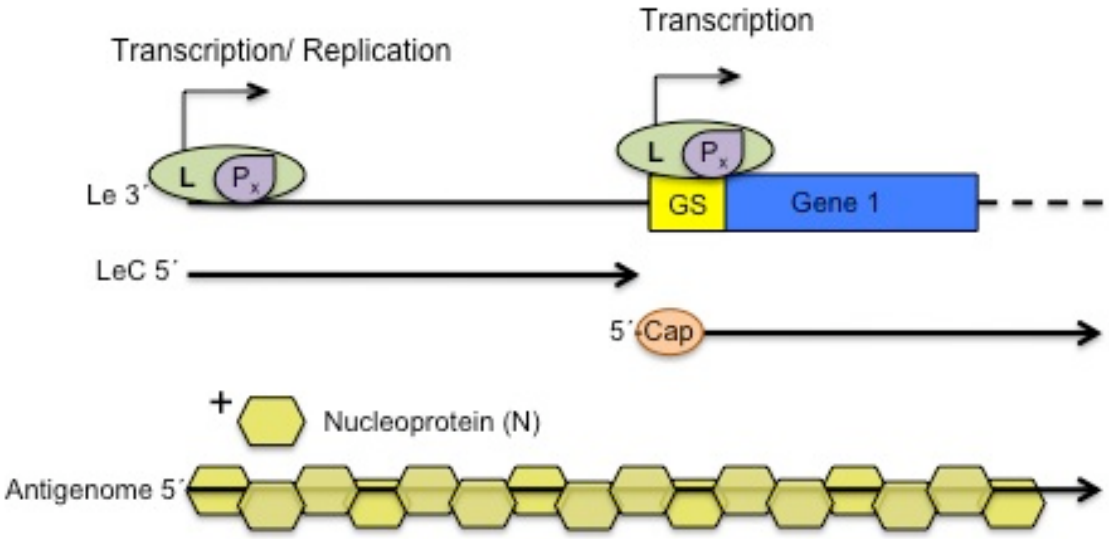


Figure 17; Model for transcription initiation for SeV. The same polymerase is responsible for carrying out both transcription and replication. The polymerase always enters the template at the 3´-end of the genome and transcribes the Le. Upon reaching the end of the Le, the polymerase stops and scans to locate the first GS signal, where it reinitiates transcription. During replication, the polymerase is influenced by the levels of N protein and generates encapsidated antigenome.

It is possible that transcription initiation is different for rhabdoviruses and paramyxoviruses, a possibility supported by the observation that the promoter regions of these viruses appear to be organized differently (149; 163; 266). A unified model was proposed to explain the disparate results obtained for VSV and SeV, in which the polymerase always enters at the 3´-end of the template, but does not always initiate RNA synthesis (46). This model suggests that during transcription the polymerase scans the Le region to locate the first GS signal, and implies that N protein is sequentially displaced as the polymerase traverses the Le, allowing recognition of the first GS signal (46; 124).

While elements of both models may apply to transcription initiation by the RSV polymerase, neither model entirely explains the findings for RSV. One tenet of the SeV model is that N protein is an important factor regulating the switch from transcription to replication. However, in RSV it has been shown that increasing the amount of N protein in the minigenome system does not result in a decrease in the levels of transcription, although replication was augmented (63). The fact that sequence within the first 12 nucleotides of the RSV Le have been shown to be critical for transcription argues against the VSV model of direct initiation at the first GS signal (64). In addition, NNS virus genomes are completely encapsidated in N protein and therefore the GS should not be directly accessible (2; 78). Thus, the VSV model raises several questions about how the polymerase identifies and accesses the first GS signal. Addressing the idea that

the polymerase does not always initiate in the Le but can scan to the first GS signal, scanning of the Le region of RSV (or any NNS virus) has not been directly demonstrated. The RSV polymerase has been shown to be capable of scanning at gene junctions, which range from 1 to 56 nucleotides in length (33; 114; 248), and at the unusual M2/L overlapping gene junction, where the polymerase must scan backwards 68 nucleotides from the M2 GE signal to locate the L GS signal (37; 61). Therefore, scanning within the Le region, which is 44-nucleotides long for RSV, is not implausible. But unlike a polymerase that encounters a gene junction and can freely diffuse along the template, a polymerase that is bound to a promoter at the 3´-end of the Le would require energy to break promoter contacts. Thus it seems unlikely that any NNS virus polymerase can scan directly from the Le.

Reconstitution of RSV RNA synthesis **in vitro**

Until recently, studying RSV polymerase activity in promoter regions was hindered by a lack of an *in vitro* assay that utilized an artificial template and purified components to examine individual stages of RNA synthesis. Intracellular assays only allow you to study stable end products of RNA synthesis; it is impossible to manipulate the cellular environment or to study unstable intermediates of transcription in a viral infection. Therefore, we developed an *in vitro* RNA synthesis assay in which RSV RNA synthesis could be reconstituted utilizing a defined RNA template and purified polymerase (186). The assay was

developed using an RNA template representing the TrC promoter. Although the template was naked RNA, the polymerase demonstrated specificity for TrC promoter in the absence of N protein. Additionally, the results of this assay recapitulated RNA synthesis events observed in RSV-infected cells, such as initiation at position +3 (186). In this chapter, we used this assay to study RNA synthesis events at the Le promoter.

The goal of the following studies was to elucidate the mechanism of transcription initiation by the RSV polymerase. Because of the similarity of the Le 3-12 sequence to the L GS, and our data showing RNA synthesis initiation from position +3 of the Le (Chapter 1), we hypothesized that position +3 may be the start site of transcription (Model, figure 18). We set out to test this model by determining whether the +3 initiation site could be accessed directly by the polymerase, or if initiation from position +3 was dependent on prior initiation at position +1. This would indicate whether all RNA synthesis initiates from position +1 and initiation from +3 is a secondary event, or if position +3 is a bona fide initiation site. We also tested the effect of mutations in the GS-like sequence on mRNA initiation from the first GS signal at position 45.

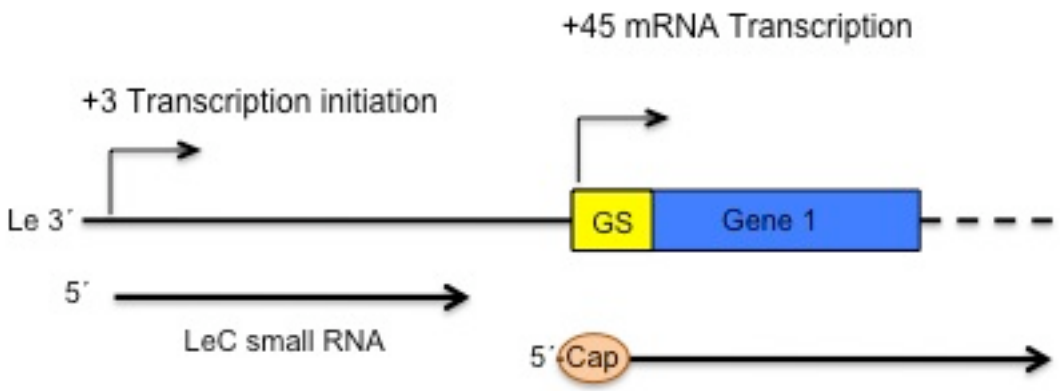


Figure 18; Proposed model for transcription initiation by the RSV polymerase. Transcription initiates from position +3 of the RSV Le region at a sequence that is highly similar to a GS sequence. A small LeC RNA is generated and this RNA is terminated after synthesis of ~25 nt. The polymerase reinitiates mRNA transcription at position +45 at the first GS signal.

Results

2.1 RNA produced in the *in vitro* RNA synthesis assay is RSV polymerase dependent and Le promoter specific.

We wanted to be able to test whether the polymerase could access the position +3 initiation site directly, or if this was a secondary event to initiation at position +1. To determine whether the *in vitro* RNA synthesis assay would be useful for examining initiation events at the Le promoter, it was important first to verify that RNA synthesis was both dependent on the presence of the RSV polymerase and specific to the Le promoter. The RNA synthesis assay was performed using an RNA template corresponding to nucleotides 1 through 14 of the wt Le promoter, and RNA synthesis on this template was tested with two forms of the L-P polymerase complex, either a wt or mutant polymerase. The mutant polymerase contains an amino acid substitution in the catalytic GDNQ motif of the L protein (N812A), which inhibits RNA synthesis. The purified polymerase complexes are shown in Figure 19.

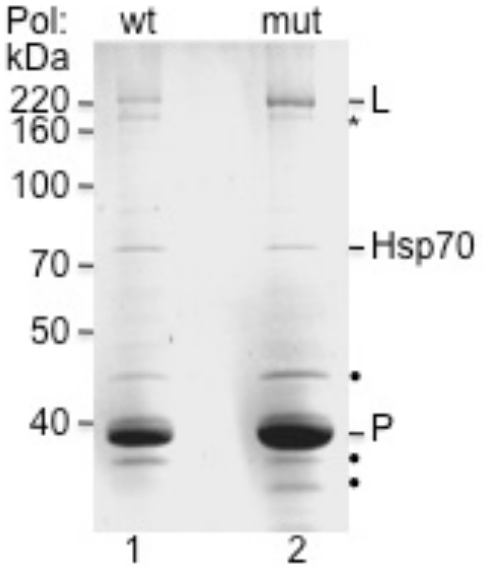


Figure 19; Analysis of isolated RSV polymerase complexes. PageBlue stained SDS-polyacrylamide gel of two RSV polymerases showing wt L/P (lane 1) and mutant L_{N812A}/P (lane 2) complexes. The bands corresponding to the expected migration patterns for L (250 kDa) and P (27 kDa) are indicated. Mass spectrometry of a representative gel showed that the band indicated with the asterisk contained L-specific polypeptides, while the bands indicated with the dot contained P-specific polypeptides. Hsp70 also purifies with the L-P complexes (as indicated). *Polymerase purification procedure was developed by Laure Deflubé* (186) *and purified polymerase was provided to me for the following experiments.*

The Le RNA was incubated with the purified polymerase complexes in a reaction mixture containing ATP, CTP, UTP and GTP, supplemented with [α-^{32}P]GTP. Following the reaction, the RNA products containing incorporated [α-^{32}P]GTP were analyzed by denaturing gel electrophoresis. Analysis of the RNA generated in the reaction containing wt polymerase yielded products ranging from 7 to 12 nt in length. These products were not detected in reactions containing no polymerase or mutant polymerase, indicating they were generated by the purified RSV polymerase (Figure 20, compare lanes 2, 3, and 4). Similarly, no products were detected in a reaction containing a template RNA representing the LeC sequence 1-14, confirming that the polymerase had template specificity for the Le promoter (lane 5). These results were similar to those obtained previously in studies of the RSV TrC promoter sequence and show that the isolated RSV polymerase was functional and had specificity for RSV promoter sequence (186).

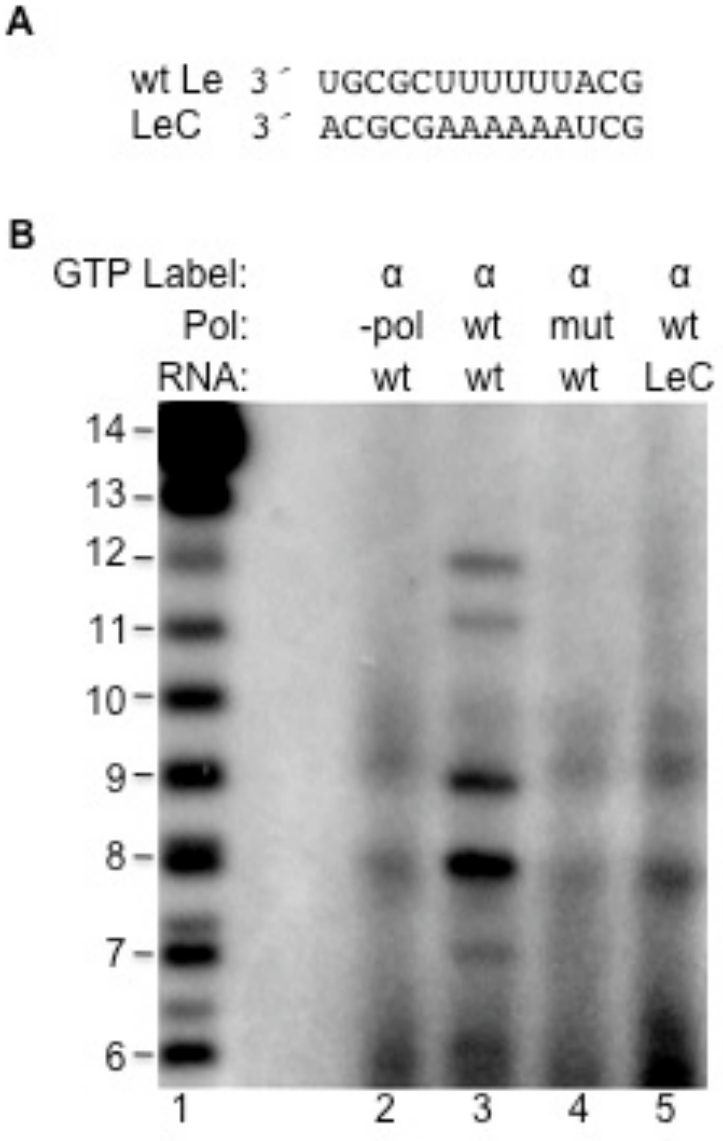


Figure 20; *In vitro* RNA synthesis is both RSV polymerase dependent and Le promoter specific. (A) Sequences of the RNA templates tested in this assay. Wt Le 1-14 represented the Le promoter, while LeC 1-14 was used as a control for polymerase specificity for the promoter sequence. (B) RNA synthesis

reactions were migrated on 20% urea-acrylamide gels alongside a ladder generated by alkaline hydrolysis of a LeC 1-14 RNA template to size the RNA products (lane 1). Le RNA was incubated without polymerase (lane 2) or with mutant polymerase (lane 4) as negative controls for RNA synthesis. The reactions were performed with all 1 mM each of all four NTPs, supplemented with [α-^{32}P]GTP to allow detection of RNA products made from the Le RNA template. *Sarah Noton generously helped with all of the RNA synthesis reactions by adding radiolabled GTP to the reactions and running the gels.*

Since the Le template is 14-nts long, the 12-nt product could represent RNA initiated from position +1 that was terminated prematurely, or it could also represent RNA initiated from position +3 and extended to the end of the template. The following experiments were performed to determine which of these possibilities was more likely.

2.2 RNA products are the appropriate size to have been initiated from position +3.

To determine whether the 12-nt product was initiated from position +1 and prematurely terminated, or from position +3 and elongated to the end of the template, a mutant template with a substitution intended to block +1 initiation was utilized. This mutant template contained a substitution at position 2 from a wt 'G' residue to an 'A' residue (2A Le). This substitution was selected based on previous results in the minigenome system indicating that a 2 G-A mutation in the Le reduced +1 initiation, but had no effect on initiation from position +3 (187). Wt Le and 2A Le 1-14 RNA templates were incubated with wt or mutant polymerase complexes in a reaction containing all four NTPs, supplemented with [α-^{32}P]GTP. Reactions with both the wt Le and 2A Le templates generated the same pattern of products, suggesting the products were all generated from position +3 (Figure 21, compare lanes 4 and 5). This result indicated that the polymerase could access the +3 site directly. However it was still possible that the polymerase was first initiating at position +1, generating 2-3 nt products, which cannot be easily

distinguished on this gel, and then reinitiating at the +3 site. To rule out this possibility, duplicate reactions were performed without UTP in the reaction mixture. The first site of UTP incorporation in the wt Le is at position 12, therefore products initiated from position +1 would be expected to be 11 nucleotides, while products initiated from position +3 would be 9 nucleotides. In contrast, the first site of UTP incorporation in the 2A Le template is at position 2, thus omission of UTP would be expected to block any initiation from position +1. If the +3 initiation site could be accessed independently by the polymerase, products generated from position +3 should not be affected, and would still be expected to be 9 nucleotides. In wt Le reactions omitting UTP, products of 8 and 9 nt were detected, but no 11 nt band indicative of initiation from position +1 was detected (lane 7). The products generated from reactions containing 2A Le without UTP were 8 and 9 nt long, and appear to be generated at the same level as the products generated from the wt Le template (lane 8). These data strongly suggest that the +3 initiation site can be accessed directly by the RSV polymerase, independent of initiation at position +1.

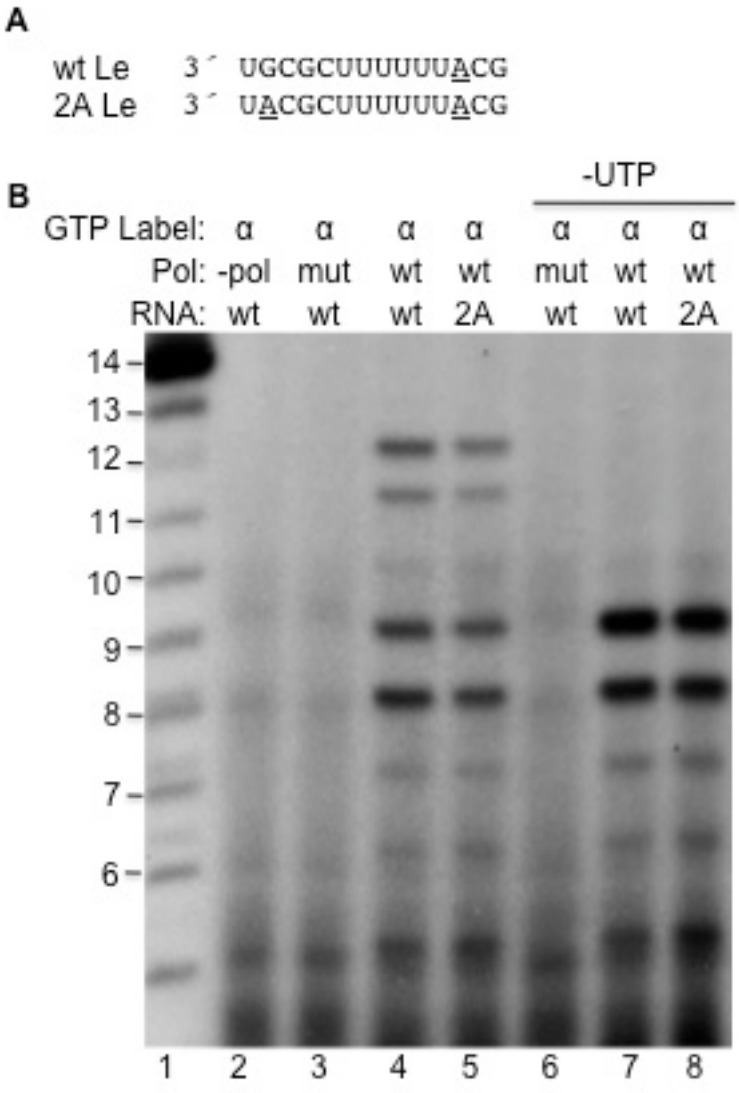


Figure 21; The RNA products detected were initiated from position +3. (A) Wt Le 1-14 template was compared to a 2A mutant template. Sites of UTP incorporation in the product are underlined. (B) Wt Le RNA was incubated with

no polymerase (lane 2) or mutant polymerase (lanes 3 and 6) as negative controls for RNA synthesis. Reactions in lanes 1-5 were performed with 1 mM each of all four NTPs, supplemented with [α-^{32}P]GTP. UTP was omitted from duplicate reactions in lanes 6, 7 and 8 to halt RNA synthesis at the first site of UTP incorporation. *Credit to Sarah Noton for help with the radioactivity and running the gel.*

2.3 End-labeling of *in vitro* RNA products indicates all detectable products are initiated from position +3.

As a final confirmation that the detectable products were initiated from position +3, wt Le or 2A Le 1-14 RNA templates were incubated with wt or mutant polymerase complexes, in reactions containing gamma-labeled radionucleotide. [γ-^{32}P]NTPs can only be incorporated at the 5´-end of RNA, allowing determination of the initiating nucleotide. In reactions performed with [γ-^{32}P]GTP, only RNA products with a 5´- 'G' residue would be labeled, eliminating detection of RNA initiated with a 5´- 'A' residue (for example, opposite position +1). In these reactions, the largest product detected from both the wt Le and 2A RNA templates was 12 nt, and the banding pattern was similar to the reactions containing [α-^{32}P]GTP (Figure 22, lanes 3 and 5). In reactions omitting UTP, bands of 8 and 9 nt were predominantly detected, and again the pattern was similar to the reaction containing [α-^{32}P]GTP, suggesting all detectable products were initiated from position 3 (lanes 7 and 9). This experiment was repeated using [γ-^{32}P]ATP in an attempt to label RNA initiated from position +1. No products were detected, confirming that initiation from position +1 is not favored in this assay (Figure 23). Note that the 14 nt band (indicated by an ellipse), which is visible in all lanes including reactions containing mutant polymerase, likely represents phosphorylated template and is not a product of RNA synthesis.

