IDENTIFICATION AND CHARACTERIZATION OF NEW AND DISTINCT FUNCTIONAL ROLES OF POSTTRANSCRIPTIONAL CONTROL ELEMENTS IN CYTOPLASMIC EXPRESSION OF RETROVIRAL RNA

DISSertation

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By

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* * * * *

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ABSTRACT

Retroviruses are dependent on host cellular machinery for synthesis, processing, nuclear export and translation of viral RNA. Cis-acting elements in the viral genome play an essential role in recruitment of cellular (and in some cases viral) trans-acting factors that facilitate viral gene expression. A portion of the primary viral transcript is spliced and exported from the nucleus by cellular splicing and export machinery, while another portion subverts this process and is exported from the nucleus independently of splicing. Maintenance of balanced expression of unspliced and spliced transcripts is necessary for appropriate viral protein production and sustained viral replication. The unspliced viral transcript exhibits dual function in the cytoplasm as genomic RNA that is packaged into progeny virions and as mRNA template for translation of Gag precursor protein and Gag-Pol polyprotein. The spliced viral transcript is the mRNA template for translation to Env protein. The central focus of this dissertation is the identification and characterization of retroviral posttranscriptional control elements that affect translation of the primary unspliced viral RNA.

We identify and characterize a new posttranscriptional control element in the Mason-Pfizer monkey virus 5’ long terminal repeat (LTR) that
modulates translational efficiency by augmentation of translational initiation. RNA and protein analysis demonstrates that MPMV RU5 has minimal effect on cytoplasmic accumulation but is necessary for cytoplasmic expression of HIV-1 gag-pol reporter RNA. MPMV RU5 also exerts a positive effect on the cytoplasmic expression of intronless luc RNA. Ribosome profile analysis demonstrates that MPMV RU5 increases the cytoplasmic localization of luc RNA with polyribosomes. MPMV RU5 functions independently of any viral proteins and instead directs functional interaction with cellular posttranscriptional modulators to facilitate translational enhancement. This work has illuminated the relationship between retroviral and cellular control of posttranscriptional gene expression. Future work will focus on the identification of MPMV RU5 cellular binding partners.

Secondly, we tested the hypothesis that combination of the MPMV constitutive transport element (CTE) and RU5 translational enhancers synergistically augments posttranscriptional gene expression. We combined the MPMV CTE and MPMV RU5 or spleen necrosis virus (SNV) RU5 on a single unspliced HIV-1 gag-pol RNA. Protein and RNA analysis demonstrated that MPMV CTE functions compatibly with MPMV and SNV RU5 to increase cytoplasmic expression of HIV-1 gag-pol reporter RNA in monkey COS, but not 293 cells. Overexpression of the CTE-interactive cellular proteins, Tap and NXT1, is necessary and sufficient to rescue increased cytoplasmic expression of HIV-1 gag-pol reporter RNA in 293 cells. This work produced the realization that differences in cellular posttranscriptional modulators dramatically affect
protein production. Future work will focus on identification and characterization of the cell-type specific cellular factors that direct productive cytoplasmic expression.

Thirdly, a necessary and important step was to extend functional analysis of the RU5 translational enhancers from heterologous reporter RNA to homologous genomic RNA. We evaluated the role of SNV RU5 on metabolism of homologous SNV genomic RNA by characterization of SNV \textit{gag-gfp} reporter RNA. Results of flow cytometric analysis indicate that SNV RU5 increases SNV Gag-GFP production. Northern blot analysis reveals that the increase in protein production is attributable, at least in part, to increased cytoplasmic accumulation of SNV \textit{gag-gfp} RNA. RU5 exerts a distinct effect on the spliced \textit{env} transcript. Deletion of RU5 has no effect on cytoplasmic accumulation of \textit{env} RNA, but significantly increases splicing efficiency. Therefore, SNV RU5 also modulates metabolism of the spliced \textit{env} RNA and is speculated to contain an RNA splicing suppressor. Future work will determine whether SNV RU5 recruits one or more cellular factors to modulate the distinct effects on unspliced and spliced SNV RNA and will characterize their importance in SNV replication.

In summary, this dissertation has identified and characterized a new posttranscriptional control element in MPMV and synergistic interactions among functionally distinct retroviral posttranscriptional control elements. This work also demonstrated an important role for the SNV posttranscriptional control element in SNV genomic RNA and discovered that SNV RU5 modulates balanced expression of unspliced and spliced SNV viral RNA.
Dedicated to my Parents.
ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Kathleen Boris-Lawrie for the opportunity to work in your lab. Your continual support and guidance have made this thesis possible. You have given me the tools to become a successful research scientist. I would also like to thank my committee members Dr. Patrick Green, Dr. Gary Kociba and Dr. Jim DeWille for advice and support. A special thank you to Dr. Michael Lairmore and Dr. Patrick Green for letters of recommendation and to all members of the Retrovirus Center for insightful comments.

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<td>3’ splice site</td>
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<td>5’ cap</td>
<td>5’ methyl-7-G(5’)pppN cap structure</td>
</tr>
<tr>
<td>5’ ss</td>
<td>5’ splice site</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
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<tr>
<td>CBC</td>
<td>cap binding complex</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>cytomegalovirus</td>
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<tr>
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<td>cyclophilin</td>
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<td>CPSF</td>
<td>cleavage-polyadenylation specificity factor</td>
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<td>CstF</td>
<td>cleavage stimulation factor</td>
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<tr>
<td>CTD</td>
<td>carboxy-terminal domain</td>
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<td>constitutive transport element</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dr</td>
<td>direct repeat element</td>
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<tr>
<td>EJC</td>
<td>exon junction complex</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>Env</td>
<td>envelope</td>
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<tr>
<td>FG</td>
<td>phenylalanine-glycine repeats</td>
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<tr>
<td>FV</td>
<td>foamy virus</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde dehydrogenase</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
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<tr>
<td>HFV</td>
<td>human foamy virus</td>
</tr>
<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus type 1</td>
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<tr>
<td>hnRNP</td>
<td>heterogeneous nuclear ribonucleoprotein</td>
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<tr>
<td>hTap</td>
<td>human Tap</td>
</tr>
<tr>
<td>INS</td>
<td>inhibitory sequence</td>
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<tr>
<td>IRES</td>
<td>internal ribosome entry sequence</td>
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<tr>
<td>LMB</td>
<td>leptomycin B</td>
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<tr>
<td>LTR</td>
<td>long terminal repeat</td>
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<tr>
<td>Luc</td>
<td>luciferase</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>mRNP</td>
<td>mRNA ribonucleoprotein</td>
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<tr>
<td>MPMV</td>
<td>Mason-Pfizer monkey virus</td>
</tr>
<tr>
<td>MuLV</td>
<td>murine leukemia virus</td>
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<tr>
<td>NES</td>
<td>nuclear export signal</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<tr>
<td>NMD</td>
<td>nonsense-mediated messenger RNA decay</td>
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<td>NPC</td>
<td>nuclear pore complex</td>
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<tr>
<td>NRS</td>
<td>negative regulator of splicing</td>
</tr>
<tr>
<td>PABP</td>
<td>poly(A)-binding protein</td>
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<td>PC4</td>
<td>positive cofactor 4</td>
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PCE       posttranscriptional control element
PCR       polymerase chain reaction
Pol       polymerase
poly(A)   poly adenosine
PRE       posttranscriptional regulatory element
PTC       premature termination codon
R         direct repeat
RanGAP    ran-specific GTPase-activating protein
RxRE      Rex responsive element
RNA       ribonucleic acid
RNAP II   RNA polymerase II
RNP       ribonucleoprotein
RPA       RNA protection assay
RRE       REV responsive element
RSL       R region stem loop element
RSV       rous sarcoma virus
sa        splice acceptor
snRNA     small nuclear messenger ribonucleic acid
snRNP     small nuclear ribonucleoprotein
SNV       spleen necrosis virus
SR        arginine/serine rich
SRV-1     simian retrovirus type-1
<table>
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<th>Description</th>
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<tr>
<td>sss</td>
<td>suppressor of src splicing</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>U3</td>
<td>unique 3' region</td>
</tr>
<tr>
<td>U5</td>
<td>unique 5' region</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>WHBV</td>
<td>woodchuck hepatitis B virus</td>
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CHAPTER 1

INTRODUCTION

Statement of the Problem and Outcomes

Retroviruses overcome several cellular quality control measures to assure efficient nuclear export and translation of their unspliced RNA by cellular gene expression machinery. The first barrier is to nuclear export because typical cellular unspliced RNA is contained in the nucleus by cellular proteins and the splicing process is necessary to recruit nuclear export proteins (35,100). In contrast, retroviruses circumvent cellular control of splicing by utilizing various RNA elements to recruit viral and cellular nuclear export proteins independently of the splicing process. The second barrier is to efficient translation of viral RNA that is unspliced because the splicing process has been shown to stimulate translation (104). Additionally, structured 5’ untranslated regions (UTRs), which are conserved among all retroviruses, typically inhibit efficient translation of cellular mRNAs by impeding ribosomes as they scan from the 5’ cap to the proximal start codon (55,101,124). Retroviruses recruit viral and cellular proteins to facilitate translation of unspliced viral RNA despite the lack of splicing and the presence of a highly structured 5’ UTR. The central focus of my dissertation is retroviral posttranscriptional control elements that facilitate translation of unspliced viral RNA.
A key outcome of this dissertation is the identification of a posttranscriptional control element in Mason-Pfizer monkey virus (MPMV) that facilitates translation of unspliced RNA. The recent identification of functionally similar posttranscriptional control elements in primate MPMV and avian spleen necrosis virus (SNV) implies that diverse retroviruses share a unique mechanism of translational control to overcome barriers to efficient translation of their unspliced RNA. This research has illuminated an essential step in viral gene expression and provides a new paradigm for understanding cellular control of the translation process. The MPMV and SNV posttranscriptional control elements also have potential application for optimization of gene expression in various systems.

Another central outcome of this dissertation is the elucidation of the relationship between nuclear export and translation of unspliced viral RNA by MPMV and SNV posttranscriptional control elements. Our data revealed for the first time that enhancement of cytoplasmic RNA expression upon combination of retroviral posttranscriptional control elements is cell-type dependent. This discovery has important implication for development of optimized gene expression systems and for retroviral vector systems.

Another key outcome of this work is the demonstration that SNV posttranscriptional control element augments production of SNV Gag from SNV genomic RNA. An additional realization is that the SNV element also modulates splicing of SNV genomic RNA. This finding is an important step
toward full understanding of SNV posttranscriptional gene expression and the virus-host interactions that choreograph retroviral replication.

The first section of the Introduction will provide an overview of the literature that has recently uncovered extensive coupling between cellular pre-mRNA processing events, nuclear export and translational control during cellular gene expression. The second section will introduce the retrovirus replication cycle and overview selected retroviral RNA elements that subvert cellular control of gene expression to assure efficient nuclear export and translation of unspliced viral RNA.

*Introduction to the cotranscriptional nature of cellular pre-mRNA processing*

DNA serves as the template for transcription of pre-messenger RNA (pre-mRNA) by RNA polymerase II (RNAP II). Upon synthesis, pre-mRNA associates with heterogeneous nuclear ribonucleoproteins (hnRNPs) to form an hnRNP complex. Within the hnRNP complex, pre-mRNA processing events generate an mRNA ribonuclear protein (mRNP) complex that is suitable for nuclear export and translation. The cellular processing machinery facilitates the acquisition of a cap structure at the 5' terminus, removal of intronic sequences from the body of the pre-mRNA and 3' cleavage and addition of a poly(A) tail. Recent studies have provided evidence that these pre-mRNA processing events occur cotranscriptionally and are coordinated by processing factors that interact with the carboxy-terminal domain (CTD) of RNAP II (34,100,119,126,127). The binding of RNA processing factors to the CTD is
regulated by site-specific phosphorylation and dephosphorylation events on the conserved heptad repeats present in the CTD (79). The elucidation of the three dimensional structure of RNAP II has revealed that the CTD is adjacent to the pre-mRNA exit groove, which positions the RNA processing factors in close proximity to the pre-mRNA emerging from the polymerase (31). The following sections briefly describe the cotranscriptional processing of pre-mRNA coordinated by the CTD and linkage to efficient nuclear export and translation.

**Cotranscriptional pre-mRNA capping**

The cotranscriptional process of 5’ RNA terminus capping is the initial pre-mRNA modification. The cap is a 7-methyl guanine residue that is attached to the 5’ nucleotide of the RNA by a novel 5’ to 5’ linkage (2). The capping enzymes include an RNA 5’ triphosphatase, RNA guanylyltransferase and RNA methyltransferase. In the nucleus, the cap structure, in association with the cap binding complex (CBC), increases mRNA stability by obstructing 5’-3’ exonucleases (9,51). In the cytoplasm, the cap stimulates cap-dependent mRNA translation (136).

Capping is concomitant with the transition from the transcription initiation phase to the transcription elongation phase and occurs after as few as 20 to 30 nucleotides of the nascent pre-mRNA are synthesized and emerge from the transcription complex (Figure 1.1) (2). The transition to the transcription elongation phase is facilitated by site-specific phosphorylation of the CTD heptad repeats (81,82,91,116,123), which triggers dissociation of transcription initiation factors and subsequent recruitment of capping enzymes (62,79,142).
Biochemical analysis has revealed that capping enzymes selectively bind to the phosphorylated form of the CTD (26,105) and deletion of the CTD reduces the production of capped transcripts (105).

Following pre-mRNA capping, dephosphorylation of the CTD triggers the release of the capping enzymes and additional site-specific phosphorylation events recruit other RNA processing factors to the CTD (25,79,142). The prerequisite of capping for efficient pre-mRNA splicing, 3' cleavage and polyadenylation suggests that coupling between capping and the start of transcription elongation is a checkpoint used by the cell to ensure that uncapped transcripts are not extended or processed (119).
Figure 1.1: Cotranscriptional processing coordinated by the carboxy-terminal domain (CTD) of RNAP II. The conserved CTD heptad repeats serve as a platform for recruitment of factors required for transcription and pre-mRNA processing. A. Prior to transcription initiation, the unphosphorylated CTD of RNAP II is associated with components of the transcriptional machinery. B. During the transcription initiation phase, partial phosphorylation of the CTD triggers release of transcription initiation factors and recruitment of capping enzymes, which cap the 5' end of the nascent transcript. C. Additional phosphorylation of the CTD during the transcription elongation phase recruits components of the pre-mRNA splicing machinery, which remove intronic sequences from the nascent transcript. D. Recognition of termination signals recruits factors required for cleavage and polyadenylation to the CTD. Some components of the 3' processing machinery are actually recruited during initiation. Figure adapted with modification from reference (119).
Cotranscriptional pre-mRNA splicing

Typical cellular pre-mRNAs require the removal of intronic sequences to qualify for nuclear export (36,59). Recent studies have also demonstrated that intron removal is required for efficient translation (104). The elimination of intronic sequences is facilitated by the spliceosome, which is composed of small nuclear ribonucleoproteins (snRNP) and non-snRNP proteins. In addition, arginine-serine rich (SR) proteins facilitate the interaction of the spliceosome with the RNA.

The coupling of the transcription elongation phase and splicing (Figure 1.1) was initially demonstrated by electron microscopy analysis, which showed excision of introns during mRNA synthesis (13,160). Biochemical data have implicated the CTD in the coupling of pre-mRNA synthesis and splicing. Several splicing factors have been shown to interact with the phosphorylated CTD (76,113,162). Deletion of the CTD from RNAP II or overexpression of a phosphorylated CTD decreases the amount of spliced transcript produced in vivo (45,106,112) and the data imply that the CTD plays an important role in intranuclear targeting of splicing factors to active transcription sites (112).

Cotranscriptional 3’ pre-mRNA processing

Another step necessary to generate a mature mRNA is processing of the 3’ RNA terminus. This process includes cleavage and polyadenylation. After RNA cleavage, poly-A polymerase adds approximately 200 adenosine nucleotides to the 3’ end of the mRNA to generate a poly(A) tail. The poly(A) tail increases the stability of the RNA and stimulates mRNA translation in
conjunction with poly-A-binding protein (PABP) (136). In the closed loop model of translation, PABP interacts with components of the cap binding complex. This circularizes the mRNA and increases translation initiation by stimulation of ribosome recruitment (136).

As demonstrated with capping and splicing, 3' processing is also a cotranscriptional process coordinated by the CTD (Figure 1.1). Deletion of the CTD from RNAP II results in defective polyadenylation of RNAP II transcripts (106). Two factors have been shown to link transcription and 3' processing: the cleavage-polyadenylation specificity factor (CPSF), which binds the conserved AAUAAA sequence, and cleavage stimulation factor (CstF), which binds to the GU rich sequence downstream of the cleavage site (23,41). CPSF and CstF are recruited to the transcription start site by basal transcription factor TFIID and the transcriptional coactivator, positive cofactor 4 (PC4), respectively. Interestingly, the start of transcription triggers the association of CPSF and the CstF-PC4 complex with the phosphorylated CTD of the elongating RNAP II (23,41,49). PC4 mediates an antitermination function to inhibit premature termination by CstF during transcription elongation and dissociates from CstF at the 3' processing site. The interaction of 3' processing factors with the CTD supports a model in which 3' processing factors associate early with RNAP II and remain attached during elongation. In summary, the pre-mRNA processing events are coordinated by the CTD of RNAP II and occur cotranscriptionally. The interesting linkage between RNA processing and nuclear export and translational control will be summarized in the following sections.
Linkage between nuclear export and splicing

Typical pre-mRNAs are fully processed before they are exported to the cytoplasm. Retention of pre-mRNA in the nucleus is a cellular quality control measure that prevents translation of intron-containing mRNAs, which could produce useless or even harmful proteins (24, 86). The process of RNA splicing has been shown to license processed mRNA for nuclear export (Figure 1.2) (92). Subsequent to intron removal by the spliceosome, a multi-protein complex assembles 20 to 24 nucleotides upstream of exon-exon junctions by a non-consensus sequence mechanism (73, 83-85). This exon junction complex (EJC) contains factors associated with splicing (RNSP1, DEK, SRm160), mRNA export (Aly/Ref) and an mRNA binding protein (Y14). Aly/Ref act to recruit the nuclear export factor Tap and its cofactor NXT1, a RanGTP-binding protein, to the EJC (83, 131, 152, 165) (14). The recruitment of Tap and NXT1 activates selective nuclear export of spliced mRNAs. Tap facilitates the translocation of mature mRNA through the nuclear pore complex (NPC) by interacting with phenylalanine-glycine (FG) repeats of nucleoporins that line the pore (7, 58, 70, 88, 158). The formation of Tap-NXT1 heterodimers enhances the association of Tap with nucleoporins (88, 158).

