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Graduate Studies

CHARACTERIZATION OF ANTIGENOMIC PROMOTER NUCLEOTIDES 28-43 OF
HUMAN PARAINFLUENZA VIRUS TYPE 3 DURING GENOME REPLICATION

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CHARACTERIZATION OF NUCLEOTIDES 28-43 OF THE HUMAN PARAINFLUENZA VIRUS TYPE 3 ANTIGENOMIC PROMOTER IN GENOME REPLICATION

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We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Biology-Clinical Microbiology concentration.

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ABSTRACT


Human parainfluenza virus type 3 (HPIV3) is a significant cause of lower respiratory tract infections in infants and young children worldwide. During replication of HPIV3, the 96-nucleotide antigenomic promoter directs synthesis of genomic RNA. Previous work showed that nucleotides 1-12 (from the terminus) were critical in promoting replication of an HPIV3 minireplicon, but the role of nucleotides 13-96 was not investigated. However, the 13-96 region of the genomic promoter was recently characterized and found to have elements involved in promoting replication. Therefore, to clarify the role of antigenomic promoter nucleotides 13-96 in replication, a series of mutations were introduced that collectively scanned this region in an HPIV3 minireplicon. Notably, mutation of nucleotides 13-39 significantly deceased replication, suggesting that this region regulates replication. Further analysis of the 13-39 element revealed a complicated control element with both stimulatory and repressing elements. Specifically, nucleotide 28 (and possibly 21-27) was shown to repress RNA replication, while flanking sequences had a stimulatory effect. The precise role of the 13-39 region is not known, but the differences between this region of the antigenomic promoter and an analogous region of the genomic promoter may contribute to the increased activity of the antigenomic promoter in directing genome replication.
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CHAPTER 1.

INTRODUCTION

Background

Human parainfluenza virus type 3 (HPIV3) is an enveloped, nonsegmented, negative-sense RNA virus within the order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae (Fig. 1) (Lamb and Kolakofsky, 2001). Other families within the Mononegavirales include the Rhabdoviridae (rabies virus) and Filoviridae (Ebola virus). These three families possess similar genome organizations and mechanisms of gene expression. Several well-known human pathogens, in addition to HPIV3, are present in the Paramyxoviridae including measles virus, mumps virus, and respiratory syncytial virus (RSV) (Lamb and Kolakofsky, 2001). Additionally, emerging human and zoonotic pathogens such as human metapneumovirus (hMPV), Nipah, and Hendra viruses have been classified within this family. Collectively, the disease and economic burden of these viruses is extensive, making the Paramyxoviridae one of the most costly virus families in terms of human suffering worldwide.
Mononegavirales

A.

- Vesicular Stomatitis Virus
- Rabies Virus
- Respiratory Syncytial Virus
- Measles Virus
- HPIV3
- HPIV1
- Sendai Virus
- Newcastle Disease Virus
- HPIV2
- Mumps Virus
- HPIV4
- Marburg Virus
- Ebola Virus

- Rhabdoviridae

- Paramyxoviridae

- Filoviridae

B. Family Paramyxoviridae
   - Subfamily Paramyxovirinae
     - Genus Respirovirus
       - Sendai virus
       - HPIV1 and 3
     - Genus Rubulavirus
       - Simian virus 5
       - HPIV2 and 4
     - Genus Morbillivirus
       - Measles virus
     - Genus Henipavirus
       - Nipah virus
   - Subfamily Pneumovirinae
     - Genus Pneumovirus
       - Human respiratory syncytial virus
     - Genus Metapneumovirus
       - Avian pneumovirus
       - Human metapneumovirus