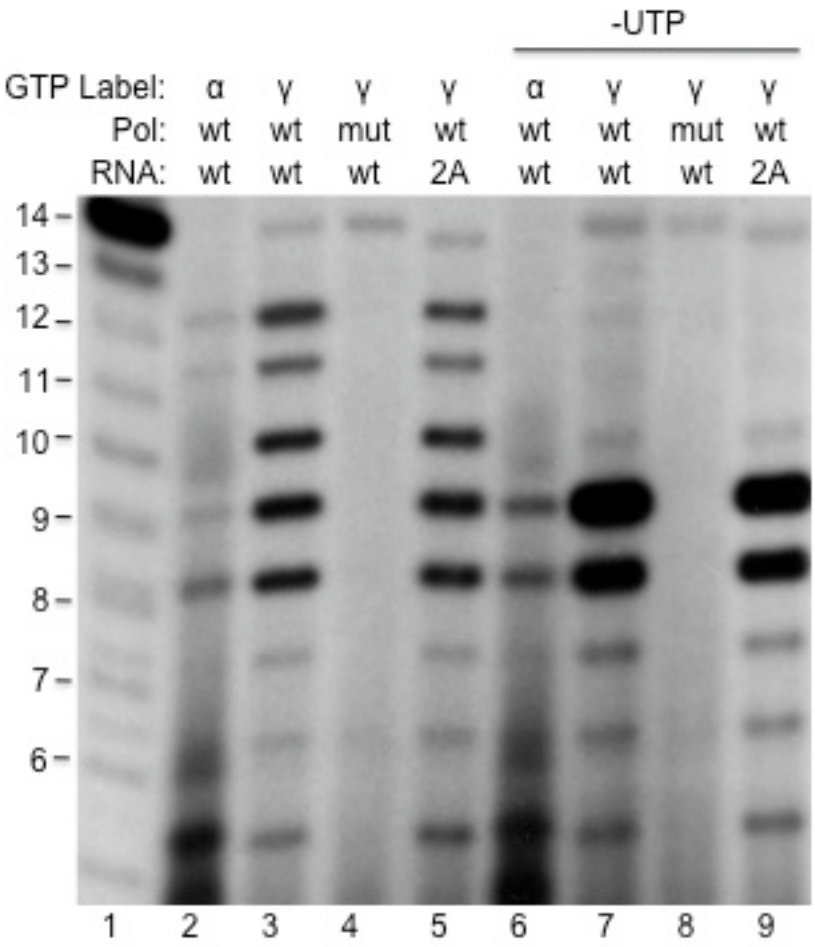


Figure 22; Products of RNA synthesis were initiated with a 5´- 'G' residue. Wt Le and 2A Le 1-14 RNA templates were incubated with 1mM each ATP, UTP, CTP, and 10μM GTP, supplemented with [γ-^{32}P]GTP to detect only RNA initiated with a 5´-'G' residue (lanes 3-5). Duplicate reactions were performed omitting UTP (lanes 7-9). Wt Le was incubated with all four NTPs and [α-^{32}P]GTP as in previous experiments, as a control. Wt Le RNA was incubated with mutant polymerase as a negative control for RNA synthesis (lanes 4 and 8). *Credit to Sarah Noton for help with the radioactivity and running the gel.*

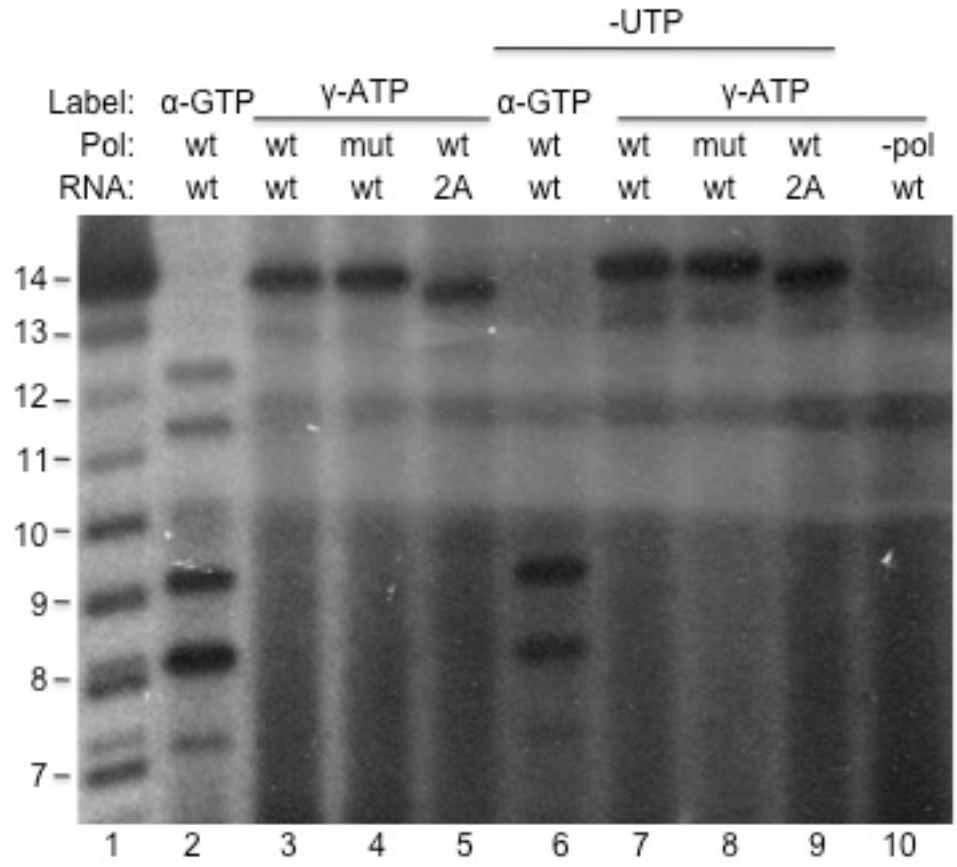


Figure 23; No initiation is detected from position +1. Wt Le and 2A Le 1-14 RNA templates were incubated with 1mM each GTP, UTP, CTP, and 10μM ATP, supplemented with [γ-^{32}P]ATP to detect RNA initiated with a 5´-'A' residue (lanes 3-5). Duplicate reactions were performed omitting UTP (lanes 7-9). Wt Le was incubated with all four NTPs and [α-^{32}P]GTP as in previous experiments, as a control. Wt Le RNA was incubated with mutant polymerase (lanes 4 and 8), and incubated without polymerase (lane 10) as negative controls for RNA synthesis. *Credit to Sarah Noton for help with the radioactivity and running the gel.*

Collectively, these data show that the RNA generated from the Le promoter under these reaction conditions was initiated from position +3, and that initiation from position +1 was not detectable. Importantly, these data demonstrate that the polymerase is able to initiate RNA synthesis at position +3 independently of initiation at position +1, confirming that the position +3 is a bona fide initiation site, consistent with the model illustrated in Figure 18.

2.4 Mutations that make Le3-12 identical to the L GS signal result in extension of the RNA initiated at position +3, and decreased initiation at position +45 at the first gene start.

The results presented thus far show that the RSV polymerase can generate short transcripts from a sequence resembling the L GS sequence at nucleotides 3 to 12 of the Le region. This is consistent with previous findings that showed that nt 3, 4, 5, 8, 9, 10, 11 and 12 are required for transcription initiation (64). However, these findings raise the question of why RNA initiated at position +3 is terminated after synthesis of only ~25 nucleotides, whereas the L mRNA can be extended several hundred nucleotides. One possible explanation is that initiation sequence may impact polymerase processivity; there is a two-nucleotide difference between Le nucleotides 3 to 12 and the L GS signal. To investigate if the sequence at Le 3-12 can alter elongation of RNA initiated at position +3, experiments were carried out using the minigenome system to study Le substitution mutants. The minigenome system is a cell-based assay that allows

for the investigation of cis-acting sequences involved in transcription and replication, enabling the study of mutations to the promoter regions that would otherwise be lethal in the virus. A minigenome plasmid typically contains one or two reporter genes flanked by GS and GE signals, and bordered by 3´- Le and 5´- Tr regions. The minigenome is expressed from a T7 promoter at its 5´-end, and the 3´-end is generated by a ribozyme. Minigenome plasmids are transfected into cells along with "helper" plasmids, which express N, P, M2-1, and L proteins, and T7 polymerase is provided in *trans* by coinfection with a modified vaccinia Ankara virus engineered to express T7 (MVA-T7).

In the following experiments, two mutant minigenomes with nucleotide substitutions in Le 3 to 12 that increase similarity to the L GS signal were utilized. The first minigenome contained a substitution at position 4 from G to C ("4C"), increasing identity of the Le to L GS sequence to 9 out of 10 nucleotides, while the second minigenome contained both the 4C substitution and a substitution at position 7 from U to G ("4C7G"), effectively recreating the L GS sequence from positions 3 to 12 of the Le (Figure 24, panel A). The minigenomes contained a chloramphenicol acetyltransferase gene (CAT) split into two cistrons by the N-P gene junction, and flanked by the NS1 GS and the L GE sequences. These minigenomes also possessed a mutation in the Tr region rendering them replication incompetent to ensure that the effect of mutations on the levels of replication could not influence transcription (64). Wt (4G), and the 4C and 4C7G

minigenomes, were transfected into cells and total intracellular RNA was isolated for analysis. RNA from transfection of wt minigenome without the L plasmid (-L) was used as a negative control for RNA synthesis.

Primer extension analysis of RNA isolated from transfections with the mutant minigenomes was performed using primers that correspond to genome positions 15-39, 45-68 and 56-75. The first two primers were used to detect initiation from positions +1 and +3, while the third primer was used to detect initiation at the first GS signal at position +45. Primer extension analysis with g15-39 shows RNA initiated from position +3 could be detected from the wt (4G) and mutant minigenomes (Figure 24, panel B, i). Interestingly, the ratio of +3 to +1 initiation in the 4C mutant was higher than the wt, suggesting the +3 site is used with greater frequency in this mutant, or that the RNA that is made is more stable (panel B (i), lane 3, and panel C). It should be noted that initiation at position +1 (replication) is also increased in the 4C mutant, a finding that has been recognized in previous studies (35; 64; 84). It is not currently understood why this is the case, and the present study does not attempt to address this phenomenon. No RNA initiated from position +1 was detected from the 4C7G minigenome, consistent with previous studies showing this mutation strongly reduces replication (64), but levels of +3 initiation were still high (panel B (i), lane 4). Analysis with the g45-68 primer showed that initiation from position +1 was still detectable from wt and the 4C mutant. However, while initiation at position

+3 decreased in the wt, the relative levels of RNA initiated from +3 detected from the 4C and 4C7G mutants were similar to those detected with the g15-39 primer. This result indicated that while the RNA initiated at +3 on the wt template was terminated prior to the GS signal, the RNA initiated at +3 of the mutant minigenomes was efficiently elongated beyond the first GS signal (panel B (ii), lanes 2, 3 and 4).

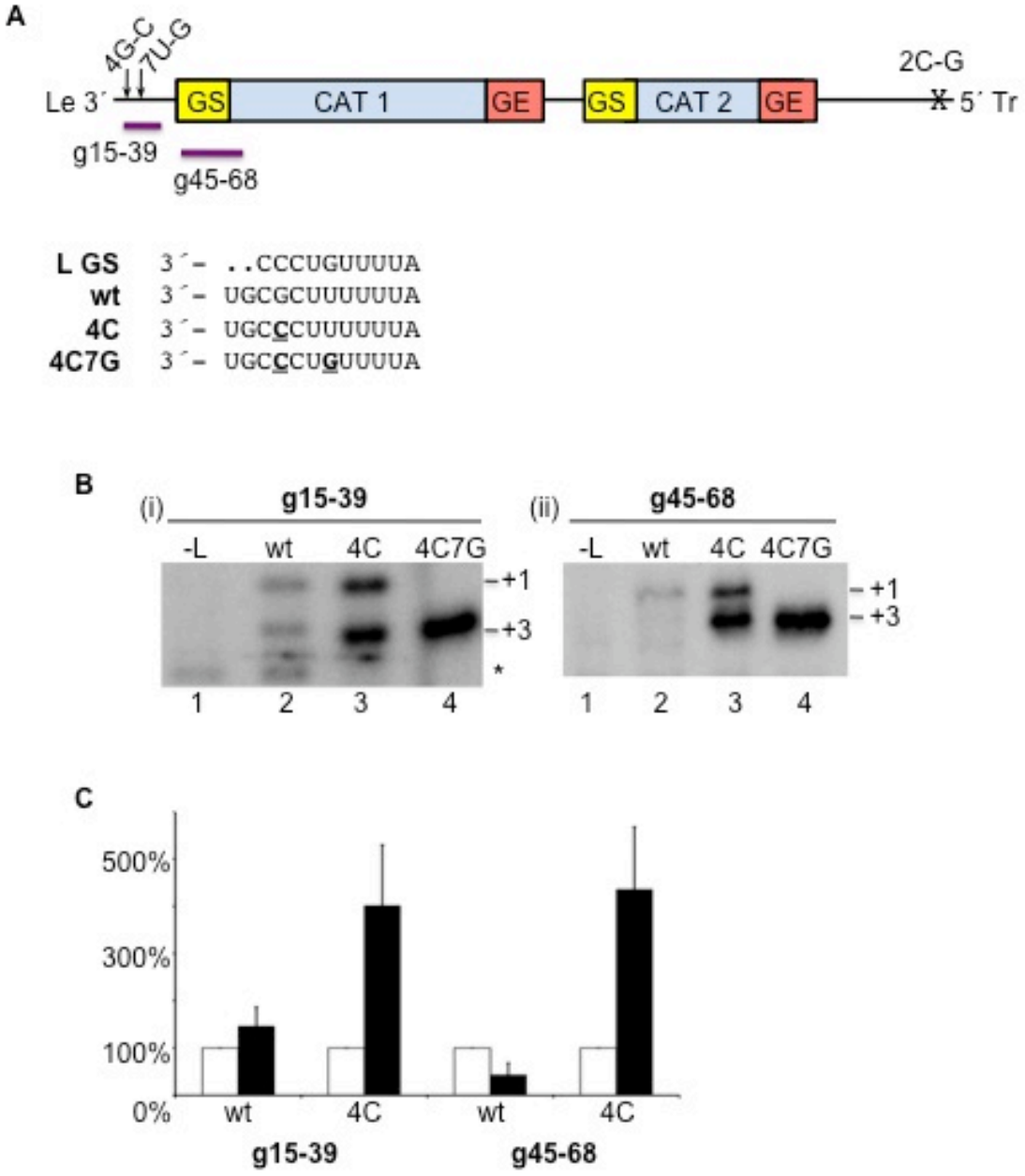


Figure 24; Single nucleotide substitutions of the Le promoter allowed RNA initiated from position +3 to be efficiently extended past the first GS. (A) Minigenome constructs containing substitutions in the Le promoter. Mutations

made were to position 4 G-C and position 7 U-G. These mutants were compared to a minigenome containing a wt 4G Le sequence. Minigenomes contained a 2 C-G substitution in the Tr region rendering them non-replicating. Genome positions the primer corresponded to are shown in purple. Le1-12 sequences of the wt and mutants compared to the L GS are shown below. Substituted nt are bold and italic. (B) Primer extension analysis of RNA isolated from transfections with wt (4G) and mutant minigenomes. The RNA was analyzed using primers g15-39 (i) and g45-68 (ii) to assess extension of RNA initiated from positions +1 and +3, as indicated. Lane 1 in each panel contains a negative control of RNA from cells transfected with wt minigenome, but lack the L plasmid. The asterisk in panel (i) indicates a non-specific background band. (C) Quantification of replicates of these experiments, showing RNA initiated from +3 (black bars), compared to RNA initiated from +1 from wt normalized to 100% (white bars). Bars show the mean of three experiments, with standard error indicated.

Because the RNA initiated from position +3 of the mutant minigenomes was extended into the first GS signal, we wanted to determine if transcription initiation at position +45 was impacted. Primer extension analysis with primer g56-75 showed an almost 2-fold reduction in initiation at the first GS signal for the 4C and 4C7G mutants as compared to wt (Figure 25, panel B (i), and C). Longer exposures of the same primer extension gel allow for direct comparison of the levels of initiation at the GS and initiation from position +1 and +3 (panel B, ii). While transcription initiation at position +45 was clearly impacted by mutations that affect elongation of the RNA initiated at position +3, the RNA initiated from +45 was still much more abundant than the RNA initiated from within the Le. This indicates either that transcription initiation occurs more frequently than initiation at position +3, or that the RNA generated at the first GS is more stable. Remarkably, this result demonstrates that mutation of the initiation sequence in the Le has an affect on initiation nearly 40 nt downstream at the first GS signal.