Human Tap (hTap) has been implicated as the global activator of human mRNA export. hTap is the functional homolog of the yeast Mex67p protein, which facilitates yeast global poly(A) mRNA nuclear export in conjunction with cofactor Mtr2p (137, 145). Exogenous expression of hTap and NXT1 rescues a Mex67p and Mtr2p deficient yeast strain, which validated that Tap and NXT1
are sufficient to facilitate mRNA export (72). Overexpression of Tap and NXT1 stimulates nuclear export of mRNAs that are normally retained in the nucleus in *Xenopus* oocytes and mammalian cells, which demonstrated that these proteins function in mRNA export in higher eukaryotes (17). The association of Tap with the EJC functionally links cotranscriptional pre-mRNA splicing with mRNA nuclear export.

Figure 1.2: pre-mRNA splicing licenses mRNA for nuclear export. In the nucleus, splicing of pre-mRNA results in recruitment of the exon junction complex (EJC) upstream of the exon-exon junction. Components of the EJC include SRm160, Y14, Aly/Ref, RNPS1 and DEK. After splicing, Tap-NXT1 heterodimers associate with the EJC to activate mRNA nuclear export. Several factors of the EJC dissociate during the export process. Y14 and RNPS1 remain associated with the mRNA in the cytoplasm and mark exon-exon junctions. Figure adapted with modifications from reference (83).
Linkage between splicing and translation

Upon export to the cytoplasm, mRNA acts as the template for translation of protein. Recently, pre-mRNA splicing in the nucleus has been shown to influence the translational fate of mRNA in the cytoplasm of both *Xenopus* oocytes and mammalian cells. In *Xenopus* oocytes, reporter RNA with an intron positioned 5' of the open reading frame stimulates translational efficiency over an intronless reporter RNA (104). In contrast, reporter RNA with an intron positioned 3' of the open reading frame is translationally repressed compared to an intronless reporter RNA. Matsumoto and colleagues postulate that splicing of the 5' intron recruits an RNP complex that stimulates translation, while excision of the 3' intron recruits an RNP complex that represses translation. This model predicts that the same mRNA is utilized differently in the cytoplasm as a consequence of packaging into distinct RNP complexes. Their data demonstrate that the position-dependent excision of an intron influences the translational fate of the reporter RNA and functionally links pre-mRNA splicing to translation efficiency. Y14 is a nucleocytoplasmic shuttle protein of the EJC that associates with spliced mRNA in the nucleus and remains associated in cytoplasm (Figure 1.2) (73,78). Because Y14 remains associated with the mRNA upon ribosome loading, Dostie and colleagues speculate that Y14 may be responsible for the stimulation of translation in response to excision of a 5' intron (44). Verification of this hypothesis would strengthen the linkage between pre-mRNA splicing and translational efficiency.
Additionally, pre-mRNA splicing has recently been linked to translational control in mammalian cells by the process of nonsense-mediated messenger RNA decay (NMD) (Figure 1.3). NMD is a cellular surveillance mechanism used to selectively degrade mRNAs with premature termination codons (PTC) (2). NMD is important to prevent the production of C-terminal truncated proteins that may be deleterious to the cell. The protein complex that executes NMD in mammalian cells consists of hUpf1, hUpf2 and hUpf3 (139). NMD requires both pre-mRNA splicing and translation and is activated when a PTC terminates translation more than 50 to 55 nucleotides upstream of the last exon-exon junction (20,102,114,156). The EJC marks the position of exon-exon junctions on the processed mRNA (83-85). Pre-mRNA splicing was linked to NMD by the observation that hUpf3, which is a nucleocytoplasmic shuttle protein that resides predominately in the nucleus, interacts with the EJC (77,95). During nuclear export, the RNP complex associated with the mRNA rearranges and two members of the EJC, Y14 and RNPS1, plus hUpf3 remain associated with the mRNP complex. Both Y14 and RNPS1 are postulated to communicate to cytoplasmic factors the location of exon-exon junctions (77,95). Additionally, perinuclear localized hUpf2 protein is postulated to associate with hUpf3 during mRNA export (94). A pioneer round of translation, which is thought to occur concurrently with export, is also necessary for triggering NMD (69,87). The literature posits the model that the leading ribosome on the mRNA template will dislodge the Y14-RNPS1-hUpf3-hUpf2 complexes as it traverses the mRNA (94,139). If the mRNA does not contain a PTC, the ribosome will
dislodge all the complexes because the natural stop codon is positioned in the last exon. In contrast, if the mRNA contains a PTC more than 50 to 55 nucleotides upstream of the last exon-exon junction, translation is terminated before all the Y14-RNPS1-hUpf3-hUpf2 complexes are displaced. The remaining complex(es) recruit hUpf1 and trigger mRNA degradation (94). NMD illuminates another mechanism where pre-mRNA splicing is linked to translational control.

Figure 1.3: Premature termination codons (PTC) activate nonsense-mediated mRNA decay (NMD). In the nucleus, the process of pre-mRNA splicing facilitates the formation of exon junction complexes (EJC) with exon-exon junctions. The EJC includes DEK, Aly/Ref, SRm160, Y14, and RNPS1. The Tap-NXT1 heterodimer and NMD factor hUpf3 associate with the EJC in the nucleus. During nuclear export the RNP architecture rearranges and results in an Y14-RNPS1-hUpf3-hUpf2 complex associated with exon-exon junctions. The leading ribosome will dislodge the Y14-RNPS1-hUpf3-hUpf2 complex(es) upstream of the PTC. The remaining Y14-RNPS1-hUpf3-hUpf2 complex(es) recruit hUpf1 and triggers NMD. Figure adapted with modifications from reference (139).
These initial sections of this literature review have highlighted control of cellular posttranscriptional gene expression that is enforced by tight linkage between transcription, pre-mRNA processing, mRNA export and translation. The remaining sections will focus on mechanisms utilized by retroviruses to subvert this tight linkage and achieve efficient export and translation of viral pre-mRNA. These sections will begin with a brief review of retrovirus genomic structure and replication cycle.

**Retrovirus genomic structure and replication cycle**

Retroviruses are a diverse group of enveloped RNA viruses. The viral particle contains two copies of genomic RNA that is 7 to 12 kb in length, of positive polarity, linear, single-stranded, and nonsegmented (30). The hallmark of retroviral replication is the ability to reverse transcribe the RNA genome into a linear double stranded DNA copy that is subsequently integrated into the host DNA as a provirus. The provirus is then disguised as a typical cellular gene and is dependent on host cellular machinery to express viral genes and genomic RNA. Retroviruses are classified as either genetically simple or complex based on their genomic structure (Figure 1.4). All retroviruses encode the three common structural and enzymatic proteins: Gag, Pol and Env. Gag provides the matrix, capsid and nucleocapsid structures inside the virion and Pol includes reverse transcriptase, integrase and protease enzymes, which are also virion proteins. Env includes both the surface and transmembrane domains of the viral envelope protein. Complex retroviruses encode additional regulatory and accessory proteins that are required for virus replication.
Figure 1.4: Genomic structure of simple and complex retroviruses. A. Representative genome of the genetically simple Mason-Pfizer monkey virus (MPMV). MPMV contains three viral genes, \textit{gag}, \textit{pol} and \textit{env}. The long terminal repeats (LTRs) include the unique 3' region (U3), direct repeat region (R) and unique 5' region (U5). B. Representative genome of the genetically complex human immunodeficiency virus type 1 (HIV-1). HIV-1 also contains two regulatory genes, \textit{rev} and \textit{tat}, and four accessory genes, \textit{vif}, \textit{vpr}, \textit{vpu} and \textit{nef}. PBS, primer binding site; E, encapsidation signal; 5' ss, 5' splice site; 3' ss, 3' splice site; PPT, polypurine tract.
Retrovirus replication (Figure 1.5) is initiated by the attachment of the Env protein to a specific cell surface receptor(s), subsequent fusion with the plasma membrane and entry of the virion core (30). In the core, the RNA genome is reverse transcribed into linear double stranded DNA. The double stranded DNA copy is transported into the nucleus within a pre-integration complex and integrated into the host genome as a provirus by virally encoded integrase. Once the provirus is integrated into the host genome, RNAP II synthesizes the pre-mRNA. Interestingly, consistent with the cotranscriptional processing events of cellular pre-mRNA, retroviral unspliced pre-mRNA is capped and polyadenylated. However, cotranscriptional splicing is modulated by the retrovirus. A portion of the unspliced RNA is exported to the cytoplasm independently of splicing while another portion undergoes splicing by cellular splicing machinery with subsequent export to the cytoplasm. Therefore, nuclear export of unspliced viral RNA is one obstacle that retroviruses must overcome to produce progeny virions. The cytoplasmic unspliced RNA acts as a template for the synthesis of the Gag-Pol polyprotein and as genomic RNA that is packaged into virions. The spliced RNAs act as a template for the synthesis of Env, and in complex retroviruses, the regulatory and accessory proteins.

A second obstacle that must be overcome is cap-dependent ribosome scanning of the unspliced RNA despite the presence of a long and highly structured 5’ UTR. The UTR is a biologically conserved feature necessary for RNA packaging and other steps in viral replication. After translation of viral proteins, assembly of the progeny virions takes place at the plasma membrane.
Two copies of the unspliced RNA are encapsidated in each infectious virion. As the progeny virions bud from the cell, the virally-encoded protease cleaves the immature Gag-Pol polyprotein to generate a mature, infectious virion.

Figure 1.5: Retrovirus replication cycle. The major steps of the retrovirus life cycle are represented. Briefly, the parental virus attaches to a specific cell surface receptor and the virion core enters the target cell. In the core, the RNA genome is reverse transcribed into a double stranded DNA intermediate, which is transported into the nucleus and integrated into the host genome as a provirus. Cellular transcription machinery transcribes a primary transcript that is either exported from the nucleus or spliced and subsequently exported. Both unspliced and spliced RNAs act as template for synthesis of viral proteins by host translational machinery. In addition, the unspliced RNA functions as genomic RNA and two copies are packaged into assembling particles at the plasma membrane. After the progeny virions bud from the cell, the viral protease cleaves the immature viral proteins to generate mature, infectious virions.
The following sections will describe four mechanisms retroviruses utilize to subvert the tight linkage between host cell splicing and nuclear export and ultimately facilitate nuclear export and translation of their primary unspliced RNA. The interaction of viral RNA elements with viral and cellular regulatory proteins orchestrates the ability of intron-containing retroviral RNA to override nuclear retention.

**Rev/RRE activate the nuclear export of intron-containing HIV-1 RNA**

Nuclear export of unspliced retroviral RNA is required for production of progeny virions. The genetically complex human immunodeficiency virus (HIV-1) encodes the posttranscriptional regulatory protein Rev, which interacts with a complex RNA secondary structure in the intronic envelope region termed Rev responsive element (RRE) [reviewed in (15,36,63,125)]. In the absence of Rev, cis-acting inhibitory sequences retain the viral mRNA in the nucleus (29,96,140,143,144). In the presence of Rev, nuclear export of unspliced and incompletely-spliced viral RNAs is activated by Rev binding to RRE (28,59,60,97-99). Rev binding to RRE also increases the stability and translational efficiency of RRE-containing RNAs (Figure 1.6) (5,39,97). Rev is a 116 amino acid nucleocytoplastic shuttle protein that contains an arginine-rich nuclear localization signal (NLS), which facilitates nuclear import and subsequent binding to the RRE (110,154). Rev also contains a leucine-rich nuclear export signal (NES) that is the binding site for the cellular CRM-1/exportin 1 nuclear export receptor (36,98,110,115). The CRM-1 nuclear export pathway is normally reserved for 5S ribosomal RNA and cellular proteins...
with leucine-rich NESs (15,36,50). Multiple Rev monomers bind to RRE-containing RNA through multimerization domains present on Rev and recruit CRM1 to the Rev/RRE complex. In the nucleus, CRM1 associates with RanGTP and binds to the Rev NES. CRM1 binds to nucleoporins of the NPC to facilitate the translocation of the Rev/RRE complex to the cytoplasm. Once in the cytoplasm, hydrolysis of RanGTP by cytoplasmic Ran-specific GTPase-activating protein (RanGAP) induces the release of the Rev-CRM1-Ran complex from the RRE-containing RNA. These viral and cellular export factors recycle back to the nucleus and target additional newly synthesized RRE-containing viral RNAs (68).
Figure 1.6: Retroviral posttranscriptional control elements modulate various steps of posttranscriptional gene expression. Human immunodeficiency virus type-1 (HIV-1) Rev/Rev responsive element (RRE) complexes associate with the cellular nuclear export receptor CRM-1 to activate nuclear export of unspliced viral RNA. HIV Rev also modulates the stability and translation of RRE-containing RNAs. Mason-Pfizer monkey virus (MPMV) constitutive transport element (CTE) associates with cellular factors Tap and NXT1 to facilitate nuclear export of unspliced viral RNA. Rous sarcoma virus (RSV) direct repeat elements (dr) associate with an unidentified cellular protein(s) to facilitate unspliced viral RNA stability, nuclear export and particle assembly. Spleen necrosis virus (SNV) RU5 associates with a cellular protein(s) that augments the association of unspliced RNA with the translational machinery. Figure adapted with modification from reference (15).
**CTE is a functional homolog of RRE**

Although genetically simpler retroviruses also require nuclear export of unspliced viral RNA, they lack an analogous Rev-like regulatory protein. The genetically simpler type D Mason-Pfizer monkey virus (MPMV) and simian retrovirus type-1 (SRV-1) contain a functional homolog of RRE, termed the constitutive transport element (CTE). The CTE is a 160-nucleotide cis-acting structured RNA element located in the 3' UTR that functions in an orientation- and position-dependent manner and independently of any viral protein (19,46,129,135,155). Instead, CTE recruits a Rev-like cellular protein to facilitate nuclear export (Figure 1.6). CTE and host cell proteins can functionally substitute for Rev/RRE to facilitate the nuclear export of intron-containing HIV-1 RNA. Additionally, CTE can sustain replication of a Rev – (minus) HIV-1 proviral clone, although replication efficiency is severely weakened when compared to wild type HIV-1 in human CD4+ cells (19,167).

Importantly, in the context of a MPMV proviral clone, CTE is required for nuclear export of MPMV unspliced *gag-pol* RNA and viral replication in a monkey COS cell line (46). Consistent with reports of CTE-mediated nuclear export in mammalian cells, CTE induces nuclear export of a lariat reporter RNA in *Xenopus* oocytes (56,122). These data validated *Xenopus* oocytes as a surrogate model for biochemical analysis of CTE nuclear export.

**Tap facilitates CTE-mediated nuclear export**

Because CTE facilitates export of unspliced MPMV and SRV-1 RNA independently of any viral protein, CTE function implicitly requires a host
protein. In *Xenopus* oocytes, excess CTE RNA blocked the nuclear export of mRNA, but not tRNA, snRNA or CRM-1 dependent Rev/RRE RNA (122). These results implied that CTE RNA sequestered and utilized proteins required for the nuclear export of mRNAs. Experiments with HeLa nuclear extracts initially identified the CTE-interactive cellular protein to be Tap (56). As previously discussed, extensive experimentation has implicated Tap as the global mRNA export receptor.

The hTap protein is a nucleocytoplasmic shuttle protein that contains an NLS, NES, nucleoporin-, NXT1- and CTE-binding domains (8,18,27,71). Tap can directly interact with CTE RNA in vitro (27,56) and tethering Tap to unspliced reporter RNAs activates nuclear export in vivo (88,88). Similar to the mechanics of mRNA nuclear export, Tap facilitates translocation of CTE RNA to the cytoplasm by binding to various nucleoporins (CAN/Nup214, Nup98, p62, CG1, Nup153) (7,70). Formation of Tap-NXT1 heterodimers enhances the interaction of Tap with nucleoporins in vitro and tethering Tap-NXT1 heterodimers to unspliced reporter RNAs increases nuclear export in vivo (27,70,88,158). These results demonstrate that recruitment of Tap and NXT1 to CTE is sufficient to facilitate nuclear export of unspliced RNA. Recruitment of Tap-NXT1 heterodimers to CTE functionally replaces splicing as a prerequisite for nuclear export. Whereas the EJC contains Aly/Ref, which recruits Tap to spliced RNA, CTE recruits Tap directly to unspliced RNA.
Rous sarcoma virus dr element modulates various posttranscriptional events

Rous sarcoma virus (RSV) is a genetically simple oncogenic retrovirus (30). The RSV genome contains four open reading frames: gag, pol, env and viral-src (v-src) (30). RSV contains two cis-acting direct repeat (dr) elements that exhibit approximately 80% sequence homology (57). The upstream dr element is positioned in between the env and src genes and the downstream dr element is in the 3’ UTR. Both the upstream and downstream dr elements include two subelements, dr1 and dr2. Each dr is an RNA element that forms a redundant stem-loop structure (117). The dr elements have been shown to modulate various steps in RSV replication (Figure 1.6). Deletion and mutational analysis demonstrated that dr is necessary for stability and cytoplasmic accumulation of unspliced RNA (117,118,120,147). Cytoplasmic accumulation of dr-containing transcripts is independent of the CRM-1 and Tap nuclear export receptors that are utilized by HIV Rev/RRE and CTE, respectively (120). The identity of the cellular protein(s) that modulates dr-mediated export is currently unknown. Analysis of additional dr mutants revealed a role for dr in RNA packaging and viral particle assembly. These dr mutants also exhibit reduced release of progeny virions (6,146,147,150). Boris-Lawrie et al speculated that mutation of dr disrupts association of important posttranscriptional modulators that target dr-containing RNA to a permissive cytoplasmic microenvironment necessary for particle assembly and release (15).
Murine leukemia virus R modulates cytoplasmic RNA levels

While MPMV CTE and RSV dr elements are positioned at the 3’ end of the unspliced viral RNA, murine leukemia virus (MuLV) utilizes a 5’ posttranscriptional control element to increase cytoplasmic accumulation of unspliced RNA. MuLV R contains a cis-acting RNA element that enhances MuLV or CAT reporter protein production by increasing cytoplasmic RNA levels (37,38,157). The MuLV cis-acting element maps to the first 28 nucleotides of the 5’ R region and is termed the R region stem loop element (RSL) (38). In the MuLV provirus, deletion of RSL reduced the level of cytoplasmic unspliced viral RNA, but did not affect the level of spliced viral RNA (157). In a nonviral cat reporter plasmid, which lacks an intron, deletion or mutation of the RSL reduced both CAT activity and cytoplasmic cat RNA levels (37,157). These results confirmed that MuLV R functions to increase unspliced cytoplasmic RNA levels. Possible explanations are that MuLV R increases unspliced cytoplasmic RNA by facilitating nuclear export, impeding splicing or increasing stability (157).