Fig. 1. Viral taxonomy. (A) Phylogenetic tree of the Mononegavirales order (B) Classification of viruses within the family Paramyxoviridae.
HPIV3 is one of four serotypes of parainfluenza viruses (types 1-4) known to cause respiratory infections in humans (Chanock, Murphy, and Collins, 2001). These viruses are major causes (behind RSV and hMPV in this regard) of lower respiratory tract diseases such as pneumonia, croup, and bronchiolitis in infants and young children (Durbin and Karron, 2003; Henrickson, 2003; Thomazelli et al., 2007). HPIVs have also been recognized as a cause of serious respiratory illness in elderly, immunocompromised, and chronically ill individuals (Glezen et al., 2000; Henrickson, 2003; Loeb et al., 2000; Nichols et al., 2004). While lower respiratory tract disease is a significant manifestation of HPIV infection, these viruses more often cause mild upper respiratory tract disease in children and adults (Chanock, Murphy, and Collins, 2001; Hall, 2001).

All four serotypes have been associated with both upper and lower respiratory tract disease, but individual serotypes possess distinct clinical and epidemiological characteristics. HPIV4 causes respiratory infection in children, but is not frequently isolated and therefore is not as thoroughly characterized as types 1-3. HPIV1 typically infects children aged 7 to 36 months, and is a common cause of croup (26-74% of cases) (Henrickson, 2003). Outbreaks of HPIV1 occur in the autumn of odd-numbered years, though this serotype is present at low levels throughout any given year. HPIV1 is also a common cause of bronchiolitis (10-15% of cases) and pneumonia (10% of cases). HPIV2 also causes croup (10% of cases) and outbreaks tend to occur in the autumn and winter of even-numbered years (Fry et al., 2006; Hall, 2001). Children aged 1 to 2 years of age are most commonly infected by HPIV2. HPIV3 appears to be unique among the HPIVs in its ability to infect infants younger than 6 months of age. HPIV3 is the second leading cause of bronchiolitis and pneumonia, behind RSV, in this age group (Chanock,
Murphy, and Collins, 2001; Henrickson, 2003). Approximately 18,000 infants and young children are hospitalized in the United States annually due to HPIV3 infection (Henrickson et al., 2004). HPIV3 typically causes spring and summer outbreaks, but, like HPIV1, HPIV3 is present at low levels year-round. Nearly all children have been infected by HPIV3 by two years of age and by HPIV1 and HPIV2 by five years of age (Hall, 2001). HPIV3 appears to be the most virulent serotype, as it is the most frequent serotype isolated from patients hospitalized with pneumonia or bronchiolitis.

No vaccines or antiviral drugs are currently available to prevent or treat illness caused by HPIV3 infection although several live-attenuated vaccines are in development. A cold-attenuated strain of HPIV3 (cp-45) has shown promise as a vaccine candidate in infants as young as one month. Thorough understanding of the HPIV3 life cycle will be crucial to develop additional potential vaccines or antiviral drugs against HPIV3 infection.

**HPIV3 life cycle**

The first event of the life cycle of HPIV3 (Fig. 2) is attachment of HPIV3 particles to a host cell. The hemagglutinin-neuraminidase (HN) protein binds sialic acid residues present on host cell surface proteins. The F (fusion) protein then mediates fusion of the host cell and viral membrane, allowing the contents of the viral particle to enter the host cell cytoplasm.
Fig. 2. The HPIV3 life cycle.

The contents of the HPIV3 particle (Fig. 3) include the 15,462 nucleotide negative-sense RNA genome that is helically encapsidated by the nucleocapsid (N) protein, forming the N-RNA complex. Two additional viral proteins present in the particle, phosphoprotein (P) and large (L) protein, form the viral RNA-dependent RNA polymerase (RDRP) and associate with the N-RNA, forming the ribonucleoprotein complex (RNP). This complex, not the naked RNA, is the minimal requirement for transcription and genome replication.
Fig. 3. Structure of the HPIV3 particle and genome organization.