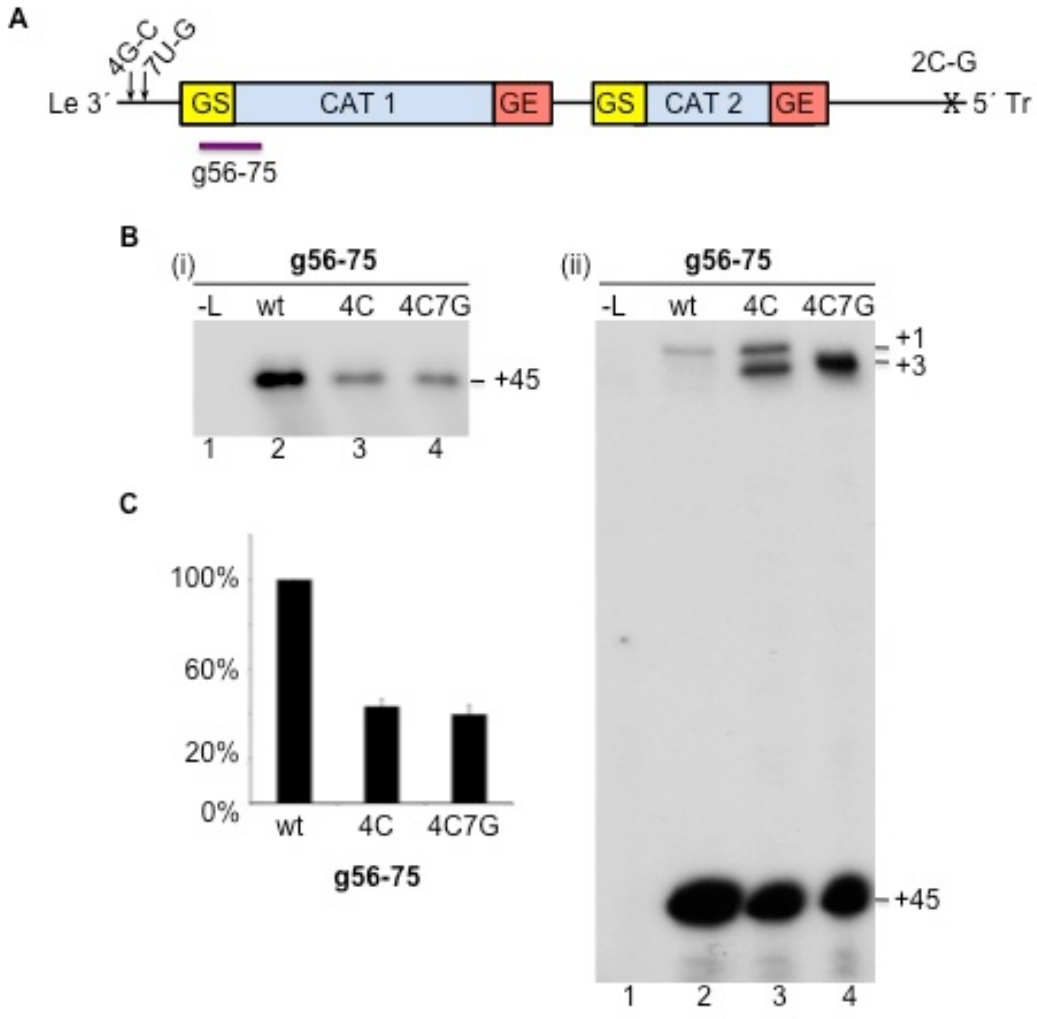


Figure 25; Mutation of the Le sequence impacts transcription initiation at position +45. (A) Position of genome the primer corresponded to is shown in purple. (B) Primer extension analysis of RNA isolated from wt and mutant minigenome transfections, analyzed with primer g56-75, to examine initiation at the first GS signal at position +45. Panel (ii) is a longer exposure of panel (i). Although we did not use markers corresponding to +1 and +3 initiated RNA, we

can infer that the products at the top of the gel are initiations from position +1 and +3 based on the banding pattern. This allows for direct comparison with initiations from +45 (bottom). (C) Quantification of initiations at positions +45 determined from replicates of panel (i). The data are normalized to the level of RNA from wt minigenome at 100%.

To determine how far RNA initiated from position +3 was extended, the RNA generated from the Le mutants was further analyzed by Northern blot. Using a probe to detect RNA initiated from within the Le region (g7-34), antigenome but no other LeC-containing RNA was detected from the wt minigenome, as expected (Figure 26, panel B). However, in addition to antigenome, LeC-containing RNA that was the appropriate size to be LeC-CAT 1 read through mRNA was detected from both the 4C and 4C7G mutants. Additionally, both mutants generated a heterogeneous smear of LeC-containing RNA, ~200 nucleotides in length (lanes 3 and 4). Blots were also probed for positive sense RNA using a negative-sense CAT-specific RNA probe, to compare levels of antigenome, CAT1 mRNA and CAT2 mRNA. Both the 4C and 4C7G mutants produced lower levels of CAT1 and CAT2 mRNA compared to wt, consistent with the primer extension analysis (panel C) and previously published data (64). As a control, a positive-sense CAT specific probe was used to detect levels of input minigenome, confirming the same amount of minigenome was transfected for each condition (panel D).

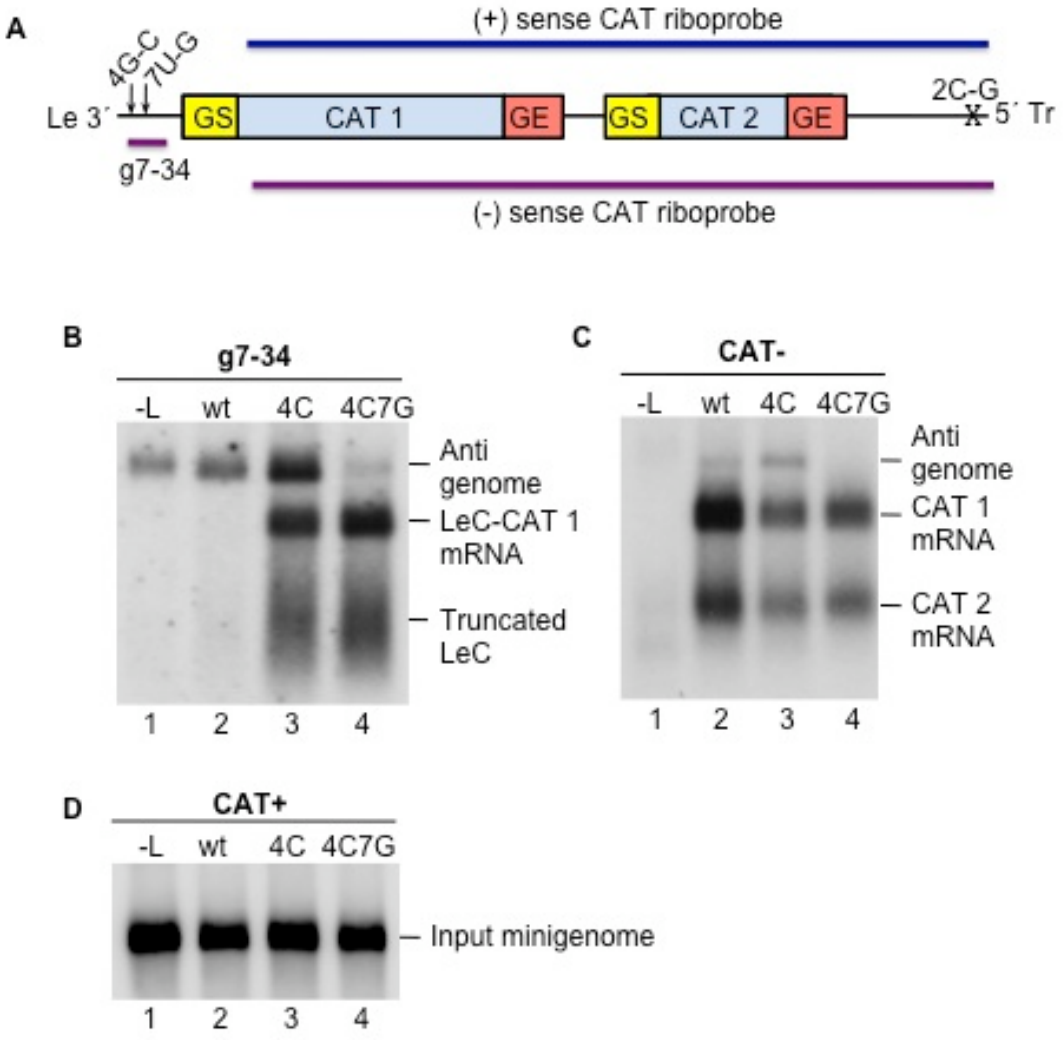


Figure 26; Substitutions within the Le promoter result in the generation of a Le-CAT 1 readthrough mRNA. (A) Minigenome schematic showing hybridization positions of the Le-specific oligo and (-) sense CAT riboprobe (bottom, purple), and the (+) sense CAT riboprobe (top, blue). RNA isolated from minigenome transfections was migrated on 1.5% agarose-formaldehyde gels. (B) Blot probed with g7-34 to detect antigenome and LeC-containing RNA. Antigenome, Le-CAT 1 mRNA and truncated LeC are indicated. Note that a

background band that comigrates with antigenome is visible in the –L control (lane 1). (C) Blot was probed with a negative-sense CAT-specific riboprobe to detect antigenome and mRNA (as indicated). (D) Blot was probed with a positive-sense CAT riboprobe to detect input minigenome, as a transfection control. Lane 1 in all blots is negative control of RNA from cells transfected with wt minigenome without the L plasmid.

Taken together, these results show that mutation to the sequence at positions 3-12 of the Le can alter polymerase processivity and result in efficient elongation of the RNA initiated from position +3. Additionally, these mutations had an impact on transcription initiation at the first GS signal, indicating that events at the promoter can affect events downstream.

2.5 The polymerase that initiates within the Le is capable of sequential downstream transcription of gene 2.

Because a single 4C substitution resulted in elongation of the RNA initiated from position +3 beyond the first GS signal, we wanted to determine if the polymerase could engage in downstream transcription after releasing the RNA initiated from +3. To facilitate analysis of the RNA initiated from +3, the first GS signal of mutant minigenomes containing either a wt Le (4G) or the 4C substitution was deleted to ablate production of CAT 1 mRNA (Figure 27, panel A). These minigenomes also contained a wt Tr region and were capable of multi-cycle replication, to enhance the signal from CAT 2. A wt minigenome with an intact CAT 1 GS was used to size the RNA generated from the mutant minigenome.

Northern blot analysis of RNA from the mutant minigenomes recapitulated the result from Figure 25, in that the 4C mutant generated a LeC-CAT 1 read through mRNA, and was the appropriate size to have been terminated at the first GE signal (Figure 27, panel B, compare lane 3 to lane 2). This RNA was not

detected from the wt minigenome (lane 4). Moreover, CAT 2 mRNA could be detected at ~50% of wt levels from the 4C minigenome, indicating that the polymerase that initiated at position +3 was capable of sequential downstream transcription (panel B, and quantitation in panel C). The RNA was also treated with nuclease to determine the encapsidation status of the RNA initiated within the Le. As expected, antigenome was not degraded by nuclease treatment, but the LeC-CAT 1 read through mRNA was ablated, indicating that this RNA was not encapsidated (panel B, lanes 5-8). Finally, primer extension analysis of these minigenomes using a primer corresponding to genome positions 24-50 detected a doublet at position +3 (panel D, doublet indicated with an ellipse). The reverse transcriptase tends to fall off the RNA when it encounters a 5´-cap, resulting in two cDNAs that migrate as a doublet (208; 223). Thus, this result can be taken as evidence that the RNA initiated from position +3 of the 4C minigenome was capped.

Taken together, these data suggest that polymerase processivity from position +3 can be influenced by the initiation sequence. More significantly, mutations at positions 4 and 7 can have an effect on initiation further downstream. When the sequence is highly identical to a GS signal, the polymerase can cap and extend the RNA initiated from position +3, and respond to downstream transcription signals, which fits with a model in which the transcribing polymerase must initiate at position +3 (Figure 18).

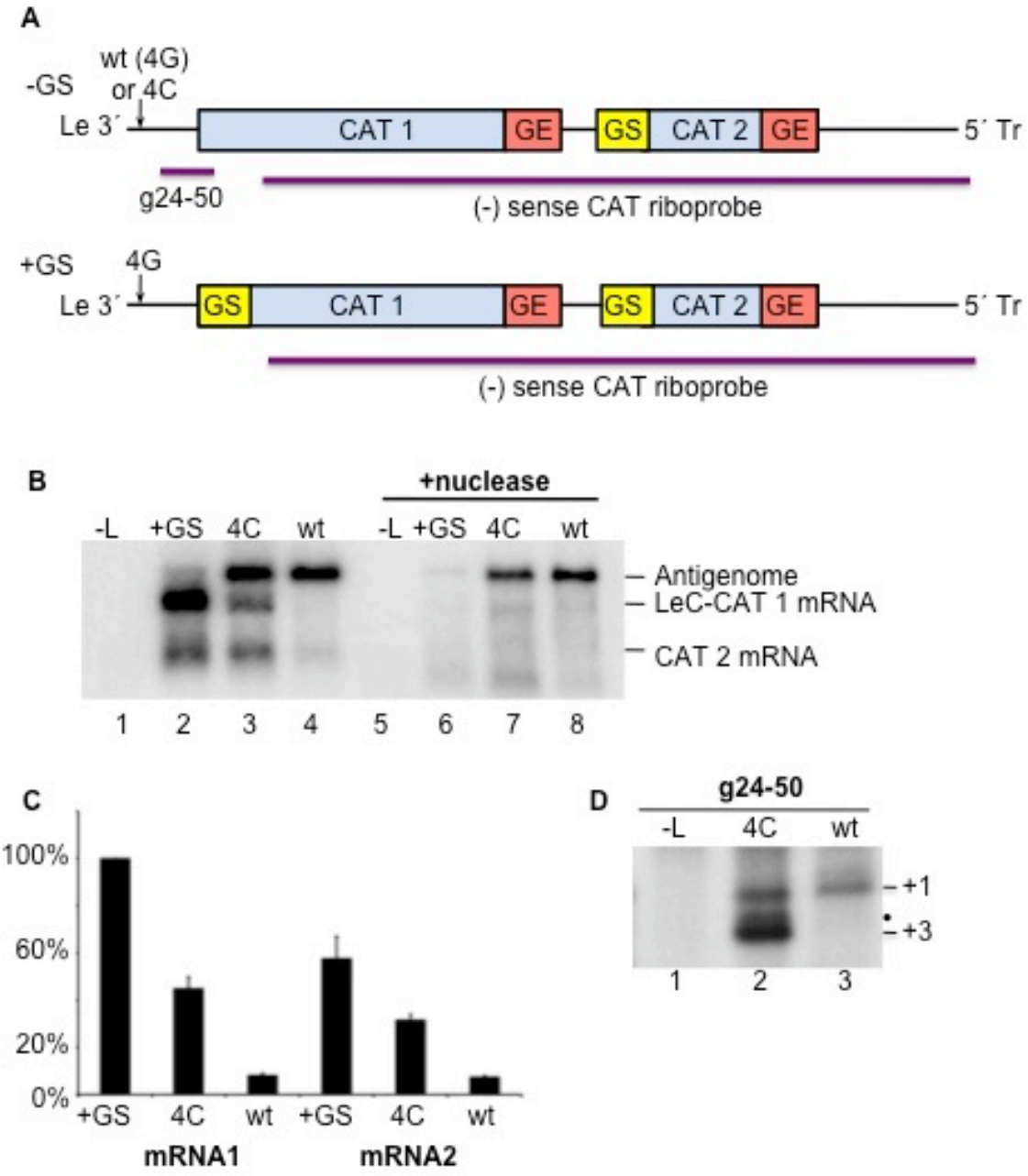


Figure 27; The polymerase that initiates from position +3 of the Le is capable of downstream transcription. (A) Replicating minigenomes containing either a 'G' or 'C' residue at position 4 of the Le, and with the first GS sequence deleted to ablate production of CAT 1 mRNA (-GS). A wt 4G minigenome with

an intact GS sequence was used as a size marker for RNA produced from the mutant minigenome (+GS). Hybridization positions of probes and primers are indicated. (B) Northern blot analysis of RNA isolated from transfected cells. RNA was prepared in duplicate, such that RNA in lanes 5-8 was treated with nuclease to digest unencapsidated RNA. RNA isolated from transfections with a wt minigenome with an intact GS signal was migrated as a size marker (+GS, lanes 2 and 6). RNA isolated from transfections with wt minigenome without L plasmid was used as a negative control (lanes 1 and 5). (C) Quantification of the CAT 1 and CAT 2 mRNAs normalized to the level of CAT 1 mRNA from +GS minigenome. Data is represented as the mean of three experiments and standard error bars are indicated. (D) Primer extension analysis of the 4C and 4G (wt) minigenomes lacking the first GS signal. The primer hybridized from positions 24-50 of the LeC. Initiations from positions +1 and +3 are indicated (markers not shown), and doublet representing capped +3 product is indicated with a dot (lane 2). RNA from a wt minigenome transfection without the L plasmid was used as a negative control (lane 1).

Discussion

Summary of Results

We have been able to reconstitute Le RNA synthesis *in vitro* using purified RSV polymerase and an RNA template corresponding to Le nucleotides 1 to 14. RNA synthesis was both polymerase dependent and promoter specific, and all detectable RNA products were initiated from position +3. No initiation from position +1 was observed. Mutation of the Le sequence to increase its similarity to the L GS signal resulted in elongation of RNA initiated from position +3 beyond the Le region, and a decrease in initiation at position +45 at the first GS signal. The resulting RNA was apparently terminated at the first GE signal, was not encapsidated, and the polymerase that initiated at position +3 was able to reinitiate transcription downstream at the next GS signal. These results suggest that transcribing polymerase is able to initiate at the 3´-end of the genome and is not constrained to initiate at the first GS signal. Additionally, the results of the minigenome experiments show that a single nucleotide substitution is sufficient to increase elongation of the RNA initiated from position +3 and impact the level of downstream initiation, indicating that the sequence of the initiation signal may play a role in polymerase processivity.

Capping as a checkpoint for RNA elongation

The results presented here demonstrate that the sequence of the template can influence polymerase activity. Substitution of Le position 4G to C results in stable elongation of RNA initiated from position +3, and the results of primer extension analysis and nuclease treatment suggest that the RNA is a product of a transcriptase, in that it is unencapsidated and potentially capped. This result raises the question of why the +3 RNA initiated from the wt Le sequence is not efficiently extended beyond 25 nucleotides. Due to the heterogeneous size of the small RNA, it is most likely that the polymerase generating the RNA reaches a checkpoint in RNA synthesis, rather than a defined termination signal. As previously discussed in Chapter 1, failure to become encapsidated by N protein is one possible checkpoint. Another checkpoint could be the failure of the nascent RNA to become capped, although these checkpoints are not mutually exclusive. Capping of viral mRNA is required for mRNA stability and allows the mRNA to be translated on the ribosome. Previous studies of an RSV capping inhibitor showed that an mRNA that does not become capped is terminated after synthesis of less than 50 nucleotides (152). It is known for VSV that initiation of RNA synthesis and capping of the nascent RNA are both directed by separable elements within the GS sequence (234). In the VSV study, mutation of nucleotides 1 or 3 of the GS sequence inhibited transcription, while mutation of nucleotide 2 did not inhibit RNA synthesis, but prevented capping and polyadenylation of the RNA (234). Similarly, the wt sequence from 3-12 of the Le

may be sufficient for directing the polymerase to initiate RNA synthesis at this site, but the two-nucleotide difference could ablate the signal for capping. This idea is supported by the minigenome data presented here, in that a single nucleotide substitution at position 4 results in elongation of the +3 RNA, and the primer extension data suggests this RNA is capped. During wt RSV infection, initiation at this site would serve to allow the polymerase to gain access to the first GS signal. In this model, elongation of this RNA due to capping would be detrimental to downstream transcription, and therefore does not occur from a wt promoter sequence.

In VSV, RNA is capped after synthesis of 31 nucleotides (243). It is thought that this is the minimum length necessary to allow the 5´- end of the RNA to be extruded from the polymerase active site and contact the putative capping domain (243). In RSV, experiments with a polymerase mutant that is defective for capping show that failure of mRNA transcripts to become capped result in mRNA being truncated around 45 to 50 nucleotides ((48), also see Appendix 2). No 25 nucleotide RNA was detected, in contrast to the Le studies. However, it is difficult to determine whether the RNA made from the Le is aborted because it fails to become capped, or if it fails to become capped because it is not elongated far enough for the 5´-end of the RNA to contact the polymerase capping domain. While the result with the L capping mutant may suggest that the checkpoint in the Le is therefore not capping, it may also indicate that there

are differences in the elongation abilities between a polymerase that initiates near the 3´-end of a template and one that initiates at the GS signal.