Translational control by retroviral 5’ posttranscriptional control elements

Retroviruses utilize the host translational machinery for production of viral proteins. Cytoplasmic unspliced viral RNA must overcome two barriers for efficient translation: a highly structured 5’ UTR and lack of intron removal. Translation is reduced by the presence of a highly structured 5’ UTR because the structure impedes ribosomes as they scan from the 5’ cap to the initiation codon (55,101,124). Translational efficiency is also theorized to be reduced by lack of intron removal due to recruitment of unproductive RNP complexes (92).
5' RNA sequences have been identified in at least three divergent retroviruses that modulate translation of unspliced viral and non-viral reporter RNAs (22,67,130,134). The following sections will describe posttranscriptional control elements (PCEs) that enhance cytoplasmic expression of viral and nonviral RNAs.

**Spleen necrosis virus RU5 augments translational efficiency**

Long terminal repeats (LTRs) are characteristic of all retroviruses and are composed of three distinct regions: U3, R and U5 (30). In the 5' LTR, U3 contains promoter and enhancer sequences and RU5 corresponds to the 5' terminus of the viral RNA. The avian spleen necrosis virus (SNV) 5' LTR contains a posttranscriptional control element (PCE) in RU5 that increases the translational efficiency of unspliced reporter RNAs (Figure 1.6) (22,130). SNV RU5 is the first example of a cap-dependent retroviral translational enhancer. SNV RU5 functions in an orientation- and position-dependent manner to augment Rev/RRE-independent HIV-1 Gag production and non-viral Luc production in the absence of any SNV protein (15,130). The significant increase in protein production in response to SNV RU5 is not attributable to a proportional increase in the steady state level or cytoplasmic accumulation of *gag* or *luc* reporter RNA. Reporter gene assays with bicistronic reporter RNAs eliminated the possibility that SNV RU5 functions as an internal ribosome entry site (IRES) and implied that RU5 increases translation by a distinct mechanism (130). Ribosome sedimentation assays determined that RU5 augments the abundance of *gag* transcripts that cosediment with ribosomes (15). Higher
resolution ribosomal profile analysis demonstrated that SNV RU5 augments cytoplasmic luc RNA association with polyribosomes, which indicated that RU5 enhances translational efficiency by augmentation of translational initiation (130). Results of both ribosome sedimentation and ribosomal profile assays indicate that RU5-interactive factor(s) program the cytoplasmic transcript for productive interaction with the translational machinery. This dissertation will detail the discovery of a related element in the RU5 region of the MPMV 5' LTR.

**Nuclear interactions are necessary for SNV RU5 translational enhancement**

A combination of in vitro and in vivo experiments were performed to address the cellular requirements for SNV RU5 translational enhancement (40). In vivo RNA transfection and in vitro translation assays demonstrated that cytoplasmic factors are not sufficient for translational enhancement by SNV RU5 and imply that nuclear factors program the RNA for enhanced translation in the cytoplasm. Leptomycin-B (LMB) treatment of transiently transfected cells, which covalently inactivates CRM-1, indicated that the nuclear export pathway accessed by SNV RU5 is independent of the CRM-1 nuclear export receptor utilized by HIV-1 Rev/RRE. Two other possible nuclear export pathways for RU5-containing RNA are either the Tap- and NXT1-dependent mRNA or the tRNA export pathways. This dissertation will evaluate the role of Tap and NXT1 on the functional activity of SNV RU5. The potential role of the tRNA pathway remains to be examined experimentally.

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Dangel et al performed competition experiments in which SNV RU5 and HIV-1 Rev/RRE were combined on a single HIV-1 gag reporter RNA; Rev/RRE was observed to sequester the reporter RNA to the CRM-1 dependent nuclear export pathway and abrogate translational enhancement by SNV RU5. The data indicate that either Rev/RRE act upstream of SNV RU5 or disrupt interaction between SNV RU5 and nuclear posttranscriptional modulators that program SNV RU5-containing RNA for enhanced translation. The interaction of SNV RU5 with nuclear factor(s) establishes a functional linkage between nuclear interactions and cytoplasmic translation in simple retroviruses.

**Human foamy virus R facilitates Gag and Pol production**

Foamy viruses (FV) are unique complex retroviruses because they are nonpathogenic in their natural hosts (89). In addition, the Gag and Pol structural and enzymatic proteins are expressed from separate mRNAs and reverse transcription can initiate in cell-free virus particles (89,161). A cis-acting RNA element in the R region of the 5’ LTR of human foamy virus (HFV) has been shown to be necessary and sufficient for HFV Gag and Pol production (61,134). The 5’ proximal R region functions in an orientation- and position-dependent manner to facilitate HFV Gag and Pol production from the unspliced and spliced viral RNA, respectively (134). Deletion of the R region downstream of the major 5’ splice donor site abrogates detection of the spliced pol transcript and implies that the deleted R sequences are necessary for utilization of the pol splice site (61,134). In contrast, unspliced gag RNA levels were not reduced, despite abrogation of Gag production. The mechanism by which R facilitated
Gag production was investigated by a bicistronic reporter assay (134). HFV R activation of the bicistronic gagIREScat RNA produced linear increases in Gag and CAT protein. These results led Russel and colleagues to speculate that HFV R programs R-containing transcripts in the nucleus for subsequent targeting to translationally active regions in the cytoplasm (134). At present, the effect of HFV R on ribosome association has not been directly analyzed, but this is necessary step to determine whether or not HFV R modulates ribosome association and translation initiation.

**Woodchuck hepatitis virus posttranscriptional regulatory element increases cytoplasmic RNA expression**

Woodchuck hepatitis B virus (WHBV) is a DNA virus of the hepadnavirus family. The HBV genome encodes four viral proteins: Polymerase, Core, Surface and X (52). Although the four viral mRNAs are generated from separate promoters, all mRNAs contain a common 3’ terminus and are primarily exported from the nucleus as unspliced RNA (153). Comparable to simple retroviruses, WHBV also contains a cis-acting element that functions independently of a WHBV trans-acting protein to modulate cytoplasmic expression of the HBV viral and nonviral unspliced mRNAs (43). The WHBV posttranscriptional regulatory element (PRE) is located approximately 1 kb from the 3’ terminus in all four viral mRNAs. WHBV PRE is composed of three distinct elements that function cooperatively to increase HBV Surface and nonviral CAT protein production in an orientation- and position-dependent manner. The increase in CAT production correlates with an increase in the
steady state level of unspliced cat RNA in both the nucleus and cytoplasm. Interestingly, transcription, stability and overall proportion of cat RNA accumulated in the cytoplasm are not increased in response to PRE. Hope and colleagues have recently shown that WHBV PRE increases the poly(A) tail length of reporter RNAs (64). Modulation of the poly(A) tail length in the nucleus is postulated to facilitate increased levels of unspliced nuclear and cytoplasmic RNA, which subsequently results in enhanced protein production. Interestingly, WHBV PRE also stimulates cytoplasmic expression of luc and green fluorescent protein (gfp) reporter RNAs in the context of retroviral and adeno-associated virus vectors (90,168).

In contrast to WHBV PRE, the human HBV PRE increases reporter RNA expression by facilitating nuclear export (42,65,66,148). Overall, these results demonstrate that in addition to retroviruses, hepadnaviruses also utilize viral posttranscriptional control elements and cellular trans-acting proteins to assure efficient cytoplasmic expression of unspliced viral RNAs. These retroviral and hepadnavirus RNA elements affect various steps of posttranscriptional gene expression.
ABSTRACT

Retroviruses utilize an unspliced version of their primary transcription product as an RNA template for synthesis of viral Gag and Pol structural and enzymatic proteins. Cytoplasmic expression of the \textit{gag-pol} RNA is achieved despite the lack of intron removal and the presence of a long and highly structured 5’ untranslated region that inhibits efficient ribosome scanning. In this study, we have identified for the first time that the 5’ long terminal repeat (LTR) of Mason-Pfizer monkey virus (MPMV) facilitates Rev/Rev responsive element-independent expression of HIV-1 \textit{gag-pol} reporter RNA. The MPMV RU5 region of the LTR is necessary and directs functional interaction with cellular post-transcriptional modulators present in human 293 and monkey COS cells, but not quail QT-6 cells, and does not require any viral protein. Deletion of MPMV RU5 decreases the abundance of spliced mRNA but has little effect on cytoplasmic accumulation of unspliced \textit{gag-pol} RNA despite complete elimination of detectable Gag protein production. MPMV RU5 also exerts a positive effect on the cytoplasmic expression of intronless \textit{luc} RNA and ribosomal profile analysis demonstrates that MPMV RU5 directs subcellular...
localization of the *luc* transcript to polyribosomes. Our findings have a number of similarities with reports on 5’ terminal post-transcriptional control elements in spleen necrosis virus and human foamy virus RNA and support the model that divergent retroviruses share 5’ terminal RNA elements that interact with host proteins to program retroviral RNA for productive cytoplasmic expression.

**INTRODUCTION**

Retroviruses need to subvert typical cellular post-transcriptional control mechanisms to produce progeny virions. Typical pre-mRNAs are assembled in ribonucleoprotein (mRNP) complexes during transcription and RNA maturation that qualify processed transcripts for nuclear export and efficient cytoplasmic expression (35,85,92,104). By contrast, retroviral pre-mRNA recruits viral or cellular post-transcriptional modulators that activate efficient nuclear export and cytoplasmic expression despite lack of intron removal (15,36,88,158). Once in the cytoplasm, the unspliced viral transcript exhibits dual function as mRNA template for translation of Gag precursor protein and Gag-Pol polyprotein and as genomic RNA that is packaged into progeny virions (21). The RNA packaging signal is a series of RNA structural motifs within the 5’ untranslated region (UTR) that are recognized by the Gag nucleocapsid protein, which directs assembly of the unspliced RNA into progeny virions (12,128). Structured 5’ UTRs typically inhibit efficient translation by steric hindrance of ribosome scanning from the 5’ cap to the proximal start codon (55,101,124). Mutational analysis of structural motifs in the human immunodeficiency virus
type 1 (HIV-1) 5’ UTR has verified that they inhibit efficient translation (53,111,121). Thus, the unspliced viral RNA subverts typical barriers to both nuclear export and efficient translation to achieve productive cytoplasmic expression of viral structural and enzymatic proteins.

HIV-1 is a complex retrovirus that encodes the viral post-transcriptional regulatory protein Rev. Rev interacts with newly synthesized viral RNA (68) at the Rev responsive element (RRE) present in distal intronic sequences to activate nuclear export despite lack of intron removal (28,59,60,98,163). Rev connects RRE-containing RNA to the CRM1/exportin 1 nuclear export receptor, which is typically utilized by 5s rRNA and shuttling proteins that contain a leucine-rich nuclear export sequence (NES) (50,115). Genetically simpler retroviruses lack a viral Rev protein. However, the type-D Mason-Pfizer monkey virus (MPMV) and simian retrovirus type 1 contain a functional homolog of RRE, which is designated the constitutive transport element (CTE). The CTE is a redundant stem-loop structure that is positioned in the 3’ UTR and facilitates nuclear export of intron-containing RNA in transfected cells (19,46,47,135,155). CTE facilitates nuclear export by interaction with Tap and cofactor NXT1, which have been implicated as global modulators of the nuclear export of fully processed cellular mRNAs (17,88,158). Recently, studies of two other retroviruses have identified 5’ proximal RNA sequences encoded by the long terminal repeat (LTR) that facilitate cytoplasmic expression of intron-containing viral RNA, but do not function solely as nuclear export elements.
The spleen necrosis virus (SNV) 5’ LTR contains a positive post-transcriptional control element (PCE) that facilitates Rex/Rex responsive element-independent expression of bovine leukemia virus (BLV) structural and enzymatic proteins in studies of hybrid SNV-BLV genomes (16). Analysis of hybrid SNV-HIV-1 reporter plasmids indicates that the SNV 5’ LTR also facilitates Rev/RRE-independent expression of the intron-containing HIV-1 gag-pol reporter RNA and that the RU5 region, which corresponds to the 5’ RNA terminus, is sufficient for activity (22). Quantitative RNA and protein analysis demonstrate that low levels of gag-pol reporter RNA accumulate in the cytoplasm in the presence and absence of SNV RU5, but is not translated unless RU5 is present. SNV RU5 functions in an orientation- and position-dependent manner to facilitate the cytoplasmic expression of HIV-1 gag-pol RNA and also augment the cytoplasmic expression of nonviral luciferase (luc) RNA (22,130). Ribosomal profile analysis indicates that SNV RU5 increases the subcellular localization of these reporter RNAs with polyribosomes. Reporter gene assays with bicistronic reporter RNAs eliminate the possibility that SNV RU5 is an internal ribosome entry site and indicate that SNV RU5 enhances translational initiation by a distinct mechanism (130). Results of RNA transfection and conventional reporter gene assays demonstrate that nuclear interactions are necessary for stimulation of cytoplasmic expression by SNV RU5 (40). The nuclear export pathway of SNV RU5–containing RNA is distinct from the leucine-rich NES-dependent CRM1 pathway accessed by Rev/RRE. However, when Rev is tethered by the RRE to a SNV RU5-containing RNA,
Rev/RRE act dominantly to sequester the transcript to the CRM1 nuclear export pathway and eliminate translational enhancement by SNV RU5. The data support the model that Rev/RRE act upstream of SNV RU5 or disrupt interaction between SNV RU5 and nuclear post-transcriptional modulators that program SNV RU5-containing RNAs for efficient cytoplasmic expression.

Similar to SNV RU5, the R region in the human foamy virus (HFV) 5' LTR has been shown to confer productive cytoplasmic expression of intron-containing viral RNA (134). HFV R functions in an orientation- and position-dependent manner and is necessary and sufficient for detectable HFV Gag and Pol protein synthesis. HFV R also has minimal effect on the cytoplasmic accumulation of HFV unspliced *gag* RNA, but is necessary for detectable Gag production (134). Similar levels of HFV *gag* RNA accumulate in the cytoplasm in both the presence and absence of R, however Gag protein is exclusively expressed from the R-containing transcripts. Because HFV R is necessary for HFV Gag translation, McClure and colleagues postulate that R directs the RNA to a proper cytoplasmic environment that is necessary for expression of the unspliced *gag* RNA. The potential positive effect of HFV R on RNA localization with polyribosomes has not been determined.

Studies herein demonstrate that the RU5 region of the MPMV 5' LTR facilitates Rev/RRE-independent expression of HIV-1 *gag-pol* RNA. MPMV RU5 functions independently of any MPMV protein and is active in human and simian, but not avian cell lines, implying that MPMV RU5 recruits cellular post-transcriptional modulators. Quantitative RNA and protein analyses indicate that
MPMV RU5 has minimal effect on cytoplasmic accumulation, but is necessary for cytoplasmic expression of the intron-containing HIV-1 gag-pol reporter RNA and also augments cytoplasmic expression of intronless luc reporter RNA. Ribosome profile analysis on RU5-luc RNA demonstrates that MPMV RU5 increases the cytoplasmic localization of luc RNA with polyribosomes. Taken together, this and previous studies indicate that MPMV and SNV RU5 and HFVR sequences share a common ability to facilitate cytoplasmic expression of intron-containing RNA. We suggest that 5' terminal PCEs are a feature shared by divergent retroviruses to facilitate translation despite lack of intron removal and the presence of a long and highly structured 5' UTR.

MATERIALS AND METHODS

Plasmid construction

The construction of cytomegalovirus immediate early promoter/enhancer (CMV) and 5' SNV LTR HIV-1 gag-pol reporter plasmids was previously described (22). To create the MPMV reporter plasmids, the SNV LTR sequence in pYW100 was replaced with PCR amplification products that contain either the 5' MPMV U3RU5 (nt 1-350) or U3 (MPMV ΔRU5) (nt 1 – 105) from pSHRM (a kind gift of E. Hunter, University of Alabama, Birmingham) at NdeI and BamHI sites. The luc reporter plasmids were constructed by replacing the entire HIV-1 sequence with luc from pGL3 (Promega) at BamHI and Xbal sites. gag sequences of p37M1-4 (a kind gift of B.K. Felber, National
Cancer Institute, Frederick, MD.), which contain inactivated inhibitory sequences (INS-1) (143) were introduced into MPMV to create MPMVM1-4.