Transcription of the viral genome is the first biosynthetic event. The RDRP binds near the 3’ end of the genomic RNA and transcribes the genome via a sequential start-stop mechanism (Fig. 4). In this mechanism of transcription, individual mRNAs are produced from the viral genome in a linear, sequential manner by the RDRP. Sequences that flank each gene regulate this process. At the upstream side of each gene, a gene start site (3’-UCCUNNUUUC-5’) serves as a transcription initiation signal, while at the downstream side, a gene end site (3’-UUNAU(A/U)(U/C)UUUU-5’) serves to terminate mRNA synthesis (Hoffman and Banerjee, 2000). At each gene junction, an intergenic sequence (3’-GAA-5’) is present in between the gene end site of the upstream gene and gene start site of the downstream gene. When the RDRP arrives at a gene end sequence, it stutters at the five U residues of the gene end site to polyadenylate the mRNA. Following addition of the poly(A) tail, transcription is terminated and the mRNA is
released. The resulting mRNA is also capped at the 5’ end, but this may take place during or after synthesis of the mRNA. The RDRP normally remains associated with the genomic RNA following termination, allowing transcription to be reinitiated at the next gene start site. However, the RDRP does occasionally fall off prior to reinitiating. Because the RDRP only initiates transcription at the 3’ end of the genome, the falling off of the RDRP results in a gradient of decreasing mRNA (and encoded protein) synthesis from the 3’ end of the template (Lamb and Kolakofsky, 2001). Six mRNAs are produced from this process. Host cell ribosomes then translate the viral mRNAs, resulting in accumulation of viral proteins within the host cell cytoplasm.

**Fig. 4.** Organization of the HPIV3 genome. Positions of the viral genes and genomic termini are shown. Below is the consensus sequence for the HPIV3 intergenic junctions. Exceptions to the consensus are at the 3’ genomic end/N boundary, which lacks the gene end sequence and at the L/5’ genomic end boundary, which lacks the intergenic and gene start sequences.

The cytoplasm is also the site of the next biosynthetic event, genome replication. The RDRP binds the very 3’ end of the viral genome and synthesizes a full-length, encapsidated, positive-sense strand, termed the antigenome (Lamb, 2001). Following
antigenome synthesis, the RDRP then binds the very 3’ end of the antigenome to synthesize full-length encapsidated copies of the viral genome. These genomic RNAs, which are synthesized in higher quantities than antigenomic RNAs, serve as additional templates for transcription and genome replication or are packaged into viral particles. Encapsidation of nascent genomic and antigenomic RNAs during replication may allow the RDRP to ignore the gene end and gene start sites recognized during transcription. Additionally, cellular proteins may also bind to the RDRP complex and influence the ability of the RDRP to recognize these signals (Qanungo et al., 2004).

Following synthesis of all viral components, the particle is then assembled. The matrix (M) protein is thought to interact with other viral proteins to coordinate assembly (Lamb and Kolakofsky, 2001). Newly synthesized RNP s are brought near the cellular membrane, where F and HN proteins are expressed. The assembled viral proteins and genome then bud from the host cell, taking a portion of the host cell membrane as the envelope.

Minigenome replicon overview

Experimental study of transcription and replication of negative-sense RNA viruses, like HPIV3, has lagged behind study of positive-sense RNA viruses and some DNA viruses. However, the introduction of reverse genetics systems for studying negative-sense RNA virus replication has provided a way to make directed mutations to the viral genome and analyze the effects of such mutations. One common reverse genetics model system used in transcription and replication studies is the minigenome replicon plasmid.
Minigenome plasmids have been used extensively to define viral cis- and trans-acting elements involved in transcription and replication in eukaryotic cells (Bridgen and Elliot, 2000). The minigenome RNAs encoded by the plasmid typically contain the 5’ and 3’ ends of the viral genome, which flank a reporter gene. The use of a plasmid-based system allows mutations to be made and analyzed for transcription and genome replication. Transcription and replication products produced from minigenomes may be quantified directly through standard methods of RNA analysis, or indirectly through reporter gene expression.