Failure to detect initiation from position +1 in vitro

It is worth commenting on the failure of the *in vitro* RNA synthesis assay to detect initiation from position +1. It is possible that +1 initiation was occurring but at levels too low to be detected. Primer extension analysis of RNA initiated from the Le in wt RSV infection show there is a much higher level of RNA initiated from position +3 than from position +1. Another possibility is that +1 initiation is not occurring in this assay because it requires the presence of N protein to become concurrently encapsidated. This would be simple to test if it were possible to purify soluble N protein. This has proven technically difficult as N protein tends to self-aggregate when not in the presence of P protein (12; 181). However, an N protein mutant exists that is unable to self-assemble, and may be useful to discern whether association of N with the L-P complex has a fundamental impact on whether the polymerase acts as a replicase. Additionally, sequence beyond Le1-14 may be required for initiation at position +1 (163). Longer Le templates could be tested in the *in vitro* assay, however the difficulty with this is that longer RNA templates may adopt secondary structures and therefore be unsuitable in this assay. But the ability to use encapsidated templates in the assay may present a solution to this problem. Finally, in the minigenome system, substitutions or deletions of the template at positions 1 and

2 are largely corrected back to wt sequence in the product, suggesting that replication may be initiated with an A-C dinucleotide primer in a non-templated manner (185). A similar mechanism for non-templated replication initiation using a self-generated primer has been demonstrated in Dengue virus as well (220). Thus, it is possible that under the conditions tested here, an A-C dinucleotide primer could not be synthesized or scavenged from the cellular milieu. The idea that replication initiation from position +1 requires a non-templated A-C dinucleotide fits nicely with the idea that transcription initiates *de novo* from position +3. This theory is explored in more detail in the Concluding Remarks.

Modified model of transcription initiation by the RSV polymerase

At the beginning of this chapter we proposed a simplified model for transcription initiation by the RSV polymerase (Figure 18). The data presented here support our hypothesis that the +3 initiation site is a *bona fide* initiation site used by the transcribing polymerase. The results of the *in vitro* RNA synthesis assay indicate that the polymerase can initiate at position +3 independently of initiation at position +1. The minigenome studies showed that the polymerase that initiates at position +3 is capable of downstream transcription in the absence of the first GS signal, and that the Le initiation sequence has an affect on downstream transcription initiation events. Putting these results together with results from Chapter 1, we offer the following expanded model for transcription initiation by the RSV polymerase: The polymerase enters the genome at the 3´-end of the

template and binds to nucleotides 3 to 12 of the Le. *De novo* RNA synthesis is initiated opposite position +3 and the polymerase moves past the promoter. After the synthesis of approximately 25 nucleotides, the polymerase reaches a checkpoint and aborts synthesis of the RNA. The polymerase is now poised to scan the template to locate the first GS signal at position +45 and reinitiate RNA synthesis. The polymerase that reinitiates RNA synthesis at position +45 becomes committed to sequential mRNA transcription (Figure 28, panel A). Transcription might also be performed by a polymerase that initiates at position +1 and generates a transcript that fails to become encapsidated before the checkpoint at ~25 nucleotides. RNA replication occurs if the polymerase initiates at +1 and the RNA becomes encapsidated with N protein before the ~25 nucleotide checkpoint. Initiation at position +1 may require an A-C dinucleotide primer (panel B).

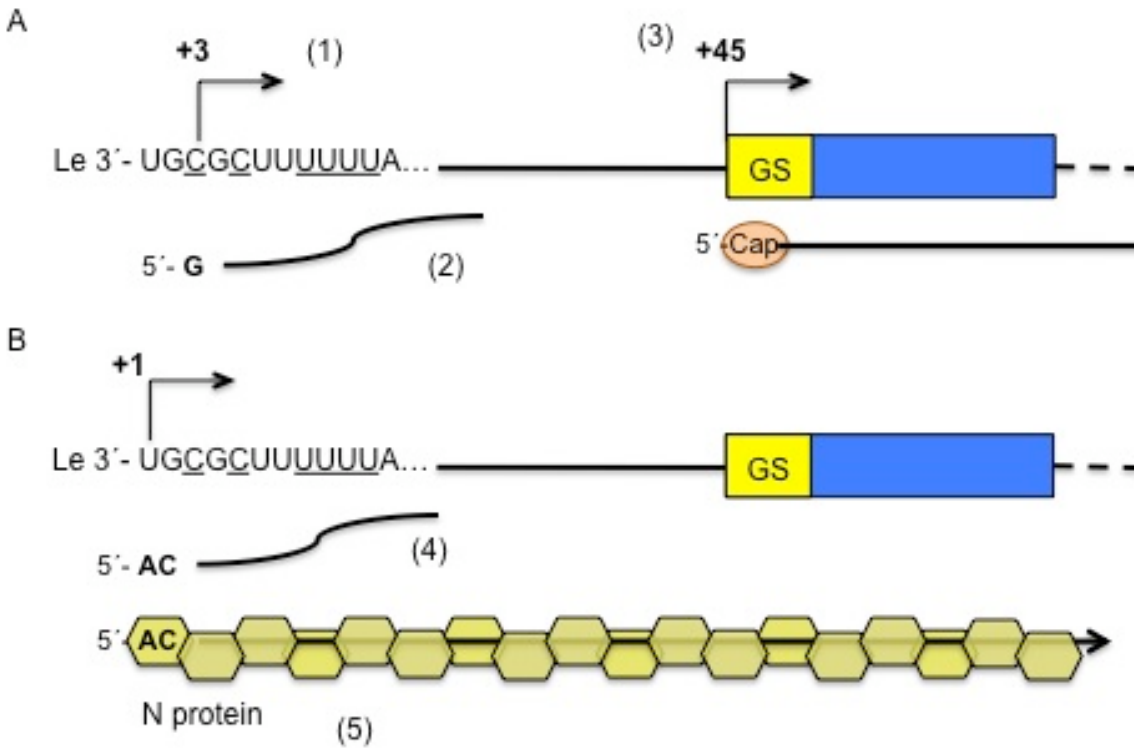


Figure 28; Modified model for transcription initiation by the RSV polymerase. (A) The polymerase enters the template at the 3´-end of the genome and binds to a promoter within the first 12 nucleotides of Le. Transcription is initiated opposite position +3 (1). The polymerase reaches a checkpoint in RNA synthesis at ~25 nucleotides and aborts the nascent RNA (2). The polymerase scans the rest of the Le region and reinitiates transcription at position +45 (3). (B) During replication, the polymerase initiates opposite position +1 of the Le. In some cases, the polymerase aborts synthesis of the RNA at ~25 nucleotides, possibly due to failure of the RNA to become encapsidated (4). Replication of full-length antigenome occurs from +1 in the presence of sufficient N protein (5).

Critique of the model

A possible criticism of this model is that even when a sequence that is identical to the L GS sequence was recreated at positions 3-12, and the RNA initiated at position +3 was efficiently elongated, transcription initiation at position +45 was not completely inhibited. In fact, as demonstrated in figure 24, initiation at position +45 is only about 2-fold lower than in the wt minigenome. There are several potential explanations for this apparent discrepancy. First, re-initiation at position +45 may be accomplished by polymerase that initiates at position +1 but terminates within the Le region due to failure of the RNA to become encapsidated, essentially an aborted replication attempt. A second possibility is polymerase that initiates at position +3 but releases the RNA prior to capping, and scans the Le to the first GS signal. Third, polymerase that initiates at position +3 and caps the nascent RNA may still terminate RNA synthesis within the first gene, as evidenced by the ~200 nt LeC-containing RNA detected by Northern blot (Figure 26). This polymerase could potentially scan backward to the first GS signal and initiation transcription.

Another possible explanation of this result, which is perhaps the most exciting, is the idea that both the +3 initiation site and the +45 initiation site are used to initiate transcription in a temporal manner. The primary initiation event on a given nucleocapsid may occur at position +3, and then once the N protein has been displaced along the Le region, all subsequent initiations on that template

are able to occur at position +45. In this model, the 3´-end of the genome may fold into a secondary structure upon N protein being locally removed, bringing the polymerase binding within the Le into close proximity with the first GS sequence, allowing initiation to occur directly at position +45. This model is appealing because it may also provide an explanation for the disparate results obtained for VSV concerning whether transcription at the GS signal required prior transcription of the Le, discussed in the introduction to this chapter (267). In those studies, UV mapping was performed on VSV nucleocapsids isolated from virions *in vitro,* and showed that Le synthesis was required for downstream transcription. However, nucleocapsids isolated from virus infected cells behaved differently, and UV mapping showed that Le synthesis was not required (267). The critical difference between these two experiments is the origin of the nucleocapsids. Virion-derived nucleocapsids are freshly synthesized and packaged into nascent virus particles and have not yet been used as a template for RNA synthesis, whereas nucleocapsids isolated from infected cells represent a population of templates that are in various stages of use by the viral polymerase. This idea suggests that for a given template, there may be a fundamentally different series of events occurring depending on what point that template is at in the replication cycle.

Does the polymerase direct transcription from the replication promoter?

Notably, initiation from position +3 leading to the generation of a small RNA is not exclusive to the Le promoter. As shown in Chapter 1, RNA synthesis is initiated at the +3 site and an ~25 nt RNA is generated from the TrC promoter in the antigenome as well. This is not surprising considering the sequence similarity between the Le and TrC promoters. However, this finding suggests the exciting possibility that both promoters can direct initiation of transcription. While only the Le promoter with downstream genes is capable of directing mRNA synthesis, the TrC promoter can synthesize a short RNA. This may suggest that the small RNAs are not just generated as a byproduct of RNA synthesis initiation and promoter escape, but may possess a viral or cellular function. This idea will be explored in Appendix 1.

Future Directions

It would be interesting to determine whether failure of the polymerase to cap the RNA is responsible for termination within the Le. One of the problems with the minigenome experiments is that the transfections were carried out with coinfection with MVA-T7 to drive expression of the minigenome and helper plasmids. The vaccinia polymerase has been shown to possess promiscuous capping activity and therefore it is impossible to determine whether capping can be attributed to the RSV polymerase. To negate the effects of coinfection with MVA-T7, these experiments could be repeated using BSR-T7/5 cells, baby

hamster kidney cells that stably express the T7 polymerase. We did attempt to perform tobacco acid pyrophosphatase (TAP) digestion of RNA from RSV infection followed by primer extension analysis to determine whether RNA initiated from position +3 was capped, however these experiments were unsuccessful and will require optimization (see Appendix 3). It would also be useful to develop a *trans*-capping assay similar to the one used for VSV, which would allow us to examine the template sequence requirements that influence capping by the RSV polymerase (148; 188). The *trans*-capping assay is a minimalist assay that would utilize purified RSV polymerase and an artificial template to distill the study of the RSV polymerase capping function to its most basic components.

Another interesting direction this work could take would be to test the model concerning temporal regulation of transcription on a given nucleocapsid. Nucleocapsids isolated from virions, and from various timepoints during RSV infection could be tested in an *in vitro* transcription assay to determine whether transcription is dependent on prior Le synthesis, similar to the studies done for VSV (267). Other experiments that could be done to test this idea would be to reexamine previously described non-replicating minigenome mutants that have substitutions at positions within the Le that are critical for downstream transcription (64). Primer extension analysis could be performed on RNA isolated from transfections with these mutants to determine whether they are

unable to utilize the position +3 initiation site. If downstream transcription were dependent on the primary initiation event occurring within the Le, then we would predict that these mutants might be unable to initiate at position +3.

In conclusion, the data presented in this chapter indicate that the +3 initiation site is accessed by the RSV polymerase independently of the +1 initiation site, and polymerase processivity from this site is influenced by the initiation sequence. These data, in conjunction with the findings of Chapter 1 have led us to propose a model for transcription initiation whereby the RSV polymerase initiates at position +3 of the Le, generates a small RNA, scans to the first GS signal and becomes committed to transcription.

Concluding Remarks

Summary of Results

The mechanism of transcription initiation by the non-segmented negative sense RNA viruses has been debated for several decades. Models proposed for related viruses were not sufficient to explain the mechanism for RSV, thus we set out to explore the behavior of the RSV polymerase at the promoter regions. We hypothesized that the sequence from Le nucleotides 3-12 serves as a recruitment site for the transcribing polymerase. This hypothesis was supported by saturation mutagenesis indicating the importance of nucleotides within this sequence for downstream transcription (64), and strong sequence similarity of Le nucleotides 3-12 with the L GS sequence (8 out of 10 nt identity). Furthermore, there was evidence for initiation from position +3 demonstrated in the minigenome system (185; 187). In Chapter 1, we were able to show that the RSV polymerase initiated RNA synthesis at position +3 of the Le and TrC promoters during virus infection. Surprisingly, RNA initiated from position +3 of the Le promoter was more abundant than RNA initiated from position +1, suggesting this site is frequently used during infection. RNA synthesis initiation at position +3 resulted in the generation of a short ~25 nucleotide RNA that was partially unencapsidated, and this RNA fractionated with subgenomic viral mRNA. These data allowed us to propose a model for transcription initiation, whereby the transcribing polymerase enters the Le region at the 3´-end of the

genome and initiates RNA synthesis from position +3, synthesizes a short RNA, and reinitiates mRNA transcription at the first GS signal. This is the first demonstration of an additional RNA initiation site that is used during RSV infection.

In Chapter 2, we tested the model that transcription is initiated from position +3 of the Le. We showed that initiation from position +3 was reproducible in the *in vitro* RNA synthesis assay using a Le RNA 1-14 template and purified polymerase. Moreover, we demonstrated that initiation from position +3 occurred independently of initiation from position +1, a finding that is consistent with previous studies in the minigenome system showing that deletion or substitution of nucleotides 1 and 2 did not affect initiation from position +3 (185; 187). This finding suggests the polymerase accesses the +3 initiation site directly, and that initiation at position +3 is not dependent on prior initiation from position +1. As mentioned earlier, the sequence from Le positions 3-12 shares eight out of ten nucleotide identity with the L GS sequence, thus we wanted to determine whether the two nucleotide difference could explain why mRNA initiated from the L GS is extended many hundreds of nucleotides, while RNA initiated from position +3 is terminated around 25 nucleotides. A single nucleotide substitution of the Le sequence (Le4C) that increased similarity to the L GS sequence altered polymerase processivity within the Le region, resulting in stable elongation of the transcripts initiated from position +3 through the first gene. The polymerase that

initiated at position +3 was able to engage in downstream transcription of the next gene. This substitution also had the effect of reducing transcription initiation at the first GS signal at position +45, indicating that the initiation sequence can affect transcription of downstream genes.

Significance

The significance of the data presented here is two-fold. First, from a basic science standpoint, the results suggesting that transcription initiates at position +3 adds to the discussion on how NNS RNA viruses initiate RNA synthesis. As demonstrated by primer extension analysis (Chapter 1) and the *in vitro* RNA synthesis assay (Chapter 2), initiation from position +3 is the dominant initiation event in the Le, suggesting the polymerase is adept at initiating internally. Thus, defining the mechanism of RNA synthesis initiation is important because the viral polymerase must faithfully replicate the genome ends, and therefore must have evolved a way to initiate right at the 3´-end of the template (at position +1). Second, understanding the behavior of the polymerase at the promoters may expose novel stages in RSV infection that could be targeted by rational drug design, and inform vaccine development. Each of these ideas is explored in more detail below.

RSV RNA synthesis initiation; challenging the paradigm

RNA-dependent RNA polymerases are known to initiate RNA synthesis predominantly by two mechanisms. The first mechanism is primer-mediated initiation, in which RNA synthesis occurs in a template independent manner and requires a protein or nucleic acid primer to initiate. Examples of this include VPg protein-primed transcription of picornaviruses (196) and cap snatching by influenza virus (202). The second mechanism of RNA synthesis initiation is *de novo* initiation, or primer-independent initiation. *De novo* initiation is facilitated through the formation of a phosphodiester bond between the initiating NTP and the second NTP, which are both specified by the template. The hepatitis C virus and rotavirus polymerases initiate *de novo* (29; 117; 118; 281). In some instances, *de novo* RNA synthesis can result in the generation of a primer, either through abortive initiation or a prime and realign mechanism. Turnip crinkle carmovirus generates short 4-8 nucleotide abortive products *de novo* that then act as primers for synthesis of negative strand RNAs (99). And some arenaviruses and bunyaviruses use a prime-realign mechanism, whereby *de novo* initiation first occurs internally. Then the nascent RNA primer is shifted back such that it aligns with the 3´-end of the template (72; 127).

Some viruses appear to utilize a combination of initiation mechanisms. For example, influenza virus replication has been shown to initiate internally *de novo* and synthesize a dinucleotide primer, which is then realigned to prime replication

(49; 280). Similarly, the polymerase of Dengue virus is able to self-generate a dinucleotide primer, which it then uses to initiate from the +1 site (220). Until recently, it was believed that the polymerases of the NNS RNA viruses initiate all RNA synthesis by a *de novo* mechanism. However, our studies suggest that for RSV, transcription initiates *de novo* at position +3 (186; 250), while replication is mediated through a dinucleotide primer corresponding to positions 1 and 2 in a nontemplated manner (185).

Primer synthesis: a possible function for the small LeC RNA

How the dinucleotide primer is generated is still under investigation. But it is possible that the small LeC RNA that is synthesized *de novo* serves as the template for the generation of an A-C dinucleotide. A sequence element at positions 15 and 16 of the LeC RNA could direct synthesis of a dinucleotide primer (5´-AC- 3´) for initiation from position +1 (Figure 29). This scenario might represent an adaptation of the prime-realign mechanism utilized by other viral polymerases as described earlier, in that the RSV polymerase might synthesize the primer from the LeC RNA, instead of from a sequence near the 3´-end of the genome (note that there are no sequence elements close to the 3´-end that could encode an A-C dinucleotide). If the small LeC RNA were necessary to generate an A-C dinucleotide primer, this could explain why initiation from position +1 was not detected in the *in vitro* RNA synthesis assay. The template used in the assay corresponded to Le nucleotides 1-14; the sequence element that can generate

the primer is found just beyond nucleotide 14, thus the primer would not be generated and initiation from position +1 would not be reconstituted.

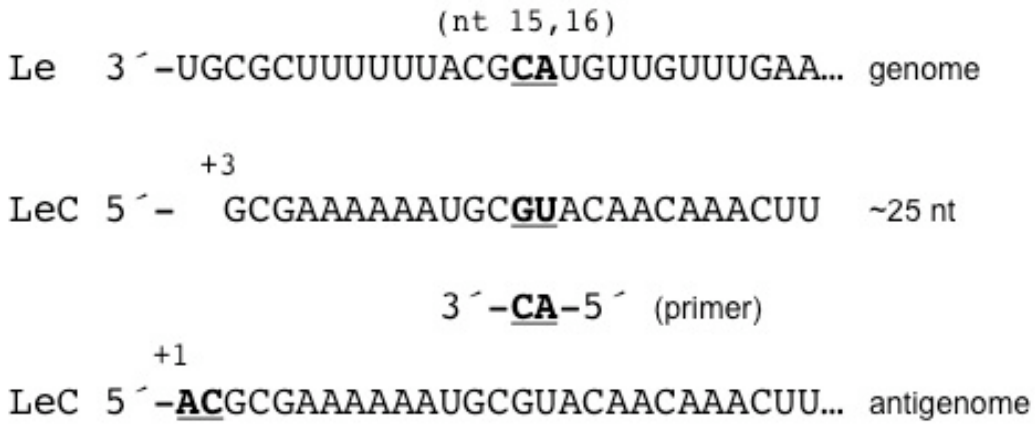


Figure 29; Schematic for the generation of a replication primer from the LeC small RNA. The first 27 nucleotides of the Le sequence from the 3´-end of the genome are shown at top. The polymerase initiates RNA synthesis *de novo* from position +3 and generates an ~25 nt RNA (middle). The sequence element at positions 15 and 16 that could encode a di-nucleotide A-C primer is underlined and in bold. The first 27 nucleotides of the LeC sequence at the 5´-end of the antigenome is at the bottom, showing incorporation of the primer from position +1.