**DNA transfection and analysis of protein production**

Triplicate reporter gene assays were performed on protein from $1 \times 10^5$ 293 cells transfected by a CaPO$_4$ protocol (22) or $3 \times 10^5$ COS or QT-6 quail cells transfected by a Lipofectamine (Invitrogen) protocol (22) in three replicate 33-millimeter diameter plates. The cells were harvested 48 h later in phosphate-buffered saline (PBS), centrifuged at 3,000 X g for 3 min and resuspended in 0.2 ml of ice-cold lysis buffer (20mM Tris-HCl [pH 7.4], 150mM NaCl, 2mM EDTA, 1% NP-40). HIV Gag levels were quantified by a Gag enzyme-linked immunosorbent assay (ELISA; Coulter Corp., Miami, Fla.) and normalized to Luc activity from cotransfected pGL3 (Promega). Luc assays were performed with 10 µl of lysate and 100 µl of Luciferase Assay Reagent (Promega) and quantified in a Luminouscount luminometer (Packard, Meriden, Conn.). Dual measurement of the Luciferases expressed from the firefly (*Photinus pyralis*) luc and sea pansy (*Renilla reniformis*) ren genes was performed with the Dual-Luciferase Reporter Assay System (Promega).

**RNA preparation**

RNA was harvested 48 h post-transfection of $1 \times 10^6$ 293 cells in 100-millimeter diameter plates. For preparation of nuclear and cytoplasmic RNA, cells were resuspended in 0.4 ml of hypotonic buffer (10mM HEPES [pH 7.9], 1.5 mM MgCl$_2$, 10mM KCL, 0.5mM dithiothreitol) and placed on ice for 10 min followed by gentle vortexing for 30 s (159). Nuclei were pelleted by
centrifugation at 3,000 X g for 2 min at 4°C. The cytoplasmic supernatant was subjected to a second centrifugation and the clarified supernatant was mixed with 3 volumes of Tri-Reagent LS (Molecular Research Center, Cincinnati, Ohio) and RNA was extracted according to the manufacturer’s protocol. The nuclear fraction was washed once in 0.5 ml of hypotonic buffer and treated with 1 ml of Trizol reagent (Life Technologies, Gaithersburg, MD). All RNA preparations were treated three times with DNase (Promega), phenol-chloroform extracted, chloroform extracted and ethanol precipitated.

For ribosomal RNA profile analysis, 1 X 10^7 293 cells in T150-cm^2 flasks were transfected and treated 48 h later with 100 µg/ml cycloheximide for 15 min at 37°C. The cells were harvested in PBS and the cytoplasmic fraction isolated by the hypotonic buffer protocol. The cytoplasmic supernatant was then layered onto a 10-ml linear gradient of 15 to 45% sucrose in 10 mM HEPES containing 10 mM NaCl, 3 mM CaCl_2, 7 mM MgCl_2 and 1 mM dithiothreitol and centrifuged at 36,000 X g at 4°C for 3.5 h in a Beckman SW41 rotor (141). Gradients were fractionated and the A_{254} was monitored using an ISCO (Lincoln, Nebr.) fractionation system. Each fraction was subjected to phenol-chloroform and chloroform extraction, and the RNA was precipitated with ethanol.

**RNA analysis**

For RNA protection assays (RPA), 15 µg of nuclear and 25 µg of cytoplasmic RNA were analyzed as previously described (22). For northern blot analysis, 10 µg of nuclear and cytoplasmic RNA were separated on 1.2%
agarose gels containing 5% formaldehyde, transferred to Duralon-UV membranes (Stratagene, La Jolla, Calif.) and incubated with either luc or cyclophilin (cp) gene DNA probes. The probes were prepared by a random-primer DNA-labeling system (Gibco-BRL) with gel purified luc or cp PCR products and $[\alpha^{-32}P]dCTP$. The hybridization products were subjected to PhosphorImager (Molecular Dynamics) analysis with ImageQuant version 4.2 (Molecular Dynamics). For ribosomal RNA profile analysis, the entire RNA sample from each fraction was used and the membranes were prepared as described above and incubated with uniformly labeled luc RNA probe synthesized with MAXscript T7 polymerase (Ambion) according to the manufacturer’s instructions. The hybridization products were subjected to quantification by PhosphorImager (Molecular Dynamics) analysis.

**RESULTS**

**MPMV 5’ LTR facilitates Rev/RRE-independent HIV-1 Gag production.**

A collection of HIV-1 gag-pol reporter plasmids was used to investigate the potential positive effect of the 5’ MPMV LTR on post-transcriptional gene expression (Figure 2.1). The reporter plasmids were analyzed for Rev/RRE-independent Gag production by HIV-1 Gag ELISA on cell-associated protein from transfected 293 cells. The MPMV reporter plasmids were compared to two previously characterized reporter plasmids (22). The negative control CMV reporter plasmid has been shown to produce less than detectable levels of Gag, while the SNV 5’ LTR reporter plasmid exhibits Rev/RRE-independent Gag
production. Three independent reporter gene assays were performed in triplicate and the results indicate that Gag is produced from the MPMV and SNV LTR reporter plasmids, but not the negative control (Table 2.1). The data indicate that the MPMV LTR facilitated a ≥100-fold increase in Gag production. For example, Rev/RRE-independent Gag production was 10.3 ± 0.5 ng/ml for MPMV, 37.9 ± 10.8 ng/ml for SNV and less than 0.1 ng/ml for CMV. Deletion of RU5 from the MPMV LTR gag-pol reporter plasmid (MPMV ΔRU5) eliminated Rev/RRE-independent Gag production (Table 2.1). These results demonstrate that MPMV RU5 is necessary for Rev/RRE-independent Gag production.

Figure 2.1: Summary of the structures of the gag and luc reporter plasmids. Labeled light gray U3, R and U5 regions of the MPMV LTR; labeled white U3, R and U5 regions of the SNV LTR; labeled 5'-terminal oval, CMV immediate early (IE) promoter-enhancer; labeled dark gray rectangle, HIV gag-pol coding region; labeled 5' and 3' ss, splice site; labeled white rectangle, luc coding region; 3' terminal oval labeled p(A), polyadenylation signal.
<table>
<thead>
<tr>
<th>Expt</th>
<th>plasmid</th>
<th>Gag (ng/ml) (mean ± SD)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>MPMV</td>
<td>10.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>SNV</td>
<td>37.9 ± 10.8</td>
</tr>
<tr>
<td></td>
<td>CMV</td>
<td>&lt;MD</td>
</tr>
<tr>
<td></td>
<td>MPMVΔRU5</td>
<td>&lt;MD</td>
</tr>
<tr>
<td>2</td>
<td>MPMV</td>
<td>24.4 ± 11.0</td>
</tr>
<tr>
<td></td>
<td>SNV</td>
<td>67.0 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>CMV</td>
<td>&lt;MD</td>
</tr>
<tr>
<td></td>
<td>MPMVΔRU5</td>
<td>&lt;MD</td>
</tr>
<tr>
<td>3</td>
<td>MPMVΔRU5</td>
<td>11.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>SNV</td>
<td>19.7 ± 2.2</td>
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<tr>
<td></td>
<td>CMV</td>
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<td></td>
<td>MPMVΔRU5</td>
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Table 2.1: Comparison of Gag protein production. Forty-eight hours posttransfection cell-associated Gag levels were analyzed from triplicate transfections of 293 cells. HIV-1 Gag levels were measured by ELISA and normalized to cotransfected Luc values. <MD, less than minimum detectable (≤0.1 ng/ml).
To determine the effectiveness of the MPMV LTR we compared its level of Gag production to that produced in response to Rev/RRE or CTE. The 293 cells were transfected with equivalent amounts of previously characterized reporter plasmids: pSVgagpol; pSVgagpol-rre in the presence and absence of pRev; and pSVgagpol-MPMV, which contains the MPMV CTE (19). As expected, the negative control pSVgagpol and pSVgagpol-rre reporter plasmids produced less than detectable levels of Gag (≤0.1 ng/ml). Cotransfection of pRev with pSVgagpol-rre or introduction of MPMV CTE to pSVgagpol (pSVgagpol-MPMV) yielded Gag levels of 57.2 ± 2.9 ng/ml and 31.8 ± 5.4 ng/ml, respectively. The data indicate that HIV-1 Gag production facilitated by MPMV RU5 is comparable in magnitude to that produced in response to Rev/RRE or MPMV CTE.

Next we analyzed whether Rev/RRE-independent Gag production by MPMV RU5 is augmented by mutation of INS-1. The cis-acting INS sequences interact with host cell factors and confer instability of HIV-1 RNA that is derepressed by Rev association with RRE or by point mutation (29,96,143,144). Consistent with published results, two independent reporter gene assays performed in triplicate demonstrated that INS-1 mutation increased Gag production from the previously characterized p37 HIV-1 control plasmid in 293 cells (130,143). For example, Gag production from p37 increased from less than 0.1 ng/ml to 27.1 ± 6.4 ng/ml upon mutation of INS-1 (p37M1-4). By contrast, INS-1 mutation had no significant effect on Gag production from the MPMV reporter plasmid (26.2 ± 2.6 and 35.8 ± 1.3 ng/ml for MPMV and MPMV
with INS-1 mutation (MPMVM1-4), respectively. These results indicate that MPMV RU5 facilitates \textit{gag} expression independently of INS-1.

\textbf{Cell type-dependent host proteins modulate MPMV RU5 activity.}

To address potential cell type-dependent differences in activity, we evaluated the MPMV LTR \textit{gag-pol} reporter plasmids in monkey COS cells, which are fully permissive for MPMV replication (46) and quail QT-6 cells, which are deficient for post-transcriptional expression of MPMV CTE reporter RNA (71). Similar to the results in 293 cells, COS cells supported Rev/RRE-independent Gag production from the MPMV LTR reporter plasmid (31.2 ± 4.2 ng/ml), but exhibited less than minimum detectable Gag (≤0.1 ng/ml) from the MPMV \textit{\Delta}RU5 reporter plasmid.

By contrast, the MPMV LTR reporter plasmid did not exhibit Rev/RRE-independent Gag production in quail QT-6 cells. Consistent with previous work in quail cells (71), CTE-containing reporter pSVgagpol-MPMV also lacked Gag production, whereas pSVgagpol-rre plus pRev exhibited Gag production (data not shown). RNA analysis was used to determine whether or not the MPMV LTR is transcriptionally active in QT-6 cells. RPA was performed on total cellular RNA from the transfected cells with a uniformly labeled antisense RNA probe that spans the HIV-1 5' UTR and detects both unspliced and spliced transcripts (Figure 2.2A). RPA analysis detected ample spliced transcript and low but detectable unspliced \textit{gag} transcript (Figure 2.2B). The data indicate that the lack of Rev/RRE-independent Gag production in QT-6 cells is not
attributable to lack of RNA synthesis. Taken together, the data indicate that MPMV RU5 facilitates Rev/RRE-independent Gag production in conjunction with host cell proteins present in human and monkey, but not quail cells.

Figure 2.2: RNase protection assay (RPA) of total cellular RNA from transfected QT-6 quail cells verifies reporter RNA synthesis. (A) Relationship between the gag-pol reporter plasmid, the uniformly labeled antisense run-off HIV-1 5’ UTR RNA probe and protected unspliced and spliced transcripts with sizes indicated. (B) Forty-eight hours post-transfection total RNA was isolated, DNase treated, 20 µg was subjected to RPA with uniformly labeled antisense HIV-1 5’ UTR and gapdh RNA probes and PAGE. Labels indicate the reporter plasmid, RNA preparation and protected transcripts.
**MPMV RU5 positively affects cytoplasmic expression of HIV-1 gag-pol RNA.**

Quantitative RPAs were used to determine the effect of MPMV RU5 on RNA processing and cytoplasmic accumulation. To assess the quality of our nuclear and cytoplasmic RNA fractionation, RNA was analyzed from 293 cells transfected with pSVgagpol-rre in the absence or presence of pRev. The RPA probe is complementary to the pSVgagpol-rre 5’ UTR and detects the unspliced gag RNA as a 290 nt RNase protection product. To assess equal sample loading, we utilized a glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) cellular RNA probe that detects a 433 nt RNase protection product. Appropriate nuclear and cytoplasmic fractionation of the gag RNA is indicated by the observation of unspliced gag RNA from pSVgagpol-rre in the cytoplasm solely in the presence of Rev (Figure 2.3A).

Nuclear and cytoplasmic RNA was harvested from 293 cells transfected with MPMV or MPMV ΔRU5 *gag-pol* reporter plasmids. We detected the expected 303 nt and 146 nt RNase protection products of the unspliced and spliced reporter transcripts, respectively (Figure 2.3B). As above, equal sample loading was confirmed by *gapdh* hybridization. The results consistently demonstrate that deletion of MPMV RU5 decreased the abundance of the spliced transcript (Table 2.2). Notably, deletion of MPMV RU5 had minimal effect on the overall steady state level or cytoplasmic accumulation of unspliced gag RNA, despite elimination of detectable Gag protein synthesis. The data indicate that MPMV RU5 effects RNA processing and increases the splicing
efficiency of the reporter transcript. Together the RPA and protein analysis results demonstrate that RU5 is necessary for productive cytoplasmic expression of the HIV-1 gag-pol reporter RNA.

Figure 2.3: RNase protection assay (RPA) of nuclear and cytoplasmic RNA from transfected 293 cells reveals that deletion of MPMV RU5 does not reduce steady state unspliced gag RNA level, but does reduce spliced RNA level. Forty-eight hours post-transfection RNAs were isolated, DNase treated, nuclear (15 µg) and cytoplasmic (25 µg) RNAs were subjected to RPA with uniformly labeled antisense 5′ UTR and gapdh RNA probes and PAGE. Labels indicate the reporter plasmid, RNA preparation and protected transcript. (A) Analysis of pSVgagpol-rre reporter RNA in the presence or absence of pRev. (B) Analysis of MPMV LTR and MPMV ΔRU5 reporter RNA.
Table 2.2: Analysis of HIV-1 \textit{gag} reporter RNA. Transfected 293 cells were analyzed for Gag production and \textit{gag} RNA expression. Nuclear and cytoplasmic RNA was extracted 48 hours posttransfection, treated with DNase, subjected to RPA with HIV-1 5' UTR and \textit{gapdh} probes and quantified by PhosphorImager analysis. The data are standardized to \textit{gapdh} RNA levels and cotransfected Luc values. Splicing efficiency (SE) is presented as the ratio of spliced RNA to unspliced RNA. Cytoplasmic accumulation (CA) is presented as the ratio of cytoplasmic RNA to nuclear RNA. Cytoplasmic expression (CE) is presented as the ratio of Gag protein to unspliced cytoplasmic RNA. Cell-associated Gag levels in nanograms per milliliter are presented normalized to cotransfected Luc values. RNA level relative to unspliced MPMV \textit{gag} reporter RNA are indicated within parentheses. <MD, less than minimum detectable.

\textbf{MPMV RU5 augments cytoplasmic expression of nonviral luc RNA.}

We used \textit{luc} reporter RNA to evaluate whether or not MPMV RU5 also augments expression of a nonviral reporter RNA. The MPMV 5' LTR was inserted upstream of the \textit{luc} open reading frame of pGL3, which is a constitutively expressed, intronless cDNA. MPMV \textit{ΔRU5 luc} reporter RNA exhibited robust and constitutive expression independently of any retroviral post-transcriptional control element (Table 2.3). The addition of MPMV RU5 increased Luc production 3 to 4-fold in 293 cells and also increased Luc production in COS cells 2 to 3-fold. As expected, robust levels of cytoplasmic \textit{luc} RNA are detected from MPMV \textit{ΔRU5} by Northern blot analysis (Figure 2.4 and Table 2.3). Addition of RU5 had minimal effect on the steady state level of \textit{luc} RNA in the nucleus or the cytoplasm. Therefore, the increase in Luc
production in response to RU5 is not attributable to increased steady state level or cytoplasmic accumulation of luc reporter RNA.

These results indicate that RU5 exerts a positive effect on the cytoplasmic expression of both nonviral luc and HIV-1 gag-pol reporter RNAs. Notably, the magnitude positive effect of RU5 is 25 to 30-fold greater in HIV-1 gag-pol RNA. We postulate that this significant difference is attributable to structural differences between the RNAs. Luc protein is produced from a cDNA that lacks a complex 5’ UTR, intronic and instability sequences that are present in HIV-1 gag-pol RNA. In subsequent experiments we sought to directly address the role of SNV RU5 in polysome loading. We chose to study the luc reporter RNA in order to evaluate translational efficiency independently of the complicating variables presented in HIV-1 gag-pol reporter RNA.

Figure 2.4: Northern blot of nuclear and cytoplasmic RNA from transfected 293 cells reveals that MPMV RU5 has minimal effect on steady state luc RNA level or cytoplasmic accumulation. Forty-eight hours post-transfection RNAs were isolated, DNase treated, 10 µg aliquots were subjected to electrophoresis on a 1.2% agarose gel with 5% formaldehyde, transferred to a Duralon-UV membrane and hybridized to α-32P-labeled DNA probes complementary to luc and cp. The blots were subjected to PhosphorImager analysis. Labels indicate the reporter plasmid and RNA fraction.
Table 2.3: Analysis of nonviral luc RNA. Duplicate transfections of 293 cells were analyzed for Luc production and luc RNA expression. Nuclear and cytoplasmic RNA was extracted 48 hours posttransfection, treated with DNase, subjected to Northern blot analysis with luc and cp probes and quantified by PhosphorImager analysis. The data are standardized to cp RNA levels. Cell-associated Luc level (relative light units, $10^3$) are presented normalized to cotransfected Ren values. Cytoplasmic accumulation (CA) is presented as the ratio of cytoplasmic RNA to nuclear RNA. Cytoplasmic expression (CE) is presented as the ratio of Luc protein to unspliced cytoplasmic RNA. RNA level relative to unspliced MPMV ΔRU5 luc reporter RNA are indicated within parentheses. ND, not determined.