A negative-sense (genomic-sense) HPIV3 minigenome plasmid, pHPIV3 MG(-), has been used in previous work to define cis-acting GP and AGP elements important for replication and transcription (Hoffman and Banerjee, 2000; Hoffman et al., 2006). This plasmid (Fig. 5) contains 97 nucleotides from the 5’ terminus of the genome (AGP region), a negative-sense firefly luciferase reporter gene, 91 nucleotides from the 3’ terminus of the genome (GP region), and a negative-sense copy of the hepatitis delta virus ribozyme. In cells, the minigenome plasmid is transcribed by T7 RNA polymerase to produce a negative-sense minigenome (Fig. 6). Transcription is mediated by promoter and termination signals for T7 RNA polymerase located upstream of the AGP region and downstream of the ribozyme sequence, respectively. In the T7-produced transcript, the ribozyme self-cleaves at the junction between the GP region and the T7 termination signal, ensuring that the minigenome has an authentic viral sequence at the 3’ end. The naked transcript is then encapsidated by free N proteins in the cell, which are derived from T7-driven plasmids encoding HPIV3 N (Durbin et al., 1997; Hoffman and Banerjee,
The newly encapsidated T7 transcript is then replicated in vivo by HPIV3 P and L proteins that are also derived from T7-driven plasmids (Hoffman and Banerjee, 2000).

**Fig. 5.** Structure of the negative-sense HPIV3 minireplicon pHPIV3 MG(-). Transcription of the plasmid begins at the T7 promoter (T7). The transcript is negative-sense and contains two nonviral G’s followed by the terminal 44 nucleotides of the 5’ genomic terminus (5’ GT), a gene end (GE), part of the 3’ nontranslated region of the L gene (L 3’ NTR), luciferase gene, part of the 5’ nontranslated region of the N gene (N 5’ NTR), a gene start (GS), the terminal 52 nucleotides of the 3’ genomic terminus (3’ GT), the hepatitis delta ribozyme (Rz), and a T7 RNA polymerase transcription termination signal (T7Φ). The genomic promoter (GP) contains the 3’ GT, GS, and part of the N 5’ NTR. The antigenomic promoter (AGP) contains the 5’ GT, GE, and part of the L 3’ NTR.
Fig. 6. Diagrammatic representation of minireplicon replication. The T7 vaccinia virus, the HPIV3 minigenome pHPIV3 MG(-), N (pN), P (pP), and L (pL) plasmids are introduced into the HeLa cell line. Once in the cell, the T7 polymerase makes transcripts of all the viral proteins (mRNA) and pHPIV3 MG(-) RNA. The viral protein mRNAs are translated into proteins (N, P, and L) by HeLa cell ribosomes. pHPIV3 MG(-) RNA is encapsidated by the N protein and viral transcription and replication occur using the viral RNA-dependent RNA polymerase. After 48 hours, the RNA is extracted and assayed for RNA products.
Research Objectives

The overall goal of this research was to characterize cis-acting elements of the HPIV3 AGP that are important for genome replication. Two replication elements, CRI (nucleotides 1-12) and CRII (nucleotides 79-96), have been defined in the HPIV3 AGP. A third element residing in nucleotides 13-39 has been mapped and thoroughly characterized in this work. This work completes the mapping of the two HPIV3 replication promoters. A fully-mapped AGP, in combination with other data, is crucial to gain a more detailed understanding of the mechanism of genome replication for HPIV3 and other related viruses. Additionally, thorough definition of AGP replication elements may reveal new targets for the development of vaccines against HPIV3.

Two main objectives were undertaken to conclusively define the 13-39 element of the HPIV3 AGP and characterize the role of this region in genome replication:

1. Clarify the role of specific regions within the 13-39 element of the HPIV3 AGP in the process of genome replication.

2. Confirm that the 13-39 element of the HPIV3 AGP is important for genome replication using an alternative replication assay system.