RSV as a model for other NNS viruses

Whether or not the mechanism of transcription initiation utilized by the RSV polymerase will serve as a new paradigm for other negative strand RNA viruses is still unclear. Studies carried out for VSV in the *in vitro* RNA synthesis assay failed to turn up evidence for internal initiation or non-templated initiation (173). This may simply suggest that the mechanism used by paramyxoviruses is distinct from that of rhabdoviruses and other NNS RNA virus families. However, the model may still hold for other members of the paramyxovirus family. It is interesting to note that all the viruses in the paramyxovirus family possess a 5´-AC at the genome and antigenome termini, suggesting they may all exploit a common mechanism for replication initiation (185). Consequently, we compared the Le sequences of other paramyxoviruses, including SeV, MuV, Newcastle disease virus (NDV), NiV, MeV, parainfluenza virus 5 (PIV5), parainfluenza virus 3 (PIV3), and human metapneumovirus (hMPV), to their conserved or consensus GS sequences to look for evidence of internal GS-like motifs in the Le region (Table 4). In some instances, internal sequences similar to GS sequences could be identified, particularly for NDV and hMPV, however none aligned as strongly as the RSV sequences (shown at the top for comparison). For some viruses, no internal alignment could be identified between the Le and GS sequences (SeV, MeV, and PIV3). Interestingly, a GS-like element can be aligned from position +4 of the VSV (*Rhabdoviridae)* Le region (bottom), despite a lack of evidence for internal initiation *in vitro* (173). It would be worthwhile carrying out primer

extension analyses on viral RNA isolated from infections with these and other viruses, to look for evidence of alternate initiation events.

Table 4; Sequence alignments of paramyxovirus Le and GS sequences. Conserved nucleotides are written in uppercase, while for non-conserved sequences, the most common nucleotide found at a given position is written in lowercase. 'N' represents any nt, 'Y' is a pyrimidine (C or U), 'K' is 'U' or 'G'. The sequence in the Le that can be partially aligned with GS sequences is underlined, and the nt that align in the GS are underlined. *(Note that VSV is a rhabdovirus)*

RSV	
Le	3´–UGCGCUUUUUUACGCAUGUU…
GS (L)	3´– CCCUGUUUUA
SeV	
Le	3´–UGGUUUGUUCUCUUGUUUGA…
GS	3´–UCCCANUUUC
MuV	
Le	3´–UGGUUCCCCUUUUACUUCUA…
GS	3´– UYCKKNYYU
NDV	
Le	3´–UGGUUUGUCUCUUAGGCAUU…
GS	3´– UGCCCAUC
NiV	
Le	3´–UGGUUUGUUCCCUCUUAUAC…
GS	3´– UCCUngGU
MeV	
Le	3´–UGGUUUGUUUCAACCCAUUC…
GS	3´–UCCcnggU

PIV5	
Le	3´–UGGUUCCCCUUUUACUUCAC…
GS	3´– UcCgggCa
PIV3	
Le	3´–UGGUUUGUUCUCUUCUCUGA…
GS	3´–UCCUNNUUUC
hMPV	
Le	3´–UGCGCUUUUUUUGCGCAUAU…
GS	3´– CCCUGUUCA
VSV	
Le	3´–UGCUUCUGUUUGUUUGGUAA…
GS	3´– UUGUCNNUAG

Other possible functions for the small LeC and Tr RNAs

As discussed, the small RNAs may serve as a template for primer generation for initiation from position +1. However, these RNAs may also have other functions (discussed extensively in Appendix 1). It has recently been shown that small viral RNAs synthesized by influenza virus bind the viral polymerase and influence a switch from transcription to replication (199; 200). Similarly, we have preliminary data suggesting the small LeC and Tr RNAs may enhance genome replication (See Appendix 1). The small RNAs may also play a role in the cellular response to RSV infection. The SeV Tr sequence has been shown to bind TIAR, a component of stress granules, and block the stress granule response to SeV infection (110). Correspondingly, it has been shown previously that RSV blocks the stress granule response to infection, and that the Tr region may play a role

(96). Additionally, virally-encoded microRNAs (miRNAs) and other small RNAs have been shown to play diverse roles in virus infection from establishing latency to evading the immune response. It is unclear whether the small RNAs generated from the Le and TrC promoters might function as miRNAs. Despite the fact that the ~25 nt RNAs are roughly the size of a fully-processed miRNA, they are synthesized *de novo* from the genome and antigenome, and do not appear to be processed in a canonical miRNA fashion. Typically miRNAs are cleaved from larger precursors in the nucleus by an endonuclease called Drosha, thus it was believed that cytoplasmic RNA viruses could not synthesize miRNAs. However, noncanonical synthesis of a viral miRNA that occurs in the cytoplasm, independent of Drosha processing has recently been described (222; 258). Additionally, it would be important to ascertain whether or not the LeC and Tr RNAs become associated with Argonaute (Ago) proteins to form a functional RNA-induced silencing complex (RISC). To that end, it would be interesting to investigate whether the small LeC and Tr RNAs have cellular or viral RNA targets. The small RNAs may also hijack cellular factors and form higher order ribonucleoprotein complexes to benefit viral infection. For example, Epstein-Barr virus encodes two small RNAs, EBER1 and EBER2, that are highly abundant, form ribonucleoprotein complexes with many host proteins, most intriguingly, La autoantigen (144), and are thought to be involved in transformation and oncogenesis of the host cell (125; 276). Such a relationship between the RSV

small RNAs and cellular proteins should be explored, since it has been shown that RSV LeC RNA also binds La protein (13).

Could the small RNAs play a role in polymerase regulation?

Taking into consideration the possible functions of the small RNAs, we can speculate on the larger question of how the polymerase is regulated between transcription and replication. The results presented in Chapters 1 and 2 allowed us to propose a modified model for transcription initiation by the RSV polymerase, in which the polymerase enters the encapsidated genome at the 3´-end of the template and binds to a recruitment site found from positions 3 to 12 of the Le. RNA synthesis is initiated *de novo* from position +3, and the polymerase escapes the promoter and synthesizes approximately 25 nucleotides of LeC RNA, before terminating synthesis. We surmised that the small RNA is not terminated at a specific stop sequence but rather that the polymerase aborts synthesis after failing a checkpoint for RNA elongation. Possible checkpoints include failure to cap the nascent RNA, or failure of the RNA to become encapsidated in viral N protein, and these may both come into play. The small LeC RNA, which likely bears a 5´-triphosphate, becomes bound either in N protein or cellular La protein to mask it from pattern-recognition receptors like RIG-I (13). After terminating synthesis of the small RNA, the polymerase is poised to scan the rest of the Le region to the first GS signal at position +45, where it reinitiates RNA synthesis and becomes committed to a transcription

program. For replication to occur, the polymerase must initiate opposite the first nucleotide of the template, and previous studies have indicated that the first two nucleotides may be non-templated (185). Thus, the small LeC RNA might serve as a template for the synthesis of the first two initiating NTPs, 5´-A-C. The polymerase may become preloaded in its active site with the A-C dinucleotide primer, and would then be able to synthesize full-length antigenome RNA from position +1 that is concurrently encapsidated, and which then serves as the template for synthesis of genome-sense RNA. Alternatively, the LeC RNA may physically associate with the polymerase and cause a change in the polymerase complex structure, altering its function, or it may act as an indicator or feedback molecule corresponding to levels of transcription. All of these possibilities may influence the polymerase behavior between transcription and replication. In addition, the polymerase can also initiate at position +3 of the TrC promoter and generate the small Tr RNA, which may function similarly to the LeC RNA. The small Tr RNA may also play a role in the cellular stress granule response to infection, which could affect viral fitness. While this is all speculation and the function of the small RNAs still needs to be investigated, based on the available data for RSV and other viruses (185-187; 199; 200; 220; 280) these scenarios are plausible (See figure 30).

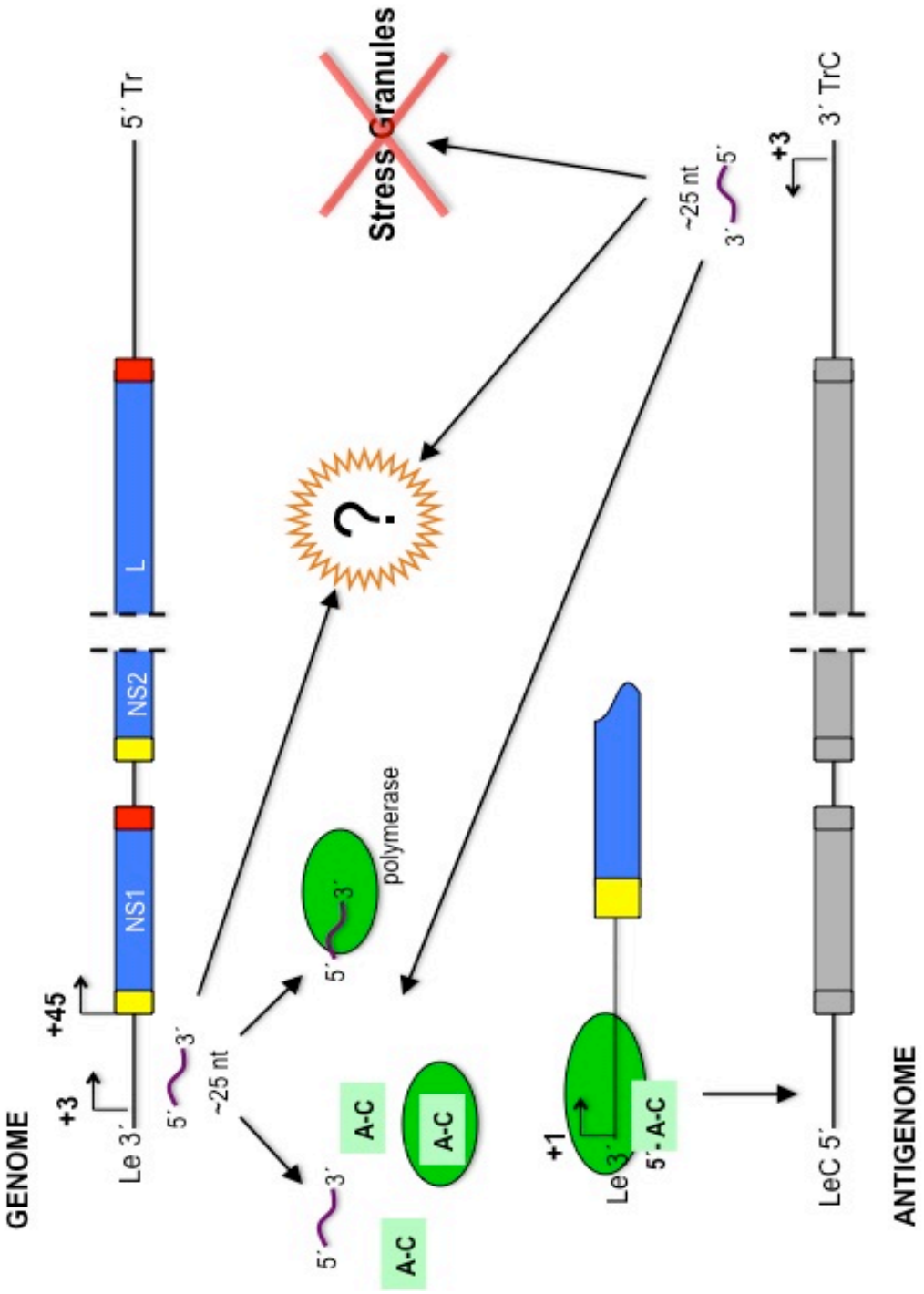


Figure 30. The small RNAs might influence polymerase behavior during infection. Schematic showing the production of the ~25 nt RNAs from the Le and TrC promoters of the genome and antigenome (shown in gray), respectively.

The polymerase is represented as a green oval. The small LeC RNA might serve as the template for the generation of A-C dinucleotide primers needed for replication from position +1. It might also physically bind the polymerase complex and influence its behavior. Both activities might lead to a shift from transcription to replication. The small Tr RNA might function similarly, and might also play a role in the stress granule response. Both RNAs might have other cellular or viral roles that should be explored (indicated by the question mark). *Not to scale*.

Application of these studies towards the development of treatments and vaccines for RSV infection

As a final note, there is a demonstrated need for RSV antiviral compounds, as treatment of severe RSV infection is currently limited to supportive care. The RSV RNA-dependent RNA polymerase is responsible for carrying out both transcription and replication from the same or overlapping promoter regions in the Le. Thus, inhibiting the RSV polymerase or blocking the interaction between the polymerase and the promoter would effectively arrest the viral replication cycle, making it an excellent target for the development of novel therapeutics. The studies presented here have improved our knowledge of how the polymerase initiates RNA synthesis and becomes committed to transcription, and could inform rational drug design. This knowledge could be applied to a couple of different novel strategies. Antisense oligodeoxynucleotides (AS-ODNs) are small molecules, 15-20 nucleotides in length that are designed to target viral sequences (201; 278). When targeted against translational regulatory sequences of NS1 and NS2 mRNAs, AS-ODNs were shown to be effective in inhibiting translation of these proteins (US patent 5 831 069). They can also be coupled with 2´, 5´-oligoA-dependent RNase L, which recruits RNase L to the RNA:DNA duplex, resulting in degradation of the target RNA (139). An AS-ODN designed to be complementary to the LeC or Tr small RNAs may have a potent effect RSV replication, as preliminary data suggests the small RNAs play a role in influencing replication (Appendix 1). A molecule representing the Le or TrC

promoter region may also function secondarily as competitor for polymerase complex, greatly reducing the pool of available polymerase for transcription and replication.

A clearer understanding of polymerase behavior at the promoters could be applied to the development of live-attenuated vaccines for RSV. Live-attenuated vaccines do not induce vaccine-enhanced disease like their killed vaccine counterparts, and are therefore a promising avenue for vaccine development (179). However, it is difficult to balance sufficient attenuation of the virus for vaccine safety and a robust ability to elicit a protective immune response. Engineering a recombinant virus with mutations to the Le promoter that promote transcription over replication may result in a vaccine strain that is strongly antigenic but is greatly impaired in viral replication. In Chapter 2, we showed that a mutation at position 4 from wt 'G' to 'C' results in elongation of RNA initiated from position +3 to the first GE signal, and reduces the level of mRNA transcription initiation at the first GS signal. A recombinant RSV with a 4C Le sequence has been rescued and has also arisen naturally through cold-passage in cell culture (67). This virus was shown to have a small plaque phenotype, and replicates to a lower titer than a wt 4G clone. However pathogenicity and virulence was not reduced in chimpanzees (112; 270). Whether this mutation alters the balance between transcription and replication has not been reported. If the RNA initiated from position +3 of the Le is elongated in a 4C virus, we would

expect the transcription gradient of the viral genes to be impacted, since there was a measurable decrease in initiation from position +45 in the minigenome system. The first viral gene is NS1, which plays a role in inhibiting the induction and signaling of type I and type III IFN. It is known that replication of a delta-NS1 RSV is attenuated in IFN-responsive cells (113; 229; 244; 245; 269). It would be interesting to determine the effect of a reduction in NS1 transcription, via generation of a Le-NS1 read through RNA, on RSV fitness and the immune response to infection, to evaluate whether this virus would be a good candidate for an attenuated vaccine.

In conclusion, we have shown that the RSV polymerase initiates RNA synthesis frequently from an additional site in the Le region during infection. Initiation from this site is independent of initiation at position +1 and may provide the polymerase with a mechanism to access the first GS signal and become committed to transcription. Additionally, small RNAs are generated from both the Le and TrC promoters in association with initiation at position +3. This is the first demonstration of an previously undescribed initiation site being used during NNS RNA virus infection, and challenges the dogma that all RNA synthesis is initiation from position +1.

Appendix 1: Functional analysis of the small RNAs generated from the viral promoters

Introduction

The interesting finding that RSV generates small non-coding RNAs from both the Le and TrC promoters (Chapter 1) led us to question whether these RNAs play a role in viral replication or the host response to infection. The following is a review of what is known about virally encoded small RNAs.

Virus-derived microRNAs

MicroRNAs (miRNAs) are small, non-coding RNAs approximately 22 nucleotides in length that play a regulatory role in gene expression in plants and animals (reviewed in (6)). MiRNAs are highly conserved and are likely to be involved in nearly every process in the cell (135; 137; 141). Dysregulation of miRNAs has now been implicated in many disorders including cancer (100; 174; 175), obesity (214; 282; 283), heart disease (240; 247; 257), heritable diseases (47; 106; 170), and schizophrenia (10; 11; 65). Canonical miRNAs originate from RNA polymerase II transcripts called primary miRNAs (pri-miRNAs). Pri-miRNAs are capped, polyadenylated and spliced RNA stem-loop structures, several hundred nucleotides in length. Pri-miRNAs are processed in the nucleus by the endonuclease Drosha in concert with a double-stranded RNA binding protein,

DGCR8 (together called the “Microprocessor”), to generate 70-80 nucleotide stem-loop precursor miRNAs (pre-miRNAs), which are then exported from the nucleus (79). In the cytoplasm, pre-miRNAs are cleaved by the enzyme Dicer, which generates an approximately 22 nucleotide imperfect miRNA:miRNA* duplex (156). The passenger strand (miRNA*) is typically degraded, while the guide strand is loaded into an RNA induced silencing complex (RISC). Argonaute (Ago) proteins are part of the RISC complex and serve to direct the guide strand to a target mRNA (211; 219). In cases of perfect complementarity between the miRNA and the mRNA target, some Ago proteins such as Ago2 can directly degrade the target mRNA (16; 151; 168; 190). When the base pairing between the miRNA and the target mRNA is imperfect, silencing of the mRNA is mediated through translational repression instead of cleavage (142; 150; 271). Recognition of a target mRNA can be achieved with only 6-8-nucleotide identity, in a region known as the “seed region” found at the 5´-end of a miRNA from nucleotides 2 through 7. (For overview, see Figure A1).

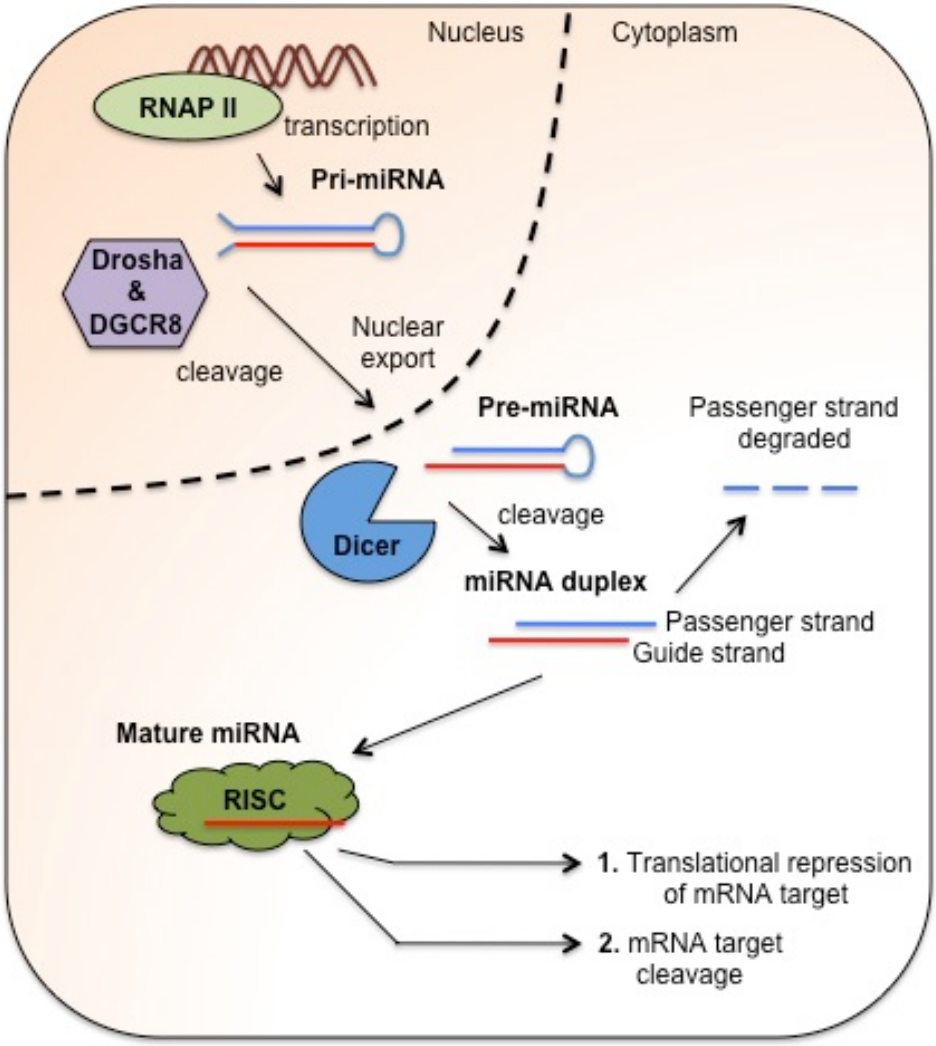


Figure A1; miRNA biogenesis pathway. Illustration showing the canonical pathway of miRNA processing.