**MPMV RU5 augments polyribosome association of luc reporter RNA.**

We performed ribosomal profile analysis to test the hypothesis that MPMV RU5 increased cytoplasmic expression of luc RNA by augmentation of translational efficiency. As expected, similar ribosomal profiles were observed from 293 cells transfected with MPMV or MPMV ΔRU5 luc reporter plasmids or mock-transfected (Fig 2.5A, representative profile from two replicate experiments). RNA was harvested from fractionated 15% to 45% sucrose gradients and subjected to Northern blot analysis with a uniformly-labeled antisense luc RNA probe. As expected, luc RNA was not detected in the fractions from mock-transfected cells (data not shown). The cytoplasm of cells
transfected with the MPMV or MPMV ΔRU5 luc reporter plasmids exhibited luc transcripts in several fractions (Figure 2.5B). PhosphorImager analysis was used to quantify the abundance of luc transcript across the gradients (Figure 2.5C). In agreement with the results of Figure 2.4, MPMV RU5 did not significantly increase the overall abundance of luc RNA in the cytoplasm. The combined luc RNA signal across the entire cytoplasmic gradient for MPMV RU5 was 6 X 10⁶ units and for MPMV ΔRU5 was 8 X 10⁶ units. In the absence of RU5, low but detectable levels of luc RNA are observed on light and heavy polyribosomes, consistent with constitutive expression of Luc protein. The presence of RU5 increased luc RNA levels on the light and heavy polyribosome fractions, indicating increased translational efficiency. MPMV RU5 increased this signal intensity by 4.4-fold, which is proportional to the increase observed in Luc protein production. The results demonstrate that MPMV RU5 increased Luc production by stimulation of ribosome loading onto luc mRNA.
Figure 2.5: Northern blot of luc reporter RNAs across the ribosomal profile reveals that MPMV RU5 redistributes cytoplasmic RNA to the light and heavy polyribosomes. (A) Representative ribosomal profile of transfected 293 cells. Cytoplasmic extracts were subjected to sucrose gradient ultracentrifugation, fractionation, and spectrophotometry ($A_{254}$). Positions of the 60S ribosomal subunit, 80S monosomes, light polyribosomes (LP; two to three ribosomes) and heavy polyribosomes (HP; four to six ribosomes) are indicated. The arrow indicates the direction of the gradient. (B) Each fraction was subjected to electrophoresis on a 1.2% agarose gel with 5% formaldehyde, transferred to a Duralon-UV membrane and hybridized to $\alpha$-P-labeled RNA probe complementary to luc. RNA levels were quantified by PhosphorImager analysis. Labels indicate the reporter plasmid and the fraction of the ribosomal profile. (C) Quantification of luc RNA levels across the ribosomal profile tallied and expressed as percentages of total PhosphorImager units. ◇, MPMV luc; ■, MPMV luc ΔRU5.
DISCUSSION

**MPMV RU5 facilitates Rev/RRE-independent cytoplasmic expression of HIV-1 gag-pol reporter RNA.**

Our results of transient transfection assays indicate that the RU5 region of the MPMV 5' LTR functions in a cell type-dependent manner and independently of INS-1 to facilitate Rev/RRE-independent expression of intron-containing HIV-1 *gag-pol* RNA. Similar to results with SNV RU5 (22) and HFV R (134), MPMV RU5 has little effect on cytoplasmic accumulation of *gag* RNA, but is absolutely necessary for detectable Gag protein production.

**MPMV RU5 redistributes cytoplasmic luc reporter RNA with polyribosomes.**

MPMV RU5 also exerts a positive effect on cytoplasmic expression of nonviral *luc* reporter RNA. Similar to the trends in HIV-1 *gag-pol* reporter RNA, MPMV RU5 has little effect on the steady state level or cytoplasmic accumulation of *luc* mRNA, but increases the production of Luc protein. Reminiscent of previous results with SNV RU5 (130), we observe that MPMV RU5 increases polyribosome loading of *luc* reporter RNA by 4.4-fold. The results imply that at least one function of MPMV RU5 is to increase translation initiation. Ribosome sedimentation analysis has previously verified that SNV RU5 increases ribosome association of HIV-1 *gag-pol* reporter RNA (22). We expect that ribosome sedimentation experiments with MPMV RU5 and HFV R will reveal a comparable result. The observation that MPMV RU5 produces a significantly greater effect in HIV-1 *gag-pol* RNA than in *luc* RNA (25 to 30-fold)
implies that RU5 exerts functional activities in addition to increasing translational efficiency.

5’ RNA termini of divergent retroviruses facilitate cytoplasmic expression.

The 5’ terminal PCEs identified share a number of similarities in addition to their absolute requirement for Gag production from unspliced viral RNA. First, deletion of MPMV or SNV RU5 (22) does not reduce steady state level or cytoplasmic accumulation of intron-containing reporter RNA. A speculative explanation for this intriguing observation is that RNP components are recruited by U3 promoter elements that facilitate cytoplasmic accumulation of the unspliced viral RNA. Previous identification of promoter-dependent recruitment of RNA splicing (32) and polyadenylation factors (41), SF2/ASF and CPSF, respectively, validates the notion that RNP factors recruited by the promoter to the pre-mRNA can modulate post-transcriptional steps of gene expression.

Another similarity between these PCEs is that MPMV RU5, SNV RU5 and HFV R increase RNA splicing efficiency, which implies that PCE activity involves recognition of splicing signals. Results with luc cDNA indicate that intron recognition is not necessary for translational enhancement by MPMV or SNV RU5. The significantly greater level of MPMV and SNV RU5 activity in HIV-1 gag-pol RNA, may be attributable to overcoming the repressive effect of lack of intron removal on post-transcriptional gene expression. Functional linkage between the process of mammalian pre-mRNA splicing and translational efficiency has been observed in the Xenopus oocyte system,
wherein the translational efficiency of reporter RNA is potently affected by the process of splicing of the β-globin intron (104).

The functional activity of these PCEs is distinct from the post-transcriptional activity identified in the R region of MuLV. While MPMV and SNV RU5 and HFV R exert little effect on the level of cytoplasmic unspliced RNA, deletion of MuLV R reduces the level of cytoplasmic unspliced RNA by a factor of 5 (157). Secondly, the positive effect on RNA splicing efficiency is not observed for MuLV R; deletion or mutation of MuLV R produces little change in the level of spliced RNA. Furthermore, in unpublished experiments, we observed that MuLV RU5 is not sufficient to confer Rev/RRE-independent expression of the HIV-1 gag-pol reporter RNA (Hull and Boris-Lawrie, unpublished findings). These results indicate that the functional activity of the MPMV, SNV and HFV PCEs is not conserved in MuLV. We speculate that the 5' PCE activity in these three divergent retroviruses provides a shared mechanism to overcome barriers to efficient cytoplasmic expression of unspliced viral RNA. We propose that MPMV PCE recruits particular RNP components to the pre-mRNA that programs their functional interaction with ribosomes and efficient cytoplasmic expression.
CHAPTER 3

COMBINATION OF 5’ AND 3’ RETROVIRAL POSTTRANSCRIPTIONAL CONTROL ELEMENTS SYNERGISTICALLY AUGMENTS HIV-1 GAG PRODUCTION IN COS, BUT NOT 293 CELLS

ABSTRACT

Typical cellular pre-mRNAs are assembled in ribonucleoprotein (RNP) complexes that couple the processes of RNA splicing, nuclear export, and translation. Retroviral pre-mRNAs use cis-acting RNA elements to recruit specialized RNP complexes that facilitate nuclear export and translation independently of RNA splicing. In this study, we hypothesized that combination of the Mason-Pfizer monkey virus (MPMV) constitutive transport element (CTE) and spleen necrosis virus (SNV) or MPMV RU5 translational enhancer on unspliced HIV-1 gag-pol reporter RNA would synergistically augment Gag production. Results of transient transfection assays demonstrate that MPMV CTE functions compatibly with SNV and MPMV RU5 to synergistically augment HIV-1 Gag production in monkey COS, but not human 293 cells. RNase protection assays revealed a comparable increase in the nuclear export of SNV RU5gag RNA by CTE in both COS and 293 cells, even though Gag protein production is solely augmented in COS cells. Gag augmentation in 293 cells was rescued by overexpression of CTE-interactive cellular proteins, Tap and NXT1. However, this result was not attributable to increased nuclear export of
the reporter RNA and instead correlated with increased expression of the cytoplasmic transcripts. Our results in 293 cells establish that increased nuclear export of SNV RU5gag RNA by CTE is not sufficient to increase Gag protein production and imply that Tap and NXT1 mediate a necessary modification of the RNP architecture that licenses productive translation of cytoplasmic RU5gagCTE RNA.

INTRODUCTION

For typical cellular mRNAs, the removal of intronic sequences from the pre-mRNA is coupled to nuclear export (92,93,165). As a consequence of intron removal, a multi-protein exon junction complex (EJC) is deposited near the 5’ exon-exon junction (84,85). The EJC facilitates interaction with the nuclear export factor Tap and essential cofactor NXT1, which translocate fully processed mRNAs across the nuclear pore (83). By contrast, retroviral pre-mRNA can achieve nuclear export independently of intron removal. Retroviruses utilize the unspliced version of pre-mRNA as a template for synthesis of the Gag and Pol structural and enzymatic proteins and also as genomic RNA that is packaged into progeny virions (21). To facilitate nuclear export of viral unspliced pre-mRNA, Mason-Pfizer monkey virus (MPMV) and simian retrovirus type 1 directly recruit the cellular export factors Tap and NXT1. These proteins are recruited to a cis-acting RNA element located in the 3’ untranslated region (UTR) that is termed the constitutive transport element (CTE) (18,56,70).
The CTE was initially identified by the ability to facilitate nuclear export of unspliced HIV-1 \textit{gag-pol} reporter RNA in transiently transfected monkey COS cells (19,167). Subsequently, human Tap was shown to be necessary and sufficient for CTE-mediated RNA export in a non-permissive quail cell line (71). Binding assays demonstrate that NXT1 enhances Tap binding to a variety of nucleoporins, including CAN/Nup214, Nup153, Nup98, p62, and CG1 (7,88,158). Furthermore, overexpression of NXT1 augments expression of reporter RNA in the context of a Tap-Rev responsive element (RRE) tethering experiment in mammalian cells (58).

The process of splicing is also coupled to translational control (78,83,104). For instance, several proteins from the EJC remain associated with the mRNP complex upon nuclear export and mark mRNAs that contain a premature translation termination codon for nonsense-mediated decay (78,83). Additionally, experiments in \textit{Xenopus} oocytes revealed that splicing of an intron located 5' of the open reading frame stimulates translational efficiency compared to an intronless reporter RNA (104). In contrast, splicing of an intron located 3' of the open reading frame represses translational efficiency compared to an intronless reporter RNA. Wolffe and colleagues speculate that the position-dependent recognition of an intron recruits a particular RNP complex that determines the translational fate of mRNA in the cytoplasm.

Retroviruses are distinct in their requirement for efficient translation of unspliced pre-mRNA. In addition to lack of intron removal, the retroviral 5' UTR contains a series of structured RNA motifs that inhibit efficient translation but
are necessary for various steps in viral replication (53,111,121,124). Divergent retroviruses contain 5’ posttranscriptional control elements (PCEs) encoded by the long terminal repeat (LTR) that enhance translation of unspliced viral RNA (22,67,134). The 5’ proximal RNA elements encoded by the RU5 sequence of spleen necrosis virus (SNV) and MPMV enhance translational efficiency of HIV-1 gag-pol and nonviral luc reporter RNAs (22,67,130). Ribosomal sedimentation and ribosome profile analyses have established that SNV and MPMV RU5 enhance translation by augmentation of ribosome loading (22,67,130). Quantitative RNA and protein analyses demonstrate that deletion of SNV or MPMV RU5 reduces Gag and Luc production, but has minimal effect on steady state level or cytoplasmic accumulation of the gag-pol and luc reporter RNAs. The results indicate that RU5-interactive host factor(s) program the cytoplasmic transcript for productive interaction with the translational machinery. RNA transfection assays and competition experiments with HIV Rev/RRE indicate that nuclear interactions are necessary for translational enhancement by SNV RU5 (40). The nuclear export pathway of SNV RU5 RNA is distinct from the CRM-1 nuclear export pathway utilized by HIV Rev/RRE (40). However, the contribution of Tap and NXT1 to the functional activity of SNV RU5 remains an outstanding issue.

We sought to test the hypothesis that SNV RU5 and MPMV RU5 function compatibly with MPMV CTE to synergistically augment protein production from an unspliced retroviral RNA. We predicted that augmentation of nuclear export by MPMV CTE, in conjunction with Tap and NXT1, would increase cytoplasmic
RNA available for translational enhancement by SNV or MPMV RU5. Secondly, we asked whether Tap and NXT1 stimulate the activity of SNV or MPMV RU5 independently of CTE. Results of transient transfection assays demonstrate that combination of SNV or MPMV RU5 and MPMV CTE synergistically augmented Gag production in COS, but not 293 cells. RNase protection assays revealed that CTE produced a similar increase in cytoplasmic accumulation of SNV RU5\textit{gag} reporter RNA in both COS and 293 cells, even though Gag production was not augmented in 293 cells. Overexpression of cellular CTE-interactive proteins Tap and NXT1 in 293 cells was sufficient to rescue Gag augmentation from SNV and MPMV RU5\textit{gag}CTE reporter RNA, but did not stimulate the reporter RNA that lacks CTE. The rescue observed for SNV RU5\textit{gag}CTE RNA is not attributable to increased cytoplasmic accumulation of the reporter RNA but to increased cytoplasmic expression of the RNA. The results imply that host proteins define the fate of SNV RU5\textit{gag}CTE cytoplasmic expression.

MATERIALS AND METHODS

\textit{Plasmid construction}

The construction of the SNV U3, SNV U3RU5, SNV U3RU5 – RRE, CMV, CMV RU5 and MPMV U3RU5 reporter plasmids has been described previously (22,40,67). To create the CTE-containing reporter plasmids, the MPMV CTE sequence was amplified by PCR from pSHRM-1 (a kind gift of E.
Hunter, University of Alabama, Birmingham) and ligated into a unique Sal I site in the 3' UTR.

**DNA transfection and reporter protein analysis**

Triplicate reporter gene assays were performed on protein from $1 \times 10^5$ human 293 or $3 \times 10^5$ monkey COS cells transfected with 2 µg of reporter plasmid by a CaPO$_4$ or a lipofectamine (Invitrogen) protocol, respectively (67). Overexpression and tethering experiments were performed with $2.5 \times 10^5$ 293 or $5.0 \times 10^5$ COS cells, 5 µg of reporter plasmid, 5 µg of pTap or pRevM10Tap and 5 µg of pNXT1 or mutant NXT1 (pmNXT1) (N48K, N50K) (kind gifts of M.-L. Hammarskjöld, University of Virginia, Charlottesville) (58). The cells were harvested 48 h post-transfection in phosphate-buffered saline (PBS), centrifuged at 3,000 x g for 3 min and resuspended in ice-cold lysis buffer (20mM Tris-HCL [pH 7.4], 150 mM NaCl, 2mM EDTA, 1% NP-40). HIV Gag levels were quantified by a Gag enzyme-linked immunosorbent assay (ELISA; Coulter Corp., Miami, Fla.) and normalized to Luc activity from cotransfected pGL3 (Promega). Luc assays were performed with 10 µl of lysate and 100 µl of Luciferase Assay Reagent (Promega) and quantified in a Lumicount luminometer (Packard, Meriden, Conn.). The student t-test was performed to determine whether the observed differences in Gag production were statistically significant with a $p$ value of $\leq 0.05$.

**RNA analysis**

RNA protection assays (RPA) were performed with 10 µg of nuclear and 25 µg of cytoplasmic RNA separated by a hypotonic buffer protocol (67). The
RNAs were analyzed with uniformly labeled antisense RNA probes that were prepared by run-off transcription of HIV 5' UTR and gapdh plasmids as previously described (22).

RESULTS

**Synergistic augmentation of Gag production by MPMV CTE and SNV RU5 in COS cells.**

To evaluate potential synergism between MPMV CTE and SNV RU5, we analyzed HIV-1 gag-pol reporter plasmids (Figure 3.1) in transient transfection assays. HIV-1 Gag production was quantified by Gag ELISA on cell-associated protein in triplicate transfections of COS cells. The reference plasmid, SNV U3, lacks both MPMV CTE and SNV RU5 and has been previously shown to support low, but detectable Gag production (22). We evaluated Gag production in response to MPMV CTE and SNV RU5 individually and in combination. As shown in two representative assays, CTE augmented Gag production 11 to 16-fold (compare SNV U3 and SNV U3 - CTE), while SNV RU5 augmented Gag production 3 to 4-fold (compare SNV U3 and SNV U3RU5) (Table 3.1). The combination of CTE and SNV RU5 produced an overall increase of 27 to 37-fold (compare SNV U3 and SNV U3RU5 - CTE). The results affirm that MPMV CTE and SNV RU5 individually facilitate Rev/RRE-independent HIV Gag production in COS cells. Furthermore, the combination of MPMV CTE and SNV RU5 produces a synergistic increase in Gag production in COS cells.
Figure 3.1: Summary of the structures of the gag-pol reporter plasmids. 5' terminal labeled white or gray rectangles, U3, R and U5 regions of the SNV LTR or MPMV LTR, respectively; 5'-terminal oval labeled CMV, CMV immediate-early (IE) promoter-enhancer; labeled black rectangle, HIV-1 gag-pol reporter gene; labeled 5' and 3' ss, splice site; oval labeled CTE, MPMV constitutive transport element; rectangle labeled RRE, HIV-1 Rev responsive element; 3' terminal oval labeled p(A), polyadenylation signal.
Comparison of Gag protein production in COS and 293 cells

<table>
<thead>
<tr>
<th>Reporter</th>
<th>Expt</th>
<th>plasmid</th>
<th>COS</th>
<th>293</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>SNV U3</td>
<td>2.6 ± 0.9 (1.0)</td>
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<td></td>
<td>SNV U3 - CTE</td>
<td>29.5 ± 4.4 (11.3)</td>
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<tr>
<td></td>
<td></td>
<td>SNV U3RU5</td>
<td>8.1 ± 1.0 (3.1)</td>
<td>47.0 ± 2.8 (5.0)</td>
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<td></td>
<td>SNV U3RU5 - CTE</td>
<td>70.9 ± 8.3 (27.2)</td>
<td>22.5 ± 3.0 (2.3)</td>
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<td>2</td>
<td>SNV U3</td>
<td>1.9 ± 0.3 (1.0)</td>
<td>9.2 ± 2.5 (1.0)</td>
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<td></td>
<td></td>
<td>SNV U3 - CTE</td>
<td>30.7 ± 8.5 (16.1)</td>
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<tr>
<td></td>
<td></td>
<td>SNV U3RU5</td>
<td>7.5 ± 0.9 (3.9)</td>
<td>48.9 ± 9.7 (5.3)</td>
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<tr>
<td></td>
<td></td>
<td>SNV U3RU5 - CTE</td>
<td>70.4 ± 11.3 (37.0)</td>
<td>24.9 ± 2.9 (2.7)</td>
</tr>
</tbody>
</table>

Table 3.1: Comparison of Gag protein production in COS and 293 cells. Forty-eight hours posttransfection cell-associated Gag levels were analyzed from triplicate transfections of COS and 293 cells. HIV-1 Gag levels were measured by ELISA and normalized to cotransfected Luc values. Gag level relative to SNV U3 reporter plasmid are indicated within parentheses.