Since miRNAs are such potent tools for the manipulation of the cellular environment, it is unsurprising that some viruses have evolved to encode their own miRNAs. The majority of viral miRNAs that have been identified thus far have been shown to be involved in inhibiting the cellular antiviral response to infection, or in downregulating the expression of certain viral proteins to establish viral latency (reviewed in (254)). Currently there is little data to support the hypothesis that virus-encoded miRNAs play a role in pathogenesis or replication of the virus, although studies in this area are ongoing (236). Table A1 lists all the currently known viruses that generate miRNAs.

Table A1; Viruses known to express viral miRNAs (*adapted from Kincaid and Sullivan, 2012* (122), and updated from miRBase (80-82; 126))

Virus Family/ Subfamily	Virus species
α-herpesviruses	Herpes Simplex Virus 1
	Herpes Simplex Virus-2
	Herpes B virus
	Herpesvirus of turkeys
	Infectious laryngotracheitis virus
	Duck enteritis virus
	Bovine herpesvirus 1
	Marek's disease virus type 1
	Marek's disease virus type 2
	Pseudorabies virus
β-herpesviruses	Human cytomegalovirus
	Mouse cytomegalovirus
	Human herpesvirus 6B
γ-herpesviruses	Epstein-Barr virus
	Rhesus lymphocryptovirus

	Kaposi's sarcoma-associated herpesvirus
	Rhesus monkey rhadinovirus
	Herpesvirus saimiri strain A11
	Mouse gamma herpesvirus 68
Polyomaviruses	Simian virus 40
	JC polyoma virus
	BK polyoma virus
	Mouse polyoma virus
	Merkel cell polyoma virus
	SA12
Retroviruses	Bovine leukemia virus
	Human immunodeficiency virus 1
Iridoviruses	Singapore Grouper Iridovirus
Ascoviruses	Heliothis virescens ascovirus
Baculoviruses	Bombyx mori nucleopolyhedrosis virus
Adenoviruses	Human adenovirus types 2 and 5
Unclassified	Bandicoot papillomatosis carcinomatosis virus type 1
	Bandicoot papillomatosis carcinomatosis virus type 2
	Heliothis zea nudivirus-1

Currently, virally encoded miRNAs have only been observed in DNA viruses or viruses that have a DNA stage in their lifecycle, such as the retroviruses. Therefore it has been theorized that RNA viruses do not generate miRNAs for two reasons; first, that many RNA viruses replicate in the cytoplasm and would not have access to the nuclear Microprocessor, and second, because miRNA processing would be detrimental to RNA virus genome and viral mRNA integrity (44; 104). However, a miRNA-like small RNA has recently been described for West Nile virus (WNV) that appears to upregulate a cellular mRNA that is important to WNV replication (107). Furthermore, nuclear and cytoplasmic RNA

viruses have been successfully engineered to express functional miRNAs with little or no negative consequence to viral replication (136; 216; 222; 258). Two cytoplasmic RNA viruses, Tick-borne encephalitis virus (TBEV) and Sindbis virus (SINV), were engineered to produce miRNAs that were processed in a non-canonical fashion (216; 222). These miRNAs were dependent on Drosha and Dicer, but independent of the Microprocessor (216; 222). This work suggested an alternative mechanism for Drosha activity such that it becomes partially localized to the cytoplasm, and two phosphorylation sites have now been identified that putatively regulate Drosha localization (221; 238). Influenza virus, an RNA virus that replicates in the nucleus, was modified to express cellular microRNA-124 (miR-124). A 500-nucleotide sequence encoding the pri-miR-124 hairpin was inserted into the viral genome. Viral replication was unaffected, and virus-generated miR-124 was completely functional (258). Additionally, the hairpin in the context of the influenza genome was not a target for cleavage by the Microprocessor, possibly due to encapsidation of the genome (258). These studies cast doubt on the theory that RNA viruses do not generate miRNAs due to deleterious effects on replication, and lack of access to nuclear enzymes. Instead, these results suggest that RNA viruses may not generate miRNAs because there is no significant evolutionary advantage to doing so.

Other virally-derived small RNAs

Despite the dearth of RNA virus-derived miRNAs, many other small non-coding RNAs have been identified. Deep sequencing of cells infected with influenza virus, hepatitis C virus, poliovirus, dengue virus, VSV or WNV has uncovered large populations of small RNAs ranging from 10 to 60 nucleotides in length, and from low to high abundance (194). In particular, the small RNAs identified from influenza virus are unusual in that they correspond to the 5´-ends of each of the eight viral gene segments and range in size from 18-27 nucleotides (199; 255). These RNAs possess a 5´-triphosphate instead of a 5´-monophosphate like a miRNA, and associate with the viral polymerase complex rather than an Ago complex (200). In the study that first described the influenza virus small viral RNAs (svRNA), the authors posit that the segment-specific svRNAs may serve to regulate the switch between transcription and replication during viral infection (199). Accordingly, the authors showed that the peak of svRNA production corresponds with a dramatic shift to replication, and that the svRNAs physically associate with the complete influenza virus polymerase (199). They suggest a model wherein the svRNA binds the polymerase and either serves to reconstitute a double stranded promoter on the viral RNA segment, or by binding the polymerase, alters the replication activity of the complex (199; 200).

Virally-encoded small RNAs have also been implicated in interfering with the cellular antiviral response to infection. In response to various forms of

environmental stress including viral infection, the cell initiates the formation of dense cytoplasmic aggregates called stress granules, which are sites of mRNA triage. SeV is known to synthesize RNA from its Tr region (143). It has been demonstrated that a mimetic of the SeV Tr region binds a cellular stress granule component, TIAR, and potentially sequesters it, preventing stress granule formation in response to SeV infection (110).

Hypothesized functions for the RSV LeC and Tr small RNAs

Similarly to the influenza virus model, it is possible that either of the small LeC or Tr RNAs may bind the RSV polymerase and behave as feedback molecules to influence the switch from transcription to replication. In addition, the small Tr RNA may also behave similarly to the Tr RNA of SeV in the context of stress granule formation. Previously it has been shown that wt RSV subverts or blocks the cellular stress granule response to infection (96). However, infection with a mutant virus that contains the 44-nt LeC region in place of the Tr region (LeC virus) leads to the formation of stress granules (96). This finding implicates the Tr region and possibly the small Tr RNA in the subversion of the stress granule response by RSV.

In the following pilot studies we set out to characterize the structure and function of the small LeC and Tr RNAs. We examined whether overexpression of the small RNAs has an effect on RSV transcription and replication. We also sought

to determine whether expression of the small Tr RNA could block stress granule formation induced by infection with LeC virus.

Results

A1.1 The small LeC and Tr RNAs can be expressed under the control of a T7 promoter in BSR-T7/5 cells.

To test whether the small LeC and Tr RNAs play a role in RSV infection, plasmids were designed to express these RNAs under the control of a T7 promoter. Le and TrC sequences from positions 3 to 27 were cloned into a plasmid between a T7 promoter and a Hammerhead ribozyme to generate the 3´-ends of the RNA, to express 25-nucleotide LeC and Tr RNAs. A scrambled sequence based on Le was cloned as a control (Sequences shown in Table 1, Materials and Methods). The plasmids were transfected into BSR-T7/5 cells, baby hamster kidney cells (BHK) stably expressing T7 polymerase (19), and the cells were coinfected with RSV or mock-infected 24 hours later. RNA was isolated 24 hpi and analyzed by urea-acrylamide Northern blot. Using probe g7-34 to detect LeC-containing RNA, the small ~25 nt LeC RNA was detected from mock-transfected and RSV-infected control cells (Figure A2, B, panel i, lane 2). When cells were transfected with the LeC overexpression plasmid and mock-infected, LeC RNA of the appropriate size was generated (compare lane 4 to lane 2). When cells were transfected with the LeC overexpression plasmid and

infected with RSV, the levels of the LeC RNA appeared to be increased, confirming overexpression of the small RNA (lane 5). RNA isolated from cells transfected with a scrambled control plasmid shows no effect on the native expression of LeC RNA from RSV (lane 3). No LeC RNA was detected from mock treated cells (lane 1). The same set of RNA samples was also probed with ag5-32 to detect Tr-containing RNA (panel ii). The ~25 nt Tr RNA was detected from mock-transfected/ RSV-infected cells (lane 2). Transfection with the scrambled control did not have an effect on the native expression of the Tr RNA in RSV-infected cells (lane 3). In cells transfected with the Tr overexpression plasmid and mock-infected, a Tr-containing RNA of the appropriate size was detected at high levels (lane 4), and overexpression of the Tr RNA was confirmed in the context of RSV infection (lane 5). These results verify that we are able to overexpress the small LeC and Tr RNAs in the context of RSV infection. Therefore we can use these overexpression plasmids to explore the effect of these small RNAs on virus and host.

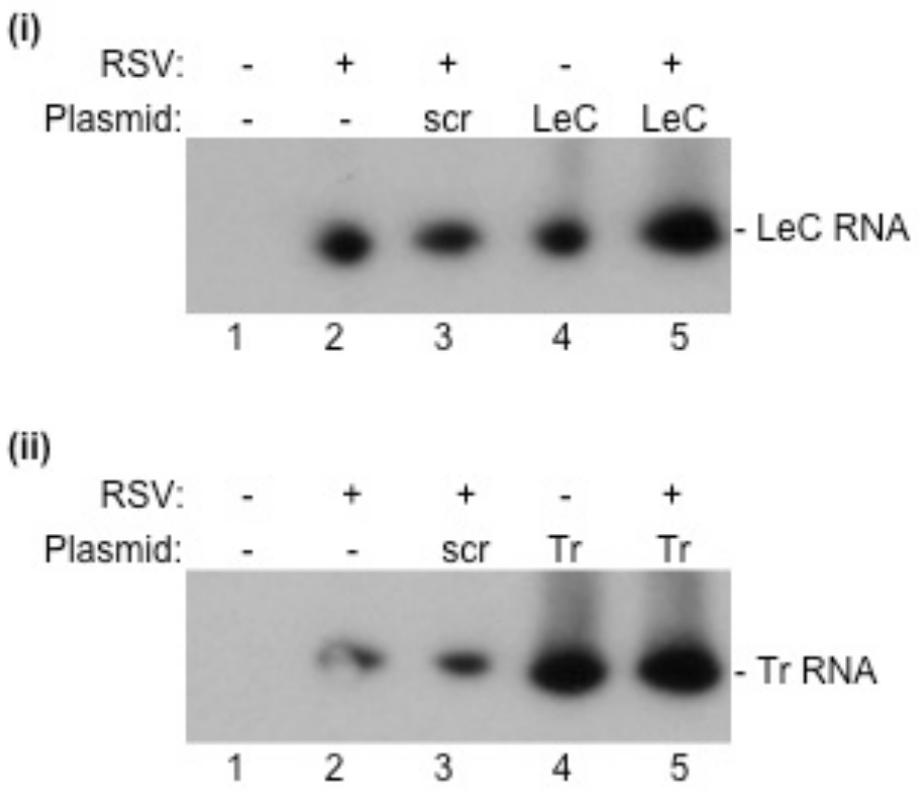


Figure A2; The small LeC and Tr RNAs can be overexpressed in BSR-T7/5 cells. RNA isolated from cells transfected with the overexpression plasmids and mock or RSV-infected (as indicated by - /+) was analyzed by urea-acrylamide Northern blot. (i) Blot was probed with g7-34 to detect LeC-containing RNA. (ii) Blot was probed with ag5-32 to detect Tr-containing RNA. RNA isolated from mock-transfected, RSV-infected cells was migrated as a control for native expression of the small RNAs (lane 2). RNA from cells transfected with the scrambled control plasmid (scr) and infected with RSV is shown in lane 3. Lanes 4 and 5 show expression of the small RNAs from plasmid alone and in the context of RSV infection, respectively.

A1.2 Expression of the small RNAs does not have a noticeable effect on cytopathic effect induced by coinfection with wildtype RSV.

Having confirmed that we can overexpress the small RNAs in BSR-T7/5 cells, we wanted to test whether overexpression of the small RNAs alone had an effect on cell morphology, or altered the cytopathic effect (CPE) induced by RSV infection. RSV spreads in culture via fusion to adjacent cells, forming large clusters of multinucleate cells called syncytia. This process is mediated by the RSV F glycoprotein, which is expressed on the surface of the budding viral particle. BSR-T7/5 cells were transfected with the overexpression plasmids (~60% transfection efficiency) and coinfected with wt virus 24 hours post transfection, and cell morphology was observed 24 hpi. Mock-transfected/ mock-infected cells are shown as a control demonstrating the normal morphology of the cells (panel i, left). Examples of syncytia are indicated with arrowheads in mock-transfected/ RSV-infected cells (Figure A3, panel i, right). If overexpression of the small RNAs had an effect on CPE, a difference in the size or number of syncytia would be observed. Transfection alone appears to have a deleterious effect on the cells, as cell rounding and thinning of the monolayer is observed (compare left panels ii and iii with panel i). However, no changes in syncytia formation were visible in infected cells overexpressing the LeC and Tr small RNAs, as compared to mock-transfected cells (compare right panels (ii) and (iii) to panel i).

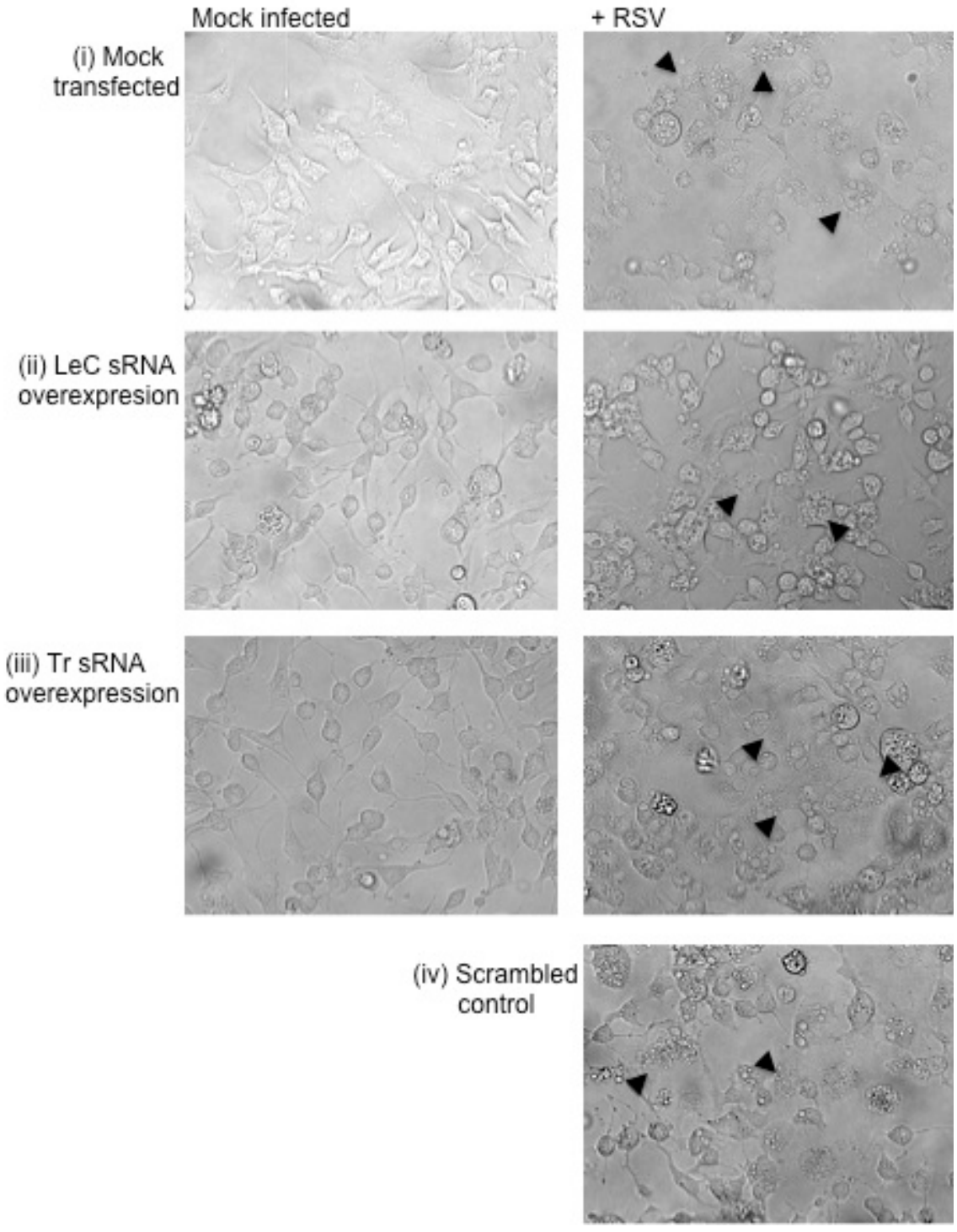

Mock infected
+ RSV
(i) Mock transfected
(ii) LeC sRNA overexpresion
(iii) Tr sRNA overexpression
(iv) Scrambled control

Figure A3; Effect of overexpression of LeC and Tr RNAs on cell morphology and RSV syncytia formation. BSR-T7/5 cells were transfected with overexpression plasmids and either mock-infected or infected with RSV at an MOI of 3, 24 hours post transfection. Cell morphology was observed by light microscopy and imaged 24 hpi with the 20X objective. Cells in left panel were mock-infected, while cells in the right panel were infected with wt RSV. Black arrowheads indicate examples of RSV syncytium formation. (i) Mock-transfected cells (ii) Cells transfected with the plasmid overexpressing the LeC RNA (iii) Cells transfected with the plasmid overexpressing the Tr RNA (iv) Cells transfected with the scrambled control plasmid.

A1.3 Overexpression of the small RNAs impacts RSV RNA and protein expression.

After observing CPE, the cells were harvested and total cell lysates were collected for protein and RNA analysis. RNA was extracted and migrated on agarose-formaldehyde gels and probed for genome, antigenome and NS1 mRNA. Note that there was no loading control for these blots, however, all the gels were run at the same time using the same RNA samples, therefore it is possible to compare the relative levels of RNA expression between blots. Overexpression of the LeC sRNA resulted in an increase in antigenome compared to the scrambled control (Figure A4, panels i and ii, compare lane 5 to lane 3). In contrast, LeC sRNA did not have a similar effect on the relative levels of genome and NS1 mRNA compared to the scrambled control (panels ii and iii, compare lane 5 to lane 3). Overexpression of the Tr sRNA did not result in an obvious increase in the levels of antigenome (panels i and ii, compare lane 7 to lanes 3), and also did not have an impact on the levels of genome and NS1 mRNA (panels ii and iii, compare lanes 7 and 3). Unfortunately, transfection with the scrambled control plasmid reduced the levels of genome, antigenome and NS1 mRNA as compared to the mock-transfected, RSV-infected controls (compare lane 3 to lane 2, all panels). Therefore these results must be interpreted with caution because the negative control was unreliable. Additionally, the T7 polymerase produces extraneous RNA from the expression vectors, as indicated by high levels of background detected throughout the lane

(panel i, lanes 4 and 5; panel ii, lanes 6 and 7), which may alter RSV infection kinetics or otherwise impact the cellular response to infection, possibly skewing the results. Therefore, it is impossible to make any firm conclusions about the data.

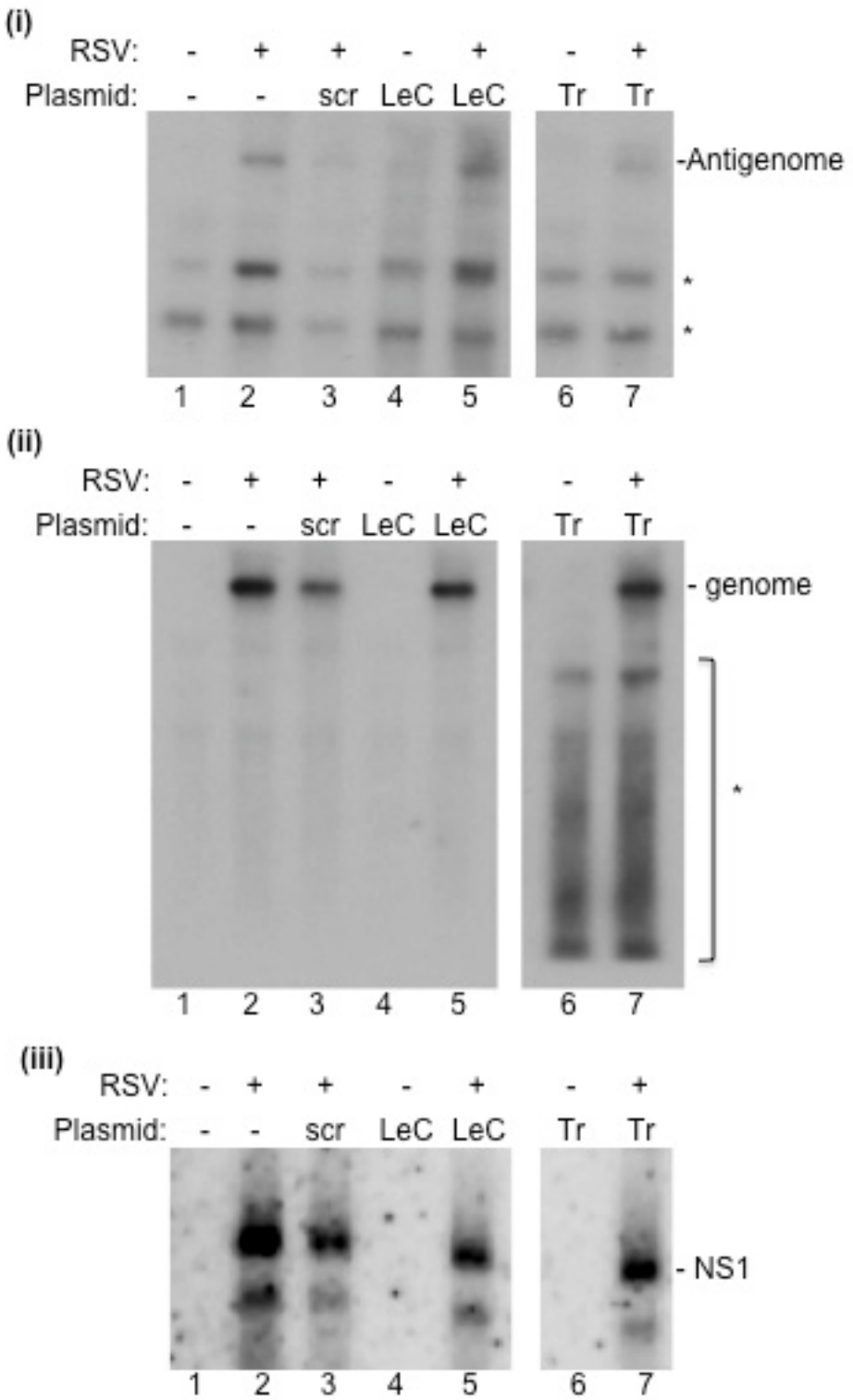

(i)
RSV: - + + - + - +
Plasmid: - - scr LeC LeC Tr Tr
-Antigenome
*
*
1 2 3 4 5 6 7
(ii)
RSV: - + + - + - +
Plasmid: - - scr LeC LeC Tr Tr
- genome
*
1 2 3 4 5 6 7
(iii)
RSV: - + + - + - +
Plasmid: - - scr LeC LeC Tr Tr
- NS1
1 2 3 4 5 6 7

Figure A4; Overexpression of the LeC and Tr RNAs appears to alter the levels of RSV transcription and replication. RNA isolated from cells transfected with the overexpression plasmids and mock-infected or infected with RSV at an MOI of 3 was analyzed by agarose-formaldehyde Northern blot. (i) Blot probed with g7-34 to detect antigenome sense RNA. (ii) Blot probed with ag5-32 to detect genome sense RNA. (iii) Blot probed with g504-540 to detect NS1 mRNA. Antigenome, genome and NS1 mRNA are indicated. Note that lanes 6 and 7 were run on the same gel for each blot. Background bands are indicated by asterisks.

Protein was extracted from the cell lysates and subjected to Western blot analysis to determine whether overexpression of the small RNAs had an effect on RSV protein expression. Blots were probed with a polyclonal anti-RSV antibody, which detects the full complement of RSV proteins. As a loading control, blots were stripped and reprobed for alpha-tubulin. RSV protein expression from mock-transfected cells infected with RSV is shown in lane 2 (Figure A5, both panels). Overexpression of the LeC RNA did not have a notable effect on RSV protein expression (panel i, lane 5). Note that although there is a decrease in RSV proteins, there is a corresponding decrease in tubulin. However, overexpression of the Tr RNA does appear to increase RSV protein expression, which is most easily discerned by comparing the band indicated with the dot in lanes 5 and 2 (panel ii). As above, interpretation of these results is hindered by lack of a good control, as transfection with the scrambled control plasmid also appears to increase RSV protein expression, although in this instance the tubulin levels also appear to be increased (panels i and ii, lane 3).

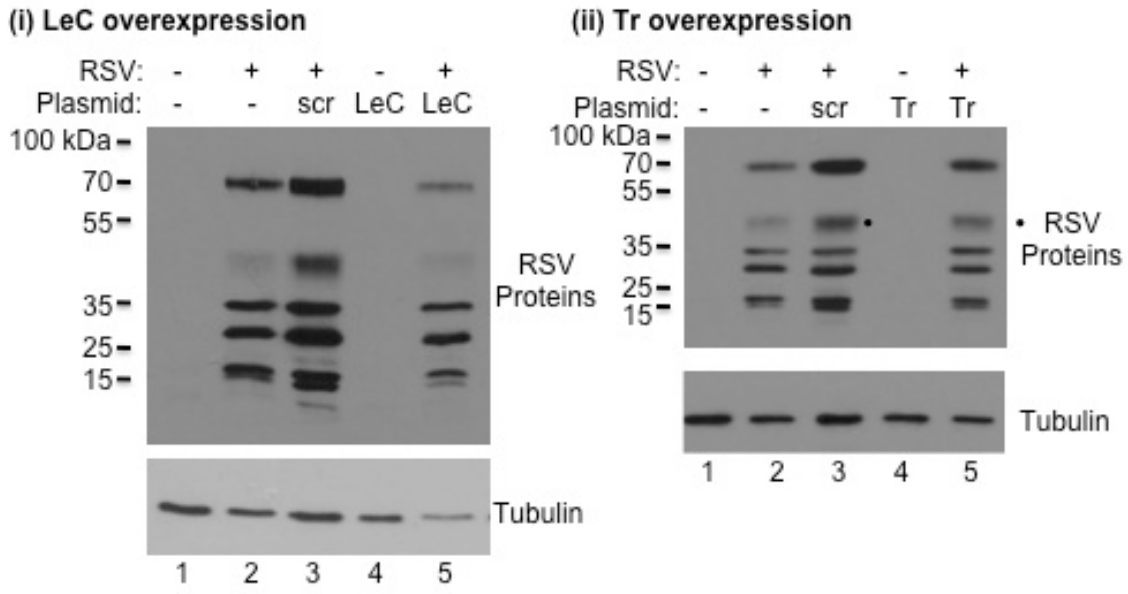


Figure A5; Overexpression of the Tr RNA appears to increase RSV protein expression. Protein was extracted from cells transfected with overexpression plasmids and infected with RSV at an MOI of 3. Protein samples were subjected to 10% SDS-PAGE and analyzed by Western blot. Blots were probed with a polyclonal RSV antibody to detect viral proteins, and reprobed with an antibody to alpha-tubulin as a loading control. Controls included protein extracted from mock-transfected, mock-infected cells (lane 1), mock-transfected, RSV-infected cells (lane 2), and cells transfected with the scrambled control plasmid and RSV-infected (lane 3). (i) Protein extracted from cells overexpressing LeC RNA and either mock (lane 4) or RSV-infected (lane 5). (ii) Protein extracted from cells overexpressing Tr RNA and either mock (lane 4) or RSV-infected (lane 5).

A1.4 LeC virus does not appear to induce stress granules in BSR-T7/5 cells.

Previously it was shown that wt RSV blocks the stress granule response during infection. A mutant virus containing LeC sequence in place of the Tr region (LeC virus) was shown to have a reduced ability to subvert the stress granule response compared to wt RSV (96). Additionally, the Tr sequence of SeV has been shown to be able to bind to TIAR, a stress granule protein, and block stress granule formation (110). Therefore, we wanted to explore the relationship between the small Tr RNA and the cellular stress response by testing whether overexpression of the Tr RNA could rescue the LeC virus' ability to block stress granule formation. These experiments were performed in BSR-T7/5 cells because we already showed that the Tr RNA could be overexpressed in these cells without the need for coinfection with MVA-T7, as vaccinia virus has been shown to induce antiviral granules (227). The cells were either mock-treated or infected with LeC virus at an MOI of ~1, fixed at 24- and 48-hpi, permeabilized, and stained for eIF3, a stress granule marker, RSV N protein to detect infected cells, and a nuclear stain, DAPI. As a control for stress granule formation, one well of mock-infected cells was treated with sodium arsenite prior to fixation to induce oxidative stress. Unfortunately, 2-hour arsenite treatment causes significant cell death and rounding, while inducing eIF3-containing granules (Figure A6, panel ii). A shorter, 30-minute arsenite treatment was also tested, however, while the cells still maintained a healthy morphology, no eIF3-

containing granules were observed (data not shown). Normal diffuse cytoplasmic distribution of eIF3 is observed in mock treated cells (panel i). Unexpectedly, virtually no stress granules could be detected in LeC virus-infected cells at either time point (24 hpi- panels iii and iv, 48 hpi- panel v). Some punctate eIF3 staining can be observed in panel (iv), however it is unclear if these are stress granules or a different type of antiviral granule.

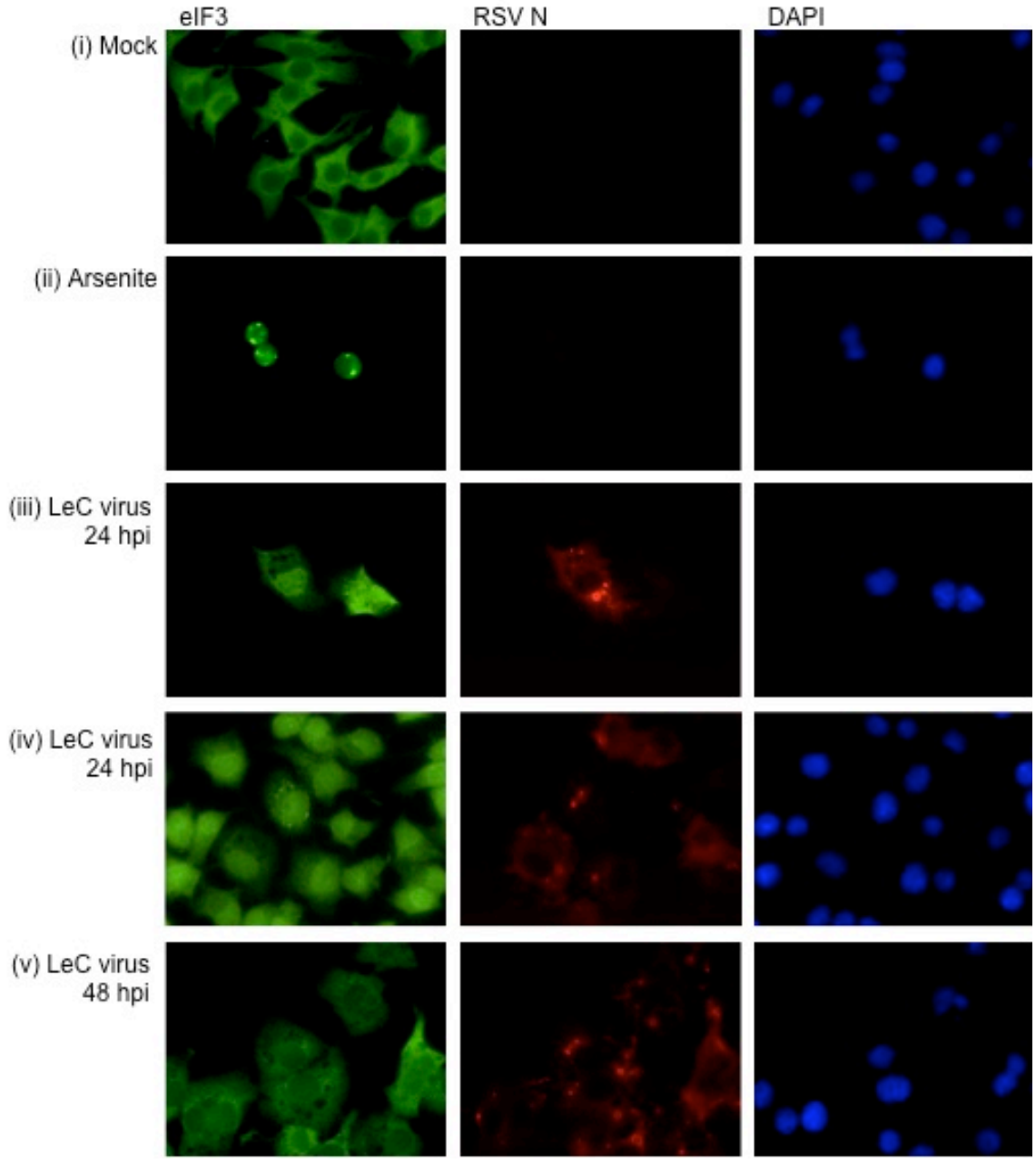


Figure A6; LeC virus does not induce eIF3-containing stress granules in BSR-T7/5 cells. Cells were mock-treated or infected with LeC virus at an MOI of

~1. Cells were fixed at 24- and 48- hpi, permeabilized, and stained with an anti-eIF3 antibody (green), and an anti-RSV N antibody for RSV infection (red). Cells were incubated with a fluorescent secondary antibody cocktail containing DAPI to stain the nucleus (blue). (i) Mock treated cells showing the diffuse cytoplasmic distribution of eIF3. (ii) Cells treated with 0.5 mM sodium arsenite for 2 hours prior to fixation, showing punctate staining of eIF3. (iii) LeC virus infected cells showing RSV N-containing viral inclusion bodies (bright aggregates), 24 hpi, first field, and (iv) second field. (v) LeC virus infected cells, fixed 48 hpi. Images are individual slices taken on a Zeiss Axiovert fluorescence microscope using the 40X objective with oil immersion.

Discussion

Summary of Results

In these preliminary experiments, we demonstrated that it is possible to overexpress the small LeC and Tr RNAs under the control of T7 in BSR-T7/5 cells. Overexpression of these RNAs did not have a significant impact on RSV syncytium formation, eliminating CPE as an easily observable phenotype to discern the effect of these RNAs on RSV infection. However, we showed that the LeC and Tr RNAs might have an effect on levels of RSV replication and protein expression. Unfortunately, we were unable to explore the relationship between the Tr RNA and stress granule formation since the LeC virus failed to induce stress granules in BSR-T7/5 cells.

The effect of the LeC small RNA on transcription and replication

One possible model for the function of the LeC small RNA is that it functions as a feedback molecule that binds the polymerase and influences the switch between transcription and replication, similar to the model proposed for influenza virus small RNAs (199). If this model is correct, overexpression of the LeC RNA in the context of RSV infection would be expected to result in an increase in replication, as measured by the level of antigenome and correspondingly, genome, while the levels of transcription might remain unchanged or even decrease. As compared to the scrambled control, an increase in antigenome was detected, as well as a

modest increase in genome. No appreciable change in the level of transcription was observed (panel iii). One explanation for this is that the RNA was isolated at 24 hpi; at this time point in infection transcription products will be saturated. Moreover, during infection transcription is the dominant event, thus a minor decrease in the level of transcription may not be distinguishable. Alternatively, rather than influence a switch between transcription and replication, the LeC RNA may somehow interact with a nonviable replicase, allowing it to become active. If this was the case, then the level of transcription would not be affected, but the level of replication would increase. Silencing the LeC RNA may therefore be expected to drastically reduce levels of genome. This could be pursued by transfecting in an LNA/DNA oligonucleotide that is complementary to the LeC RNA in order to bind and block native expression of the RNA during infection.

The effect of the small Tr RNA on stress granule formation

Concerning the hypothesized role of the Tr RNA in the stress response, unfortunately, we were unable to test whether the Tr RNA is involved in subverting the stress granule formation. Despite documented eIF3-containing granule formation in response to LeC virus infection in HEp-2 cells (96), we were unable to recapitulate this effect in BSR-T7/5 cells. This disparity may point to a host-specific response to infection. In addition, it has not been demonstrated that BSR-T7/5 cells are able to form stress granules, although they are derived from BHK cells, which are known to form stress granules. Another possibility is that

the LeC virus phenotype reverted to wt, a phenomenon observed due to instability of the LeC phenotype, and accomplished by a single substitution at position 12 relative to the 5´-end of the genome, from ‘A’ to ‘U’ (96).

Future Directions

Because we were unable to confirm whether the BSR-T7/5 cells were able to mount a stress response, it is important to repeat the experiments performed here in a cell line that is known to form stress granules. The A549 human airway cell line is more authentic than the BSR-T7/5 cells with regards to RSV infection. This would necessitate recreating the small RNA expression vectors under the control of a different promoter, such as the cytomegalovirus (CMV) promoter. In addition, because of the toxicity of lipofectamine transfection, it would be important to perform a transfection time course for small RNA expression to determine the best time to subsequently infect the cells, to minimize the length of time the cells are in culture. The peak of sRNA expression may fall within 12-18 hours, thus cells could be infected much earlier. Additionally, the infection could be halted and the cells fixed between 12 and 18 hpi, as stress granules have been shown to form within this time frame (96). The sodium arsenite treatment must also be optimized such that stress granules are induced but the cells still maintain a normal morphology. Increasing the concentration of sodium arsenite and decreasing the length of time of the treatment may achieve this.