**Combination of MPMV CTE and SNV RU5 does not augment Gag production in 293 cells.**

We sought to determine whether or not the combination of MPMV CTE and SNV RU5 also augments Gag production in 293 cells given that previous characterization of SNV and MPMV RU5 was performed in this cell line (22,67,130). Consistent with previous results, SNV RU5 augmented Gag production 5-fold in 293 cells (compare SNV U3 and SNV U3RU5) (Table 3.1) (22). Unexpectedly, CTE did not augment Gag production either in the absence
or presence of SNV RU5. For example, in a representative triplicate transfection, Gag production from SNV U3RU5 decreased from 47.0 ± 2.8 ng/ml to 22.5 ± 3.0 ng/ml in the presence of CTE.

The functional activity of MPMV CTE in the 293 cell transfection assay was verified by analysis of pSVgagpol and pSVgagpolMPMV CTE reporter plasmids that were used in the original identification of CTE in COS cells (19). Our results demonstrate that CTE increased Gag production from less than the minimum detectable (≤0.1 ng/ml) to 13 ± 3 ng/ml in 293 cells (Table 3.2, compare pSVgagpol and pSVgagpolMPMV CTE). Results of the reporter gene assays implied that SNV U3 impeded Gag augmentation from the SNV U3 – CTE and U3RU5 – CTE reporter plasmids in 293 cells. To directly address whether SNV U3 was responsible, this sequence was replaced with the CMV immediate early promoter in four SNV U3-containing reporter plasmids to generate CMV, CMV – CTE, CMV RU5, CMV RU5 – CTE (Figure 3.1). Results of triplicate transient transfection assays demonstrated that CTE augmented Gag production from both CMV derivatives in 293 cells (Table 3.2). The combination of MPMV CTE and SNV RU5 rescued an additive increase in Gag production. The results indicated that the lack of Gag augmentation for the SNV U3 – CTE and SNV U3RU5 – CTE reporter plasmids is attributable to the SNV U3 sequence.

We postulated that the synergistic augmentation of Gag production upon combination of SNV RU5 and MPMV CTE in COS cells is attributable to increased nuclear export of unspliced RU5gag RNA by CTE, which increases
cytoplasmic RNA available for translational enhancement by SNV RU5. By contrast, in 293 cells, the lack of synergy is postulated to be attributable to SNV U3 impeding nuclear export by CTE, which would produce no increase in cytoplasmic RNA available for translational enhancement. An explanation could be that cellular factors recruited to the mRNP complex by SNV U3 may not function in coordination with Tap and NXT1, which are recruited to MPMV CTE.

<table>
<thead>
<tr>
<th>Expt</th>
<th>plasmid</th>
<th>Gag (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pSVgagpol</td>
<td>&lt;MD</td>
</tr>
<tr>
<td></td>
<td>pSVgagpolMPMV CTE</td>
<td>13.0 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>CMV</td>
<td>&lt;MD</td>
</tr>
<tr>
<td></td>
<td>CMV - CTE</td>
<td>12.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>CMV RU5</td>
<td>7.0 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>CMV RU5 - CTE</td>
<td>17.0 ± 2.0</td>
</tr>
<tr>
<td>2</td>
<td>pSVgagpol</td>
<td>&lt;MD</td>
</tr>
<tr>
<td></td>
<td>pSVgagpolMPMV CTE</td>
<td>16.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>CMV</td>
<td>&lt;MD</td>
</tr>
<tr>
<td></td>
<td>CMV - CTE</td>
<td>8.0 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>CMV RU5</td>
<td>5.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>CMV RU5 - CTE</td>
<td>13.0 ± 2.0</td>
</tr>
</tbody>
</table>

Table 3.2: Gag production in response to CTE in 293 cells. Forty-eight hours posttransfection cell-associated Gag levels were analyzed from triplicate transfections of 293 cells. HIV-1 Gag levels were measured by ELISA and normalized to cotransfected Luc values. <MD, less than minimum detectable (≤0.1 ng/ml).
CTE facilitates similar levels of nuclear export of SNV RU5gag RNA in COS and 293 cells.

To address the level of nuclear export of RU5gag reporter RNA in the absence and presence of CTE, quantitative RPAs were performed on nuclear and cytoplasmic RNA. Cells were fractionated by a hypotonic buffer protocol that was used previously to effectively separate Rev-dependent HIV-1 gag-pol reporter RNA into nuclear and cytoplasmic fractions (67). RPAs were performed with a uniformly labeled antisense HIV-1 RNA probe that detects both unspliced and spliced HIV-1 transcripts (Figure 3.2A and 3.2B). The data were quantified and are presented relative to gapdh signal and cotransfected Luc values in Table 3.3. CTE reduced the steady state level of unspliced nuclear RU5gag RNA and produced a corresponding increase in the steady state level of unspliced cytoplasmic RNA in both COS and 293 cells. These results demonstrated that in contrast to our prediction, CTE produced a similar and reproducible 3-fold increase in cytoplasmic accumulation of the RU5gag reporter RNA in both COS and 293 cells, as defined by the ratio of unspliced gag RNA in the cytoplasm to that in the nucleus plus cytoplasm. Surprisingly, comparison of cytoplasmic gag RNA level to Gag protein level indicated that MPMV CTE increased expression of cytoplasmic SNV RU5gag RNA 5-fold in COS cells and reduced cytoplasmic expression by a factor of three in 293 cells. The results demonstrate that increased cytoplasmic accumulation by CTE was manifested in augmented Gag production solely in COS cells and imply that increased nuclear export of SNV RU5gag RNA is not sufficient for augmented
Gag production. This observation may indicate a fundamental difference in the mRNP complex associated with RU5gagCTE RNA that programs the RNA for augmented Gag production in COS, but not 293 cells.

Figure 3.2: RNase protection assay (RPA) of nuclear and cytoplasmic RNA detects comparable increases in cytoplasmic accumulation in response to CTE in COS and 293 cells, despite discordant Gag production. (A) Relationship between the gag-pol reporter plasmid, the uniformly labeled antisense run-off HIV-1 5' UTR RNA probe and protected unspliced and spliced transcripts with sizes indicated. (B) Forty-eight hours post-transfection RNAs were isolated and treated with DNase. Nuclear (15 µg) and cytoplasmic (25 µg) RNAs were subjected to RPA with uniformly labeled antisense HIV-1 5' UTR and gapdh RNA probes and polyacrylamide gel electrophoresis. The RPAs were subjected to PhosphorImager analysis. Labels indicate the reporter plasmid, RNA preparation, protected transcripts and cell line.
<table>
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<tr>
<th>RNA preparation</th>
<th>PhosphorImager units (10^5)</th>
<th>CA</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells Plasmid</td>
<td>Gag unspliced spliced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COS SNV U3RU5</td>
<td>11.0 325.1 438.2 54.7 483.6</td>
<td>0.1 0.5 0.2</td>
<td>(1.0)</td>
</tr>
<tr>
<td>COS SNV U3RU5 – CTE</td>
<td>72.0 145.7 148.9 73.2 147.1</td>
<td>0.3 0.5 1.0</td>
<td>(5.0)</td>
</tr>
<tr>
<td>293 SNV U3RU5</td>
<td>46.0 277.1 675.4 77.5 468.0</td>
<td>0.2 0.4 0.6</td>
<td>(1.0)</td>
</tr>
<tr>
<td>293 SNV U3RU5 – CTE</td>
<td>18.0 103.9 142.2 93.1 143.8</td>
<td>0.5 0.5 0.2</td>
<td>(0.3)</td>
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</table>

Table 3.3: Analysis of HIV-1 gag reporter RNA in COS and 293 cells. Transfected COS and 293 cells were analyzed for Gag production and gag RNA expression. Nuclear and cytoplasmic RNA was extracted 48 hours posttransfection, treated with DNase, subjected to RPA with HIV-1 5’ UTR and gapdh probes and quantified by PhosphorImager analysis. The data are standardized to gapdh RNA levels and cotransfected Luc values. Cytoplasmic accumulation (CA) is presented as the ratio of cytoplasmic RNA relative to nuclear plus cytoplasmic. Cytoplasmic expression (CE) is presented as the ratio of Gag protein to unspliced cytoplasmic RNA. Cell-associated Gag levels in nanograms per milliliter are presented normalized to cotransfected Luc values.

**MPMV U3RU5 also supports Gag augmentation by CTE in COS, but not 293 cells.**

When reporter plasmids were evaluated that combined the MPMV LTR and MPMV CTE (Figure 3.1), similar trends were observed in Gag production. Results of multiple triplicate reporter gene assays in COS cells demonstrated that Gag production from MPMV U3RU5 was augmented 5 to 7-fold in response to the MPMV CTE (Table 3.4). For example, Gag production was 10.0 ± 2.0 ng/ml and 57.0 ± 18.0 ng/ml from MPMV U3RU5 and MPMV U3RU5 – CTE, respectively. Comparable to results with SNV U3RU5 reporter plasmids, no augmentation of Gag production by MPMV CTE was observed in 293 cells. Gag production remained similar in the absence or presence of CTE at 10.0 ± 0.6 ng/ml and 10.0 ± 0.9 ng/ml, respectively. The results indicate that...
SNV and MPMV sequences display functional similarity and share increased Gag production in response to CTE in COS, but not 293 cells.

<table>
<thead>
<tr>
<th>Expt</th>
<th>Reporter</th>
<th>Gag (ng/ml)</th>
<th>COS cells</th>
<th>293 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MPMV U3RU5</td>
<td>10.0 ± 1.8</td>
<td>10.0 ± 0.6 (1.0)</td>
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<tr>
<td></td>
<td>MPMV U3RU5 - CTE</td>
<td>57.0 ± 18.1 (5.7)</td>
<td>10.0 ± 0.9 (1.0)</td>
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<td>2</td>
<td>MPMV U3RU5</td>
<td>4.00 ± 0.8</td>
<td>11.0 ± 1.0 (1.0)</td>
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<tr>
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<td>MPMV U3RU5 - CTE</td>
<td>30.0 ± 6.7</td>
<td>11.0 ± 0.8 (1.0)</td>
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</tbody>
</table>

Table 3.4: Comparison of Gag protein production in COS and 293 cells. Forty-eight hours posttransfection cell-associated Gag levels were analyzed from triplicate transfections of COS and 293 cells. HIV-1 Gag levels were measured by ELISA and normalized to cotransfected Luc values. Gag level relative to MPMV U3RU5 reporter plasmid are indicated within parentheses.

**SNV U3RU5 does not eliminate responsiveness to Tap and NXT1.**

Taken together, the RPA and ELISA data indicate that the increased nuclear export by CTE is not sufficient to increase Gag production from SNV RU5gag reporter RNA in 293 cells. We tested whether or not overexpression of the CTE export factors, Tap and NXT1, can rescue Gag augmentation in 293 cells. We reasoned that since previous studies have shown that tethering Tap-NXT1 heterodimers to HIV-1 gag and nonviral cat reporter RNA increases protein production in 293T cells (58,88,158), Tap and NXT1 may be sufficient to rescue Gag augmentation in 293 cells. Initially, to confirm that Tap and NXT1 are sufficient to augment Gag production in the presence of SNV U3RU5, we targeted these proteins to a HIV-1 gag-pol reporter RNA by tethering.
As a control, Tap was tethered to RNA expressed from the established pSVgagpolRRE reporter plasmid by the RevM10Tap fusion protein. Consistent with published results (58,88), RevM10Tap facilitated Rev-independent Gag production in 293 cells and increased Gag production from less than the minimum detectable ($\leq 0.1\ ng/ml$) to $25 \pm 4\ ng/ml$. Cotransfection of pRevM10Tap and pNXT1 expression plasmids further increased Gag production 3.5-fold in 293 cells (Figure 3.3). Cotransfection of a mutant NXT1 (pMNXT1) that contains mutations N48K and N50K, which diminish affinity for Tap (58), reduced Gag production to baseline levels. These results are consistent with published results that targeting both Tap and NXT1 to gagpol/RRE RNA increases Gag production (58,88,158). We also document that similar results were observed when the control experiment was performed in COS cells. Next we analyzed the effect of RevM10Tap and NXT1 on the SNV U3RU5 - RRE reporter plasmid in 293 cells (Figure 3.1), which was previously shown to exhibit Rev/RRE-independent Gag production and to be Rev-responsive (40). In a representative assay, Gag production from SNV U3RU5 – RRE was increased from $70 \pm 15\ ng/ml$ to $160 \pm 20\ ng/ml$ in response to RevM10Tap. Cotransfection pRevM10Tap and pNXT1 expression plasmids further increased Gag production 9-fold (Figure 3.3). As expected, cotransfection of pmNXT1 reduced Gag production to baseline levels. The results demonstrate that targeting of Tap and NXT1 to SNV RU5gagRRE RNA augmented Gag production. The results indicate that SNV sequences can function compatibly with Tap and NXT1.
Figure 3.3: Tethering RevM10Tap and NXT1 to SNV RU5gagRRE RNA is sufficient to augment Gag production in 293 cells. The SVgagpolRRE and SNV U3RU5 – RRE reporter plasmids were cotransfected in triplicate with pRevM10Tap, pRevM10Tap and pNXT1 and pRevM10Tap and pmNXT1 (N48K, N50K). Cell-associated Gag levels were analyzed by ELISA, normalized to cotransfected Luc values and are presented relative to RevM10Tap.

**Tap and NXT1 rescue Gag augmentation in 293 cells by increasing cytoplasmic expression.**

Transfection assays with Tap and NXT1 expression plasmids were used to directly address whether or not increased availability of Tap and NXT1 could rescue Gag augmentation from SNV and MPMV RU5gagCTE reporter RNA in 293 cells in the absence of targeting. Individual expression of exogenous Tap did not increase Gag production from SNV and MPMV U3RU5 – CTE reporter plasmids, whereas combined expression of Tap and NXT1 increased Gag production by 30 and 17-fold, respectively (Figure 3.4). Cotransfection of pmNXT1 reduced Gag production to near baseline levels. By contrast, in COS cells overexpression of Tap and NXT1 did not augment Gag production further.
than what was observed in response to the CTE. The results demonstrate that exogenously expressed Tap and NXT1 are necessary and sufficient to rescue Gag augmentation from SNV and MPMV RU5gagCTE RNA in 293 cells. By contrast, exogenous expression of Tap and NXT1 is not necessary for Gag augmentation in COS cells.

Figure 3.4: Combined overexpression of Tap and NXT1 is necessary to rescue Gag augmentation in 293 cells. The SNV or MPMV U3RU5 – CTE gag-pol reporter plasmids were cotransfected in triplicate with pTap, pTap and pNXT1, or pTap and pmNXT1 (N48K, N50K). Cell-associated Gag levels were analyzed by ELISA and normalized to cotransfected Luc values.

Quantitative RNA analysis was performed to determine whether or not overexpression of Tap and NXT1 augmented Gag production in 293 cells by increasing the cytoplasmic accumulation of SNV RU5gagCTE RNA. As shown in the representative RPA (Figure 3.5) and quantified in Table 3.5, overexpression of Tap and NXT1 had minimal effect on the absolute level or overall cytoplasmic accumulation of unspliced SNV RU5gagCTE RNA, despite
augmentation of Gag production. The results of quantitative RNA and protein analyses demonstrated that exogenous expression of Tap and NXT1 facilitates increased cytoplasmic expression of SNV RU5gagCTE RNA.

Figure 3.5: RNase protection assay (RPA) of nuclear and cytoplasmic RNA reveals that combined overexpression of Tap and NXT1 does not increase cytoplasmic accumulation of RU5gagCTE RNA in 293 cells. Forty-eight hours post-transfection RNAs were isolated and treated with DNase. Nuclear (15 µg) and cytoplasmic (25 µg) RNAs were subjected to RPA with uniformly labeled antisense HIV-1 5’ UTR and gapdh RNA probes and polyacrylamide gel electrophoresis. The RPA was subjected to PhosphorImager analysis. Labels indicate the reporter plasmid, RNA preparation and protected transcripts.
Table 3.5: Analysis of HIV-1 gag reporter RNA in 293 cells. Transfected 293 cells were analyzed for Gag production and gag RNA expression. Nuclear and cytoplasmic RNA was extracted 48 hours posttransfection, treated with DNase, subjected to RPA with HIV-1 5' UTR and gapdh probes and quantified by PhosphorImager analysis. The data are standardized to gapdh RNA levels and cotransfected Luc values. Cytoplasmic accumulation (CA) is presented as the ratio of cytoplasmic RNA relative to nuclear plus cytoplasmic. Cytoplasmic expression (CE) is presented as the ratio of Gag protein to unspliced cytoplasmic RNA. Cell-associated Gag levels in nanograms per milliliter are presented normalized to cotransfected Luc values.