Implementing these controls may aid in providing a clearer picture of the Tr region's relationship to stress granule formation.

Remaking the small RNA vectors under the control of a CMV promoter may also subvert some of the problems that arose from T7 expression of these RNAs. As noted in the results, in addition to the small LeC and Tr RNAs, extraneous RNA products are generated. These RNAs are likely T7 transcripts that are not efficiently cleaved by the ribozyme. The T7 polymerase also requires a 'GGG' motif to efficiently initiate, and will therefore add sequence at the 5´-end of the small RNAs that are expressed. Another solution to these problems would be to generate PCR products using a primer that corresponds to the exact 3´-end of the small RNA, and a primer corresponding to the 5´-end with a T7 promoter sequence added, that overlap. The 'GGG' motif of the T7 promoter can be removed which will reduce efficiency of the promoter (109), however, a high concentration of the PCR product could be transfected, ensuring expression of a precise small RNA.

It is disconcerting that the scrambled control plasmid had an effect on RSV RNA and protein expression, thus a better control must be identified. A greater number of scrambled plasmids could be tested to discover one that has no effect on RSV RNA and protein expression. An empty vector control should also be included to control for the effect of DNA transfection. In general, it would also be

important to confirm that expression of the small RNAs are not activating an IFN response, which would then impact RSV infection. This could be tested by probing Western blots of protein samples from cells overexpressing the small RNAs for phosphorylated IRF-3 or phosphorylated-PKR.

Going forward there are several experiments that could be performed to better understand the role of the small LeC and Tr RNAs in RSV infection. It would be interesting to examine the kinetics of viral small RNA production as well as precise determination of the sequences of these RNAs. A timecourse of infection should be carried out to determine the peak of expression of the small RNAs, as it compares to RSV transcription and replication, and protein expression. Additionally, assumptions were made about the RNA length and sequence in order to proceed with the experiments described within this chapter. We postulated that the RNA was predominantly 25 nucleotides and initiated from position +3, with a 5´-triphosphate end. These assumptions were based on the data from the primer extension analysis and small RNA Northern blot analysis (Chapter 1). However the sequence could be determined with more accuracy using a method commonly used to clone miRNAs (107; 154). Briefly, the small RNAs could be migrated on high percentage urea-acrylamide gels, the bands could be extracted, and the RNA isolated and polyadenylated. The polyadenylated RNA would then be subjected to first strand cDNA synthesis using a poly(dT) primer. The resulting cDNA would be amplified by PCR using a

forward primer designed to sit within the predicted small RNA sequence and oligo(dT) as a reverse primer. PCR products would then be cloned and sequenced. It is likely that the 3´-ends of the RNAs are heterogeneous, however, it would be informative to confirm whether the RNAs are initiated from position +3 or position +1.

Since the model posits that LeC RNA binds the polymerase and acts as a feedback molecule for transcription, it is important to pursue whether the RSV polymerase contains a binding site for the small LeC RNA. This could be done similarly to the studies on influenza (199; 200) by cotransfecting tagged L-P and a small LeC RNA mimetic into cells, and then coimmunoprecipitating for the tag, followed by small RNA Northern blot to detect the LeC RNA. This would confirm that the polymerase does in fact bind the small RNA. If these studies are borne out, then they could be repeated using L truncation mutants to narrow down the domain, and single amino acid substitutions to narrow down the residues that are directly involved in binding the RNA.

In the event that the LeC RNA does not bind the RSV polymerase, then we could pursue whether the RNA has other cellular or viral binding partners that may implicate its function in transcription and replication. It would also be interesting to identify cellular binding partners of the Tr small RNA. One approach that has been used for West Nile virus (107) is to conduct a “fishing experiment” using

DNA oligonucleotides representing the small RNAs to fish out cellular sequences from mock and infected cell extracts. Reverse transcription polymerase chain reaction (RT-PCR) would be carried out on total cell extracts from mock and RSV-infected A549 cells using a Tr oligonucleotide that contains restriction sites for cloning. This will amplify any sequence the Tr RNA binds to. The cDNA generated would be purified, tailed, cloned, and sequenced, and sequencing hits would be analyzed using BLAST (NCBI). To identify RNA-protein binding interactions, an approach that has been used successfully for *Helicobacter pylori* (212) is aptamer-tagging of the small RNAs followed by affinity chromatography and mass spectrometry. A small tag could be added to the 3´-ends of small RNA mimetics, which would then be incubated with A549 cell extracts. Lysates would be purified on a column to bind the aptamer, RNA-protein complexes eluted, and the proteins would be migrated on a gel and analyzed by mass spectrometry. These techniques could potentially provide a reasonable list of cellular binding partners for both the Tr and LeC RNAs, whose function during RSV infection could then be fully explored.

In conclusion, the data presented in this appendix suggests that the small LeC and Tr RNAs play a role in RSV replication, by potentially influencing the activity of the polymerase. More work needs to be done to confirm and expand on these findings, and the mechanism of how these RNAs function needs to be elucidated. However, the prospect that RSV generates small RNAs that behave in a

regulatory fashion is novel in the field of single stranded, negative sense RNA viruses and should be investigated.

Appendix 2: Polymerase capping mutants terminate mRNA synthesis at ~40 nucleotides

As noted in the introduction, the RSV L protein is thought to contain all the enzymatic domains necessary for viral transcription and replication. Capping is an important activity of the polymerase that is essential to transcription. Studies in VSV have demonstrated that capping is necessary for elongation of transcripts (148; 234). Additionally, studies of an RSV capping inhibitor showed that failure of transcripts to become capped resulted in termination of mRNA synthesis at ~50 nucleotides (152). Sequence alignment of the RSV L protein with other NNS virus L proteins suggests that capping activity resides within a domain called conserved region V (20; 66; 147; 159; 178; 228). To identify the amino acid residues in conserved region V that are critical for polymerase capping activity, single alanine substitutions were made at six different residues thought to be important based on conservation of these residues in other NNS viruses (69; 148; 189; 204). These mutants were tested for RNA synthesis in the minigenome system using a non-replicating, wt minigenome construct (Figure A7, panel A). RNA from transfections with these mutants was isolated and migrated on urea-acrylamide Northern blots. The blots were probed with an end-labeled oligonucleotide corresponding to positions 12-31 of the first reporter gene to detect mRNA initiated from the first GS signal.

The majority of the products synthesized by a wt polymerase in this assay were larger than 150 nt, with a dominant band between 500 and 1000 nt, corresponding to polyadenylated CAT 1 mRNA (lane 1). As a negative control for RNA synthesis, RNA was isolated from transfections with the polymerase that has a mutation in the catalytic RNA synthesis domain (N812A, lane 2). All of the mutants displayed a varying degree of deficiency in generating full-length mRNA as compared to the wt (compare lanes 3-8 to lane 1). However, the mutants that were most noticeably deficient generated a short transcript, ~40 nt in length (lanes 3, 7, and 8). Low levels of this short RNA were also detected from wt, as well as the mutants that were still able to produce longer RNAs (lanes 1, 4, 5, and 6). Thus, this result indicates that polymerase mutants that are unable to efficiently cap mRNA synthesized from the GS signal terminate RNA synthesis at ~40 nt. This contrasts with the results presented in Chapter 1 indicating that RNA initiated from the +3 initiation site in the Le is terminated after synthesis of ~25 nt, suggesting failure to cap the nascent transcript from position +3 may not be the checkpoint that causes the polymerase to terminate RNA synthesis. Alternatively, this result may indicate that there is a fundamental difference in polymerase initiation within the Le region and polymerase initiation from a GS signal.

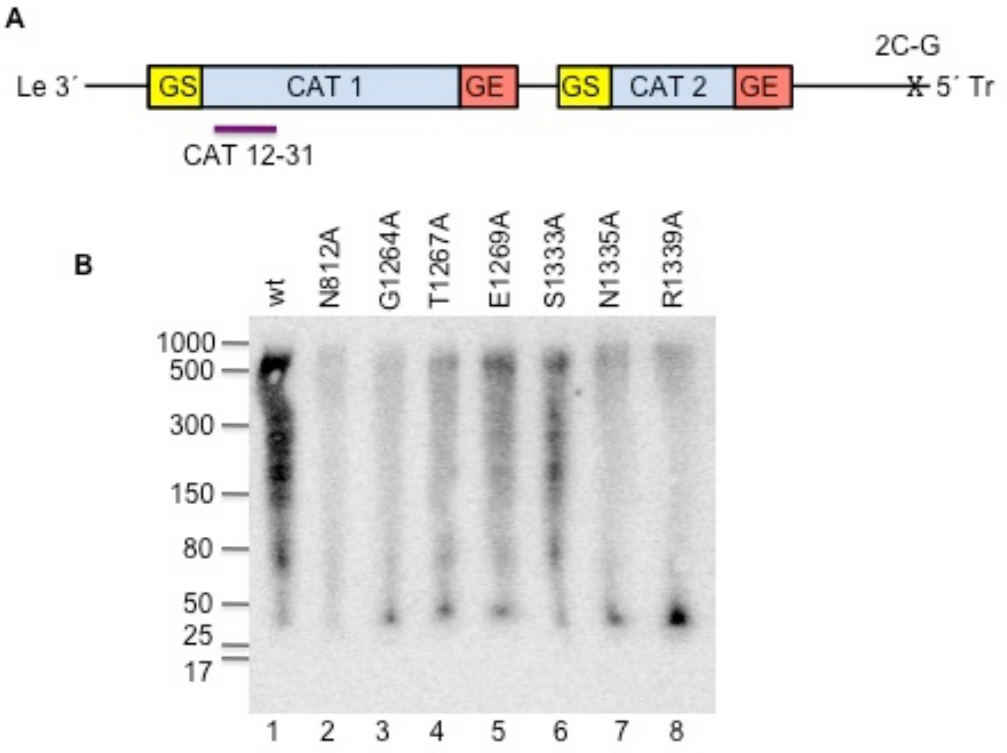


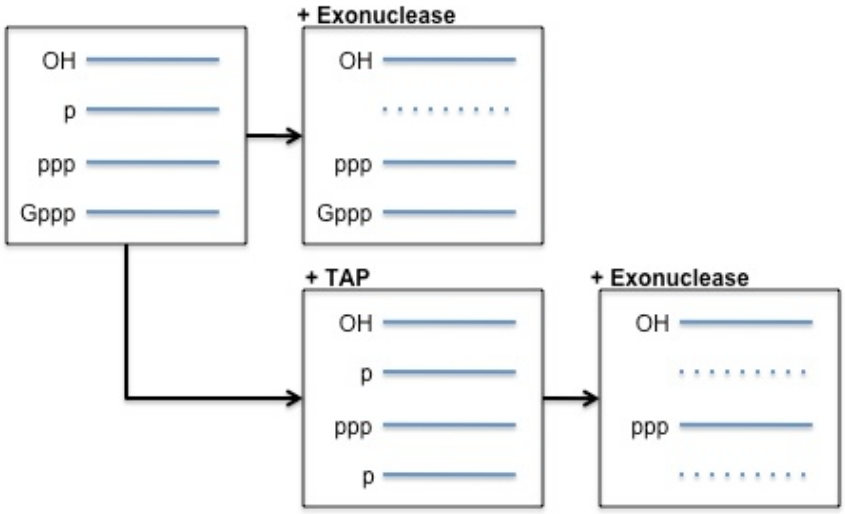
Figure A7; Determination of the size of prematurely terminated RNA initiated from a GS signal. *Adapted from Deflube et al, 2014* (48) (A) Minigenome schematic showing the hybridization position of the probe used for this Northern blot analysis. (B) Urea-acrylamide Northern blot analysis of RNA isolated from minigenome transfections with polymerase mutants containing substitutions in the putative capping domain. Gel lanes containing the markers were removed and stained with ethidium bromide, and realigned with the blot for sizing. *Laure Deflubé generated the polymerase mutants and performed the minigenome transfections and RNA isolations. I performed the Northern blot analysis.*

Appendix 3: Development of an assay to determine RNA 5´-end structures

To test the possibility that RNA initiated from position +3 is capped, we attempted to digest the RNA with tobacco acid pyrophosphatase (TAP), which is an enzyme that digests 5´-cap structures, leaving a 5´-monophosphate on the RNA. If the RNA initiated from position +3 were capped, treatment with TAP, followed by treatment with exonuclease, which digests 5´-monophosphorylated RNA, should ablate the RNA (Figure A8, panel A). To test the efficacy of these enzymatic treatments, primer extension analysis was performed on mRNA initiated from the NS1 GS signal, which is capped. As explained in Chapter 2, primer extension analysis of capped RNA often results in the detection of a doublet due to inefficient cDNA extension through the cap by the reverse transcriptase. Treatment of the mRNA with TAP would therefore be expected to ablate the upper band of the doublet (representing capped RNA), and further treatment of the RNA with exonuclease should digest the remaining RNA. As expected, mock-treated RNA migrates as a doublet (panel B, lane 2, indicated with a blue ellipse). TAP treatment reduced the upper band of the doublet, however overall levels of RNA were also reduced (compare lane 4 to lane 2). Further treatment with exonuclease did not completely ablate the RNA, suggesting that either decapping by TAP was inefficient, or exonuclease digestion was incomplete (lane 6). Because levels of RNA were very low post-treatment, and because digestion of decapped mRNA was incomplete, we were unable to use this approach to examine RNA initiated from position +3. Time did not permit

optimization of this technique, however measures could be taken to increase RNA recovery after each enzymatic treatment, which may facilitate proper analysis of treated RNA. Moreover, both TAP and exonuclease treatments can be optimized to ensure efficient digestion of RNA.

A



B

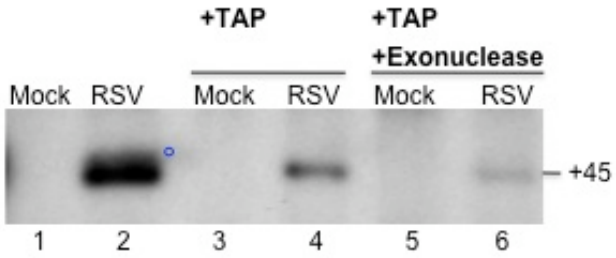


Figure A8; TAP and Exonuclease digestion is inefficient on a known capped mRNA. (A) Enzymatic digestion scheme showing four RNAs with different ends and how these RNAs would be expected to be affected by treatment. Digestion of RNA is indicated by a dashed line. (B) Primer extension analysis of RNA isolated from RSV-infected cells, treated with TAP and Exonuclease. RNA was mock-treated (lanes 1 and 2), treated with TAP (lanes 3

and 4), or treated with Exonuclease following treatment with TAP (lanes 5 and 6) and analyzed by primer extension using a primer that corresponded to genome positions 91-113 to detect initiations from the first GS at +45. Doublet band representing the cap structure is indicated with a blue ellipse (lane 2).

LIST OF ABBREVIATED JOURNAL TITLES

Acta Crystallogr D Biol Crystallogr	Acta Cystallographic, Section D: Biological Crystallography
Am J Dis Child	American Journal of Diseases of Children
Am J Epidemiol	American Journal of Epidemiology
Am J Hum Genet	American Journal of Human Genetics
Am J Hyg	American Journal of Hygiene
Am J Respir Cell Mol Biol	American Journal of Respiratory Cell and Molecular Biology
Am J Respir Crit Care Med	American Journal of Respiratory and Critical Care Medicine
Ann Intern Med	Annals of Internal Medicine
Ann N Y Acad Sci	Annals of the New York Academy of Sciences
Antimicrob Agents Chemother	Antimicrobial Agents and Chemotherapy
Antisense Nucleic Acid Drug Dev	Antisense & Nucleic Acid Drug Development
Antivir Ther	Antiviral Therapy
Antiviral Res	Antiviral Research
Arch Dis Child	Archives of Disease in Childhood
Arch Otolaryngol Head Neck Surg	Archives of Otolaryngology, Head & Neck Surgery
Arch Virol	Archives of Virology

Biochem Biophys Res Commun	Biochemical and Biophysical Research Communications
Biochemistry	Biochemistry
Biochim Biophys Acta	Biochimica et Biophysica Acta
Cell	Cell
Cell Signal	Cellular Signalling
Circulation	Circulation
Clin Exp Immunol	Clinical and Experimental Immunology
Clin Geriatr Med	Clinics in Geriatric Medicine
Clin Infect Dis	Clinical Infectious Diseases
Cold Spring Harb Symp Quant Biol	Cold Spring Harbor Symposia on Quantitative Biology
Crit Rev Immunol	Critical Reviews in Immunology
EMBO J	The EMBO Journal
Exp Biol Med (Maywood)	Experimental Biology and Medicine
Expert Opin Emerg Drug	Expert Opinion on Emerging Drugs
Expert Rev Hematol	Expert Review of Hematology
FEBS Lett	FEBS Letters
Gene Expr	Gene Expression
Genes Dev	Genes & Development
Hum Vaccin	Human Vaccines
Immunity	Immunity
J Allergy Clin Immunol	Journal of Allergy and Clinical Immunology

J Biol Chem	The Journal of Biological Chemistry
J Clin Invest	The Journal of Clinical Investigation
J Clin Microbiol	Journal of Clinical Microbiology
J Comp Pathol	Journal of Comparative Pathology
J Exp Med	The Journal of Experimental Medicine
J Gen Virol	Journal of General Virology
J Immunol	The Journal of Immunology
J Infect Dis	Journal of Infectious Diseases
J Med Virol	Journal of Medical Virology
J Mol Cell Cardiol	Journal of Molecular and Cellular Cardiology
J Pediatr	Journal of Pediatrics
J RNAi Gene Silencing	Journal of RNAi and Gene Silencing
J Virol	The Journal of Virology
JAMA	JAMA: the Journal of the American Medical Association
Leuk Lymphoma	Leukemia & Lymphoma
mBio	mBio
Methods Enzymol	Methods in Enzymology
Methods Mol Biol	Methods in Molecular Biology
Microbes Infect	Microbes and Infection (Institut Pasteur)
Mol Cell	Molecular Cell
Mol Psychiatry	Molecular Psychiatry

N Engl J Med	New England Journal of Medicine
Nat Genet	Nature Genetics
Nat Immunol	Nature Immunology
Nat Med	Nature Medicine
Nat Protoc	Nature Protocols
Nat Rev Immunol	Nature Reviews Immunology
Nat Rev Mol Cell Biol	Nature Reviews Molecular Cell Biology
Nature	Nature
Nucleic Acids Res	Nucleic Acids Research
Open Virol J	Open Virology Journal
Pediatr Pulmonol	Pediatric Pulmonology
Pediatrics	Pediatrics
Pharmacoeconomics	Pharmacoeconomics
Pharmacol Ther	Pharmacology & Therapeutics
PLoS One	Public Library of Science (PLoS) ONE
PLoS Pathog	PLoS Pathogens
Proc Natl Acad Sci USA	Proceedings of the National Academy of Sciences of the United States of America
Proc Soc Exp Biol Med	Proceedings of the Society for Experimental Biology and Medicine
Protein Eng	Protein Engineering
Respir Res	Respiratory Research

RNA	RNA
RNA Biol	RNA Biology
Science	Science
Semin Respir Crit Care Med	Seminars in Respiratory and Critical Care Medicine
Trends Biochem Sci	Trends in Biochemical Sciences
Vaccine	Vaccine
Viral Immunol	Viral Immunology
Virology	Virology
Virus Res	Virus Research

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