**SNV RU5 and MPMV RU5 do not confer responsiveness to Tap and NXT1.**

Lastly, the overexpression strategy was used to address whether or not SNV and MPMV RU5 confer responsiveness to Tap and NXT1 independently of CTE. In contrast to results with CTE-containing derivatives in Figure 3.4, exogenous expression of Tap and NXT1 has little effect on Gag production in the absence of the CTE. In a representative experiment, Gag production was 49 ± 10 ng/ml for SNV U3RU5 and 57 ± 11 ng/ml in response to Tap and NXT1 or 34 ± 4 ng/ml for MPMV U3RU5 and 30 ± 0.6 ng/ml in response to Tap and NXT1. The results demonstrated that SNV and MPMV RU5 do not confer responsiveness to Tap and NXT1 and that Tap and NXT1 are not rate-limiting factors for Rev/RRE-independent Gag production by SNV and MPMV RU5. The results also affirm that rescue of Gag augmentation from SNV RU5gagCTE RNA by Tap and NXT1 in 293 cells is attributable to interaction with CTE.
DISCUSSION

This study set out to determine whether or not MPMV CTE functions compatibly with 5’ terminal translational enhancers in the SNV or MPMV 5’ LTR to augment Rev/RRE-independent Gag production. Transient transfection assays demonstrated that the combination of CTE with SNV RU5 or MPMV RU5 on a single HIV-1 gag-pol RNA produced a synergistic increase in Gag production in monkey COS cells. However, the same combinations did not increase Gag production in human 293 cells, despite verification of CTE activity from the SVgagpolMPMV CTE reporter plasmid. Results of RPAs on nuclear and cytoplasmic RNA verified increased cytoplasmic accumulation in response to CTE in both COS and 293 cells. However, the absolute level of cytoplasmic SNV RU5gag RNA displayed only a minor increase in response to MPMV CTE. These results imply that CTE augments cytoplasmic accumulation of unspliced SNV RU5gag RNA and also licenses the cytoplasmic RNA for increased expression. In 293 cells, the defect in this licensing property is rescued by overexpression of Tap and NXT1. Interestingly, previous analysis of the CTE utilized reporter plasmids that require CTE for both nuclear export and protein production, which does not allow for assessment of the effect of CTE on cytoplasmic RNA expression (19,46). In this report, analysis of the SNV RU5gagCTE reporter RNA provided a new tool that allows for the comparison of protein production in the absence and presence of the CTE with similar amounts of RNA in the cytoplasm. This has revealed an unexpected additional property of CTE; augmentation of cytoplasmic RNA expression.
Additionally, transient transfection assays demonstrated that replacement of the CMV promoter with SNV U3 eliminated responsiveness to CTE and indicated that SNV U3 was responsible for lack of Gag augmentation in 293 cells. The results imply that SNV U3 functions upstream of CTE and cotranscriptionally recruits an RNP complex to SNV RU5gagCTE RNA in 293 cells that hinders Gag augmentation. RNA analysis eliminated the possibility that SNV U3 recruited an RNP complex that disrupted increased nuclear export by CTE because CTE produced 3-fold increases in cytoplasmic accumulation of SNV RU5gag RNA in both COS and 293 cells. The data imply that SNV U3 recruits an RNP complex that disrupts CTE-mediated cytoplasmic expression in 293 cells. In *Xenopus* oocytes, differences in cytoplasmic expression were speculated to be controlled by variations in recruitment of RNP components to position dependent exon-exon junctions (104). Excision of an intron positioned 5' of the open reading frame stimulates translational efficiency relative to a 3' intron. An interesting analogy in another step of posttranscriptional control of gene expression is the promoter-dependent cotranscriptional recruitment of splicing factors that dictates disparate patterns of alternative splicing of fibronectin RNA (32,33).

Interestingly, overexpression experiments demonstrated that Tap and NXT1 are necessary and sufficient to rescue augmentation of Gag production from SNV or MPMV RU5gagCTE RNA in 293, but does not further augment Gag production in COS cells. Quantitative RNA and protein analyses revealed that Gag augmentation was not due to increased cytoplasmic accumulation of
unspliced reporter RNA, but increased cytoplasmic expression of the SNV RU5gagCTE RNA. We speculate that overexpression of Tap and NXT1 reorganizes the RNP complex to achieve productive association with necessary factors required for increased cytoplasmic expression by CTE. By contrast, in COS cells, a productive RNP complex is constitutively recruited to RU5gagCTE RNA and reorganization by Tap and NXT1 is not necessary to achieve synergy by SNV RU5 and MPMV CTE.

Whereas the CTE effector proteins Tap and NXT1 are well studied, presently the identity of the SNV RU5 binding protein(s) is not known. The observation that SNV and MPMV RU5 do not individually confer responsiveness to Tap and NXT1 indicates that Tap and NXT1 are not rate-limiting factors for Rev/RRE-independent Gag production by MPMV or SNV RU5. The study of synergy between these functionally distinct retroviral posttranscriptional control elements has provided new awareness of cellular control of productive cytoplasmic expression of retroviral RNA. Unexpectedly, our results establish that augmentation of nuclear export is not sufficient to increase cytoplasmic expression of the SNV RU5gagCTE RNA in 293 cells. Future studies will evaluate the mRNP architecture that programs cytoplasmic transcripts for efficient translation and the regulatory effect of MPMV CTE and SNV RU5-interactive proteins.
CHAPTER 4

SPLEEN NECROSIS VIRUS RU5 MODULATES POSTTRANSCRIPTIONAL CONTROL OF SNV GENOMIC RNA

ABSTRACT

Divergent retroviruses have been shown to utilize 5’ terminal posttranscriptional control elements (PCEs) to augment cytoplasmic expression of heterologous reporter RNAs and are expected to also modulate expression of their homologous unspliced genomic RNA. Previous reports established that the RU5 region within the 5’ long terminal repeat (LTR) of spleen necrosis virus (SNV) facilitates Rev/RRE-independent expression of unspliced HIV-1 gag-pol RNA and augments expression of intronless luc reporter RNA. SNV RU5 increases ribosome loading on these reporter RNAs despite lack of intron removal and the presence of a highly structured 5’ untranslated region. In this study, we assess the effect of SNV RU5 on metabolism of homologous SNV genomic RNA. We developed SNV gag-gfp reporter RNAs to directly monitor Gag protein production in response to SNV RU5 deletion or point mutation. Results of transient transfection assays demonstrate that SNV RU5 augments production of SNV Gag-green fluorescent protein (GFP) fusion protein in human and canine cells. Northern blot analysis of nuclear and cytoplasmic RNA indicates that SNV RU5 increases cytoplasmic accumulation of unspliced SNV
\textit{gag-gfp} RNA and decreases the splicing efficiency of SNV \textit{env} RNA. The increase in unspliced SNV \textit{gag-gfp} RNA in the cytoplasm correlates with increased Gag-GFP protein production. LMB-sensitivity assays demonstrate that SNV RU5-mediated SNV Gag-GFP production is independent of the CRM-1 nuclear export receptor. The data indicate that SNV RU5 increases the availability of unspliced SNV \textit{gag-gfp} RNA in the cytoplasm and plays a necessary role in SNV genomic RNA to maintain balanced expression of unspliced and spliced transcripts.

\textbf{INTRODUCTION}

Retroviruses require nuclear export and efficient translation of their unspliced RNA independently of intron removal and the presence of a long and highly structured 5' untranslated region (UTR). Typical unspliced cellular pre-mRNA is not licensed for nuclear export and is detained in the nucleus (36,92). Furthermore, long and highly structured 5' UTRs impede ribosomes as they scan from the 5' cap to the initiation codon and preclude efficient translation initiation (55,101,124). Retroviral RNA elements interact with viral and cellular posttranscriptional modulators to facilitate nuclear export and efficient translation of their unspliced RNA. In the cytoplasm, the unspliced RNA acts as a template for synthesis of Gag and Pol structural and enzymatic proteins and as genomic RNA that is packaged into progeny virions (21). At least two divergent retroviruses contain 5' proximal RNA sequences that are encoded by
the 5’ long terminal repeat (LTR) and enhance the translational efficiency of unspliced viral and non-viral RNAs (22,67,130).

The RU5 region of spleen necrosis virus (SNV) 5’ LTR is a position- and orientation-dependent 5’ posttranscriptional control element (PCE) that increases translational initiation as demonstrated by enhanced association of cytoplasmic reporter RNA with polyribosomes (22,130). The 5’ RNA terminus of Mason-Pfizer monkey virus (MPMV) contains a functionally similar PCE in MPMV RU5 that also enhances ribosome loading (67). Both SNV and MPMV RU5 facilitate Rev/Rev responsive element (RRE)-independent HIV-1 gag-pol RNA expression and augment non-viral luciferase (luc) expression. Their effect in homologous genomic RNA remains an important unaddressed issue.

Deletion of SNV or MPMV RU5 abrogates protein production, despite minimal effect on the steady state level or cytoplasmic accumulation of the gag and luc reporter RNAs (22,67,130). Reporter gene assays with bicistronic reporter plasmids eliminated the possibility that SNV RU5 functions as an internal ribosome entry site and indicated that SNV RU5 enhances translational initiation by a distinct mechanism (130). SNV RU5 structure-function analysis has revealed two functionally redundant stem-loop structures that are necessary for functional activity (Roberts and Boris-Lawrie, submitted). Point mutations that disrupt the two RU5 stem-loops eliminate Gag production and compensatory mutations restore activity. RNA transfection and competition experiments with HIV Rev/RRE indicate that SNV RU5 requires interaction with nuclear factors to achieve translational enhancement in the cytoplasm (40).
SNV RU5 confers nuclear export independently of CRM-1, which is the nuclear export receptor utilized by HIV-1 Rev to export RRE-containing viral RNAs (40). In addition, SNV RU5 also functions independently of Tap, which modulates nuclear export of CTE and cellular mRNAs (Hull and Boris-Lawrie, submitted). A candidate for the alternate nuclear export receptor that is recruited by RU5 is the tRNA export receptor and this remains to be determined.

The R region of human foamy virus (HFV) 5’ LTR contains a PCE that is necessary and sufficient for HFV Gag and Pol production (134). Similar to results with SNV and MPMV RU5, deletion of HFV R has a minimal effect on the cytoplasmic accumulation of HFV unspliced gag RNA, despite abrogation of Gag production. Russel and colleagues speculate that HFV R directs gag RNA to a cytoplasmic environment that is necessary for expression. The potential positive effect of HFV R on increasing ribosome association has not been addressed experimentally.

Experiments in this report were designed to determine the effect of SNV RU5 on the metabolism of homologous SNV genomic RNA. To monitor SNV Gag protein levels we fused the green fluorescent protein (GFP) reporter gene in frame with SNV gag. We measured synthesis of Gag-GFP fusion protein by flow cytometry and SNV RNA expression by Northern blot. Overall, the results demonstrate that SNV RU5 augments SNV Gag-GFP production, at least in part, by increasing the cytoplasmic accumulation of unspliced SNV gag-gfp
RNA by a CRM-1 independent pathway. In addition, SNV RU5 dramatically decreases the level of spliced env RNA. These results demonstrate that SNV RU5 exerts distinct effects on SNV unspliced and spliced RNA.

MATERIALS AND METHODS

Plasmid construction

To create the SNV U3RU5 gag-gfp reporter plasmid, a PCR amplification product containing the SNV 5' LTR, 5' UTR and the complete gag gene upstream of the gag stop codon from the proviral clone pPB101 (sequence coordinates 1 - 2475) was ligated upstream and in-frame with the enhanced green fluorescent protein gene (770 bp) in pEGFP-N1 N-terminal protein fusion vector at AseI and SmaI sites. In addition, a Pvu II restriction fragment (sequence coordinates 4788-6900) of pPB101 was ligated into a unique Hpa I site downstream of gfp. The Pvu II restriction fragment includes the 3' region of SNV pol (sequence coordinates 4788-6070), the SNV env splice acceptor (sequence coordinate 5745) and the 5' region of SNV env (sequence coordinate 6010-6900). To create the SNV RU5 deletion or point mutant reporters (SNV U3 and SNV U3mRU5, respectively) the 5' LTR in the SNV U3RU5 gag-gfp reporter plasmid was replaced with PCR amplification products that contain either SNV U3 (nt 1 - 398) or SNV 5' LTR that contains nine point mutations in RU5, respectively, at Ase I and Sma I sites. The PCR templates were pYW100 and pYW233, respectively, which have been described previously (22,40).
**DNA transfection and analysis of protein production**

Triplicate reporter gene assays were performed on protein from $2.5 \times 10^5$ human 293 or $3.5 \times 10^5$ canine D17 cells transfected with 5 µg of reporter plasmid by a CaPO₄ protocol (67) or FuGENE 6 (Roche) according to the manufacturer's instructions, respectively. GFP mean fluorescence intensity (MFI) was measured from $2.0 \times 10^4$ transfected 293 or D17 cells by flow cytometry analysis after fixation with 1% paraformaldehyde. Transfection efficiency was monitored in 293 cells by Luc activity from cotransfected pGL3 (Promega) (67) and in D17 cells by pDsRed MFI from cotransfected pDsRed1-N1 (Roche). For leptomycin B (LMB) sensitivity experiments, duplicate plates of $2.5 \times 10^5$ 293 cells were transfected and twenty-four h post-transfection, 2.5 mg/ml of LMB was added to one replicate plate. Forty-eight h post-transfection, both the mock and LMB treated plates were prepared for either flow cytometry or HIV-1 Gag ELISA as previously described (22). HIV Gag levels were quantified by a Gag enzyme-linked immunosorbent assay (ELISA; Coulter Corp., Miami, Fla.).

**RNA preparation and analysis**

Northern blots analysis was performed on 10 µg of nuclear and cytoplasmic RNA that had been fractionated by a hypotonic buffer protocol (67). The RNAs were separated on 1.2% agarose gels containing 5% formaldehyde, transferred to Duralon-UV membranes (Stratagene, La Jolla, Calif.) and incubated with either an SNV probe that extends across the env splice acceptor (sa) or cyclophilin (cp) DNA probes. The probes were prepared by a random-
primer DNA-labeling system (Gibco-BRL) with gel purified sa or cp PCR products and $^{\alpha-32P]}dCTP$. The hybridization products were subjected to PhosphorImager (Molecular Dynamics) analysis with ImageQuant version 4.2 (Molecular Dynamics).

**RESULTS**

**SNV RU5 augments SNV Gag-GFP production in human and canine cells.**

To investigate the potential positive effect of SNV RU5 on SNV Gag production, we analyzed a panel of SNV gag-gfp fusion reporter plasmids in transient transfection assays. The reporter plasmids contain either the wildtype SNV 5’ LTR (U3RU5), a deletion of RU5 (U3) or point mutation of RU5 (U3mRU5) (Figure 4.1). The SNV 5’ LTR has been shown to facilitate Rev/RRE-independent HIV-1 Gag production (22). Deletion of RU5 supports low, but detectable Rev/RRE-independent HIV-1 Gag production. The point mutant contains nine nucleotide changes that disrupt two structural motifs that are necessary for functional activity (Roberts and Boris-Lawrie, submitted). This report evaluates SNV Gag-GFP production in human 293 and canine D17 cell lines because previous characterization of SNV RU5 utilized both cell types. Also, D17 cells have been shown to support SNV replication (75). SNV Gag-GFP production was measured by flow cytometry and the mean fluorescent intensity (MFI) of GFP-positive cells is presented.
Figure 4.1: Summary of the structure of SNV gag-gfp reporter plasmids and comparison to the wild-type SNV provirus. 5' terminal labeled white rectangles, U3, R, U5 regions of the 5' SNV LTR; 5' terminal labeled light gray rectangles, mutant R and U5 regions of the SNV LTR; labeled white rectangles, SNV gag, pol and env genes; labeled black rectangle, green fluorescent protein (gfp) gene; labeled 5' and 3' ss, splice site; 3' terminal oval labeled p(A), polyadenylation signal; 3' terminal labeled white rectangles, U3, R, U5 regions of the 3' SNV LTR. Sequence coordinates of SNV provirus are shown.

Three independent transient transfection assays were performed and results from two representative experiments are presented. As expected, the results of flow cytometry verified that mock-transfected cells produced a baseline signal and the positive control pEGFP-N1 parental plasmid produced a robust signal (GFP MFI of 16). The SNV U3RU5 reporter plasmid also produced GFP, which verified synthesis of the fusion protein (Table 4.1). SNV U3 and SNV U3mRU5 produced low, but detectable levels of SNV Gag-GFP.
The results indicate that SNV RU5 augments SNV Gag production in both 293 and D17 cells.

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<td>SNV U3mRU5</td>
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Table 4.1: Comparison of SNV Gag-GFP protein production. Forty-eight hours posttransfection cell-associated green fluorescent protein (GFP) mean fluorescence intensity (MFI) was analyzed from duplicate transfections of 293 and D17 cells. GFP MFI was measured by flow cytometry and normalized to cotransfected Luc values or DsRed MFI. ND, not determined.

**SNV RU5 exerts distinct effects on unspliced and spliced SNV RNAs.**

Quantitative northern blot analysis was used to determine the effect of SNV RU5 on SNV RNA metabolism. The 293 cells were transiently transfected with SNV gag-gfp reporter plasmids and cells were fractionated by a hypotonic buffer protocol that was used previously to effectively separate Rev-dependent HIV-1 gag-pol reporter RNA into nuclear and cytoplasmic fraction (67). Duplicate northern blots were probed with a DNA probe that spans the 3’ splice acceptor (Figure 4.2A) and detects both unspliced and spliced reporter RNAs.
(Figure 4.2B). The data were quantified and are presented relative to \textit{cyclophilin (cp)} signal and cotransfected Luc values (Table 4.2).

The expected 5100 nucleotide unspliced \textit{gag-gfp} RNA and 1600 nucleotide spliced \textit{env} RNA are expressed from SNV U3RU5. Deletion or mutation of SNV RU5 did not alter the steady state level of unspliced SNV \textit{gag-gfp} RNA in the nucleus, but reduced the level in the cytoplasm by a factor of 2. These results demonstrate that SNV RU5 increased cytoplasmic accumulation of the unspliced SNV \textit{gag-gfp} RNA. The data indicate that the increase in SNV Gag-GFP production in response to SNV RU5 is attributable, at least in part, to increased cytoplasmic accumulation of SNV \textit{gag-gfp} RNA.

SNV RU5 also modulated the metabolism of the spliced \textit{env} RNA, but produced a distinct effect (Figure 4.2B and Table 4.2). Deletion or mutation of SNV RU5 dramatically increased the overall abundance of spliced \textit{env} RNA, but did not affect cytoplasmic accumulation of the spliced RNA. Compared to SNV U3RU5, deletion of SNV RU5 resulted in a 10-fold increase in the level of spliced RNA in the nucleus. A similar 14-fold increase was observed in the cytoplasm. The levels also increased 3-fold in the nucleus and 5-fold in the cytoplasm in response to RU5 point mutation. Possible explanations are that reduction in the abundance of spliced \textit{env} RNA is attributable to an RNA splicing suppressor in RU5 or increased stability of the spliced RNA.
Figure 4.2: Northern blot of nuclear and cytoplasmic RNA from transfected 293 cells reveals that SNV RU5 increases cytoplasmic accumulation of unspliced genomic RNA and decreases splicing efficiency of env RNA. A. Relationship among the SNV gag-gfp reporter plasmid, the complementary splice acceptor (sa) DNA probe, and detected unspliced and spliced reporter RNA with sizes indicated. B. Forty-eight hours posttransfection RNAs were isolated and treated with DNase. Ten-microgram aliquots were subjected to electrophoresis on a 1.2% agarose gel with 5% formaldehyde, transferred to a Duralon-UV membrane, and hybridized to α-32P-labeled DNA probes complementary to the SNV 3' splice site (sa probe) and cp. The blots were subjected to PhosphorImager analysis. Labels indicate the reporter plasmid and RNA fraction.
Table 4.2: Analysis of SNV gag-gfp reporter RNA in 293 cells. Transfected 293 cells were analyzed for Gag-GFP production and gag-gfp RNA expression. Nuclear and cytoplasmic RNA was extracted 48 hours posttransfection, treated with DNase, subjected to Northern blot with an SNV probe that extends across the env splice acceptor (sa) and cp probe and quantified by PhosphorImager analysis. The data are standardized to cp RNA levels and cotransfected Luc values. Cytoplasmic accumulation (CA) is presented as the ratio of cytoplasmic RNA relative to nuclear plus cytoplasmic. Splicing efficiency (SE) is presented as the ratio of spliced RNA to unspliced RNA. RNA level relative to unspliced SNV U3RU5 reporter RNA are indicated within parentheses.

**SNV RU5-mediated SNV Gag-GFP production is CRM-1 independent.**

The genetically complex HIV-1 activates nuclear export of Rev/RRE-dependent RNA by recruitment of the cellular nuclear export receptor CRM-1, which is inactivated by the antibiotic leptomycin B (LMB) (15,36,115). Previously, we have shown that SNV RU5-mediated Rev/RRE-independent HIV-1 Gag production is not inhibited by LMB (40). These results indicated that in the context of heterologous retroviral reporter RNA, SNV RU5 does not utilize the CRM-1 nuclear export receptor. Here we used LMB treatment to determine whether or not SNV RU5-mediated SNV Gag-GFP production was dependent on the CRM-1 nuclear export receptor. As expected, LMB reduced HIV-1 Gag production from the control Rev/RRE-dependent gag-pol reporter plasmid, but not the control SNV RU5-containing Rev/RRE-independent gag-pol reporter.
plasmid (Figure 4.3). Results of replicate transfection assays demonstrate that LMB has minimal effect on RU5-mediated SNV Gag-GFP production. The results confirm previous reports and further indicate that SNV RU5-mediated SNV Gag-GFP production is not dependent on the CRM-1 nuclear export receptor.

![Bar chart showing the effect of LMB on SNV Gag-GFP production](chart.png)

**Figure 4.3:** Effect of leptomycin B (LMB) on SNV Gag-GFP production reveals that SNV RU5 functions independently of the CRM-1 nuclear export receptor. Twenty-four hours posttreatment either cell-associated HIV-1 Gag levels or GFP MFI were analyzed from triplicate transfections of 293 cells. HIV-1 Gag levels were measured by ELISA and SNV Gag-GFP MFI intensity was measured by flow cytometry. The relative level of HIV-1 Gag and SNV Gag-GFP production is presented relative to that of mock-treated control.
DISCUSSION

Previous studies revealed that SNV RU5 is a unique 5’ PCE in two types of reporter RNA. This study addresses the necessary and important issue of the role of SNV RU5 in metabolism of SNV genomic RNA. We developed SNV gag-gfp reporter plasmids to monitor SNV Gag protein production. SNV RU5 augments SNV Gag-GFP production in human 293 and canine D17 cells. Northern blot results indicate that SNV RU5 increases SNV Gag-GFP production, at least in part, by increasing cytoplasmic accumulation of gag-gfp RNA. LMB-sensitivity experiments demonstrated that SNV RU5-mediated SNV Gag-GFP production is not dependent on the CRM-1 nuclear export receptor and indicate that SNV utilizes a distinct nuclear export pathway.

SNV RU5 also decreased the steady state level of spliced SNV env RNA. Possible explanations are the presence of a splicing suppressor in RU5 or less likely, that RU5 selectively decreases the stability of the env RNA. Although additional experiments are necessary to resolve this issue, we hypothesize that SNV RU5 inhibits splicing to the 3’ env splice site to achieve a proper ratio of unspliced and spliced RNA.

Efficient retrovirus replication requires a precise balance of unspliced and spliced viral RNAs. Rous sarcoma virus (RSV) contains a novel cis-acting negative regulator of splicing (NRS) element within gag RNA (4,151). The NRS functions to repress splicing of the env and src RNAs and also inhibits splicing of heterologous introns (4,109). Similar to our results with SNV RU5 mutants, deletion of NRS increased the level of spliced env and src RNAs. Additionally,
optimization of the 3’ env and src splice sites also increased the abundance of spliced RNAs (74,164). This shift in the balance of unspliced and spliced RNA produced a drastic delay in viral replication compared to wild type RSV. Interestingly, upon prolonged culture revertant mutants evolved that had restored the inefficient 3’ env and src splice sites. These results proved that NRS modulation of splicing efficiency is necessary for efficient replication. Paradoxically, cellular splicing factors bind the NRS, but inhibit instead of activate splicing (54,107). NRS is postulated to recruit a nonproductive 5’ splicing complex that sequesters the 3’ env and src splice sites (48). This prevents interaction of the 3’ splice site with the actual 5’ splice site and reduces splicing efficiency. In future studies, we seek to test the hypothesis that modulation of splicing efficiency by SNV RU5 is necessary for efficient SNV replication. Interestingly, RSV contains another regulator of splicing termed suppressor of src splicing (sss) (10,108). Deletion of sss or point mutations that disrupt the RNA secondary structure of sss result in increased spliced src RNA (3). We will test the interesting question of whether SNV RU5 secondary structure or primary sequence is necessary for regulation of splicing efficiency.
PERSPECTIVES

Identification of a unique class of posttranscriptional control element in two divergent retroviruses

In the course of this dissertation, we have demonstrated that two divergent retroviruses, MPMV and SNV, contain 5' posttranscriptional control elements (PCEs) that enhance the translational efficiency of HIV-1 unspliced gag-pol and intronless non-viral luc reporter RNAs (22, 67, 130). The PCEs map to the RU5 region of the 5’ LTR and function by directing the subcellular localization of RU5-containing gag and luc RNA to the translational machinery. The identification of functionally similar PCEs in MPMV and SNV suggests that translation enhancers are shared feature among divergent retroviruses. This would be an interesting hypothesis to test in future experiments.

The two translation enhancers identified to date are from genetically simple retroviruses. In contrast to genetically complex retrovirus, simple retroviruses lack regulatory proteins that activate viral gene expression. For instance, the genetically complex HIV-1 encodes the posttranscriptional regulatory protein Rev that activates nuclear export and increases stability and translational efficiency of RRE-containing RNA. An attractive model is that simple retroviruses, which lack posttranscriptional regulatory proteins, utilize cis-acting RNA elements to recruit cellular posttranscriptional modulators to
activate the various levels of gene expression. In agreement with this model, MPMV and SNV RU5 recruit cellular factors that enhance translation.

**Candidate RU5 trans-acting factors**

We have established that MPMV and SNV RU5 enhance translational efficiency of HIV-1 gag and non-viral luc RNA by augmentation of ribosome loading, but the cellular trans-acting factor(s) that facilitates translational enhancement remains undefined. Both gag and luc reporter RNAs contain features that have been shown to repress translation efficiency. The HIV-1 gag RNA contains a highly structured 5' UTR and both gag and luc RNAs lack intron removal (53,104,111,121). The RU5 trans-acting factor would overcome these repressive features and facilitate translation (Figure 5.1). Three candidates for the trans-acting factor are: a translation initiation factor, an RNA helicase or a member of the EJC. A possible translation initiation factor is the cap-binding factor, eIF4E (2,103,136). eIF4E is the rate limiting translation initiation factor and is part of the multiprotein cap-binding complex (eIF4F), which is composed of eIF4E, eIF4G and eIF4A. eIF4A is the RNA helicase responsible for unwinding 5' UTR secondary structure, which creates a single stranded landing pad for ribosomes. RNAs that contain highly structured 5' UTRs are inefficiently translated because of insufficient RNA helicase activity. Overexpression of eIF4E can increase the translation of RNAs with highly structured 5' UTRs (80,149,166). The increase in translational efficiency by eIF4E is attributable to increased recruitment of the eIF4A helicase. One possibility is that eIF4E is recruited by RU5 and stimulates delivery of the eIF4A helicase, which can melt
the highly structured 5' UTR of the HIV-1 gag reporter RNA and increase translation.

Additionally, RU5 may recruit a distinct RNA helicase to facilitate the melting of the structured 5' UTR, such as RNA helicase A. Recruitment of RNA helicase A would compensate for insufficient levels of eIF4A helicase. RNA helicase A might supplement the melting of the 5' RNA secondary structure that is initiated by eIF4A and thereby increase ribosome scanning and translational efficiency. Interestingly, preliminary data from our lab has shown that RNA helicase A is an SNV RU5 binding protein. The possible role in SNV RU5 translational enhancement and association with MPMV RU5 remains to be analyzed.

Another possible mechanism of MPMV and SNV RU5 addresses the linkage between pre-mRNA splicing and translational efficiency (104). Given that MPMV and SNV RU5 increase the translational efficiency of unspliced intron-containing HIV-1 gag and intronless luc RNA, a speculative possibility is that RU5 replaces the exon-exon junction to directly recruit components of the EJC that may enhance cytoplasmic translation. A primary candidate of the EJC is the nucleocytoplasmic shuttle protein, Y14. As mentioned previously, several studies have led to the hypothesis that Y14 is a protein that links pre-mRNA splicing and stimulation of translational efficiency (44,73,78). We propose that RU5 binds directly to Y14 to stimulate the translational efficiency of RU5-containing HIV-1 gag and luc reporter RNAs.
Figure 5.1: Model for MPMV RU5 translational enhancement. A. MPMV RU5 recruits a cellular trans-acting factor (MPMV RU5-binding protein (BP)) that can melt the secondary structure of the 5' untranslated region and stimulate ribosome loading. B. Deletion of RU5 results in decreased ribosome loading due to the presence of a highly structured 5' UTR.

**Utility of translational enhancers in retroviral vectors**

Lentiviral vectors are a broadly used approach for gene transfer because of their large cloning capacity and ability to integrate into the host cell genome in both mitotic and non-mitotic cells (1). HIV-1-derived lentiviral vector systems have undergone rapid improvement in biosafety, efficiency of gene delivery and expression of the transgene in target cells (1). Currently, production of third generation lentiviral vectors requires cotransfection of four constructs: a packaging helper plasmid that encodes structural and enzymatic proteins, a
transfer vector that contains the transgene of interest, a separate plasmid that encodes the regulatory protein Rev required for nuclear export and efficient expression of the helper proteins and nuclear export of the vector RNA, and a separate env-encoding plasmid. Cotransfection of all four plasmids into human 293T cells produces high titer replication-defective vector viruses. Recently, much effort has been put forth to improve viral titer, efficiency of entry and transcription of the transgene (1,64). The posttranscriptional control elements studies in this dissertation have important potential for improvement of posttranscriptional expression of transgenes delivered by lentiviral vectors.

MPMV RU5 has important potential application in lentiviral vector systems. MPMV RU5 can be utilized to stimulate transgene expression in the target cell, which has been shown for the woodchuck hepatitis virus (WHV) posttranscriptional regulatory element (WPRE) (43). WPRE stimulates transgene expression in target cells independently of WHV protein, the nature of the transgene, cell type or delivery method (43,64,90,168). The optimization of transgene expression at the posttranscriptional level will enhance the ability of lentiviral vector systems to produce therapeutic levels of transgene protein production in the target cell. Important experimental questions must be addressed before MPMV RU5 can be most effectively utilized in gene transfer vectors: does MPMV RU5 function when integrated into the host genome as a vector provirus; does MPMV RU5 function in a wide variety of cell types; is the function of MPMV RU5 affected by properties of the transgene RNA. Results of the identification and characterization of MPMV RU5 in this dissertation imply
that newly identified 5′ posttranscriptional control elements are a promising prospect to increase the expression of lentiviral vector delivered transgenes.

**Combination of PCEs in lentiviral vectors**

Studies performed to increase transgene expression have focused on the addition of a single type of PCE. Another attractive approach is to combine PCEs that function at different levels of posttranscriptional regulation, such as nuclear export and translational enhancement, on a single transgene. In this dissertation we analyzed the effect of combining the nuclear export element, MPMV CTE, and a translational enhancer, MPMV or SNV RU5, on a single HIV-1 gag-pol reporter RNA. The results revealed that augmentation of Gag production upon combination of PCEs is cell-type dependent. A study by Schambach et al analyzed the combination of WPRE and an intron, which is also a posttranscriptional stimulator of gene expression, and the combination did not augment transgene expression (138). These results imply that further understanding of the biochemical mechanisms of posttranscriptional control elements is necessary to predict which combination of PCEs is beneficial for maximal expression of the transgene of interest. Results of these studies will determine the practicality of combining PCEs to enhance transgene expression.

In conclusion, this dissertation has identified and characterized the function of a new retroviral posttranscriptional control element in MPMV RU5 and defined parameters of synergistic interaction among functionally distinct PCEs. The results of this dissertation provide new insights into the regulation of retroviral posttranscriptional gene expression. We believe that a particularly
significant insight is that nuclear export and cytoplasmic accumulation of retroviral RNA is not sufficient for productive cytoplasmic expression of the RNA. Instead, PCE-cellular protein interactions are necessary to program the cytoplasmic transcript for efficient protein synthesis.

FUTURE WORK

**MPMV RU5 structure-function relationship**

This work identified and characterized the MPMV RU5 posttranscriptional control element. Future goals of this work will focus on the characterization of the structure-function relationship of MPMV RU5 and translational enhancement. Deletion mutagenesis studies will define the region(s) of RU5 necessary for function. Substitution mutagenesis studies will reveal if structure and/or primary sequence is required for function. Interestingly, the predicted secondary structure of MPMV RU5 contains eight potential RNA loops (Figure 5.2). Initially, substitution mutagenesis studies will target RNA loops in the RU5 secondary structure because single stranded RNA regions have been shown to be targets for binding of trans-acting factors. For instance, the HIV-1 regulatory protein Tat binds a bulge in TAR RNA (11,132,133) and Tap binds two internal loops in CTE RNA (47,56,122). Once we have identified a structural and/or sequence motif required for MPMV RU5 function, experiments will be performed to identify the MPMV RU5 binding protein(s) (MPMV RU5-BP). UV-cross-linking and RNA electromobility shift assays with wild-type and mutant MPMV RU5 RNA will be initial experimental
approaches. Subsequent to identification, biochemical analysis will be critical to verify the interaction in vivo. The role of the MPMV RU5-BP in translational enhancement will be verified by overexpression of dominant negative mutants. Identification of the MPMV RU5-BP will also be useful to analyze the role of this protein in modulating the fate of cytoplasmic reporter RNA expression.

Figure 5.2: Computer-generated secondary structure for MPMV RU5 (Free energy –26.4 kcal/mol). Structure was predicted by the M-fold RNA folding program of Zucker. MPMV RU5 RNA folds into a stem-loop structure with eight loops that are potential cellular trans-acting factor binding sites.

*Expected role of RU5 posttranscriptional control element in replication of SNV*

An additional focus will be to determine the role of RU5 in sustaining balanced expression of unspliced and spliced RNAs during SNV replication. We will compare wild-type and mutant RU5 SNV viruses for replication efficiency and the unspliced to spliced RNA ratio. Additionally, as expected, if mutation of SNV RU5 reduces viral replication, we will select revertant mutants
and use sequence analysis to locate second site mutation(s) that restore the appropriate balance of unspliced and spliced RNA and wild-type levels of replication.
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