Notes on Chapters 2 and 3

Chapter 2 is a manuscript to be submitted to Virology detailing the complete mapping of the HPIV3 AGP. As such, this chapter contains the work of LeeAnne Schwan, a previous graduate student who will be a coauthor of this paper, in addition to a portion of my thesis work. For clarification, LeeAnne conducted the entire analysis of Figure 8. Most of the analysis of Figures 7, 9, and 10 was also her work, though I repeated the analysis of these series to produce publication-quality images. The data
obtained from these replicates are included in the results for these series. I am responsible for the analysis described in Figure 1. The work described in Chapter 3 is not intended for publication at this time and is entirely my own.
CHAPTER 2.

ROLE OF NUCLEOTIDES 13-96 OF THE HPIV3 ANTIGENOMIC PROMOTER IN RNA REPLICATION

Abstract

During replication of human parainfluenza virus type 3 (HPIV3), the 96-nucleotide antigenomic promoter (AGP) of HPIV3 directs synthesis of genomic RNA. Previous work showed that nucleotides 1-12 were critical in promoting replication of an HPIV3 minireplicon, but nucleotides 13-96 were not investigated. In this analysis, the role of nucleotides 13-96 in AGP function was analyzed by creating mutations in an HPIV3 minireplicon. Cytosine residues at positions 79, 85, and 91 were confirmed to comprise an element seen in other paramyxoviruses known as conserved region II. Additionally, mutation of nucleotides 13-39 significantly decreased AGP function, suggesting that this region constitutes an additional \textit{cis}-element. Detailed analysis of the 13-39 element revealed a complicated control element with both stimulatory and repressing elements. Specifically, nucleotide 28 was shown to repress RNA replication, while flanking sequences had a stimulatory effect. A similar element exists in the genomic promoter at nucleotides 13-28. The increased size and sensitivity to mutation of
the 13-39 element of the AGP element may contribute to the increased activity of the AGP in directing replication.

Introduction

Human parainfluenza virus type 3 (HPIV3) is an enveloped, nonsegmented, negative-sense RNA virus within the Respirovirus genus of the subfamily Paramyxovirinae, family Paramyxoviridae, order Mononegavirales (Lamb and Kolakofsky, 2001). HPIV3 is a significant cause of respiratory tract infections (most notably croup, pneumonia, and bronchiolitis) in infants and young children (Chanock, Murphy, and Collins, 2001; Durbin and Karron, 2003; Henrickson et al., 2004; Thomazelli et al., 2007), and has also been recognized as a cause of serious respiratory illness in elderly, immunocompromised, and chronically ill individuals (Henrickson, 2003; Loeb et al., 2000; Nichols et al., 2004).

During the infection cycle of mononegaviruses, the viral genome, which is encapsidated by the nucleocapsid (N) protein, serves as the template for viral RNA synthesis. The RNA-dependent RNA polymerase (RDRP), composed of the viral P and L proteins, binds to the extreme 3’ end of the genome and produces an encapsidated, full-length antigenome. This antigenomic RNA serves as the template for synthesis of progeny viral genomes when the RDRP binds the 3’ end of the antigenome.

The 3’ ends of the genomic and antigenomic RNAs contain all of the sequences necessary for genome replication and are referred to as the genomic and antigenomic promoters, GP and AGP, respectively. Specific features of these replication promoters vary among the different families and subfamilies of the Mononegavirales. For the Paramyxovirinae, which includes members of the Respirovirus (HPIV3 and Sendai virus
[SeV]), *Morbillivirus* (measles virus), *Rubulavirus* (simian virus 5 [SV5]), and *Henipavirus* (Nipah virus [NiV]) genera, these features include multiple *cis*-elements in the GPs and AGPs, proper spacing between these *cis*-elements, and adherence to the rule of six (Lamb and Kolakofsky, 2001; Whelan, Barr, and Wertz, 2004).

Two critical *cis*-elements have been identified in the replication promoters of all members of the *Paramyxovirinae* studied to date: conserved region I (CRI) and conserved region II (CRII) (Hoffman and Banerjee, 2000; Murphy and Parks, 1999; Tapparel, Maurice, and Roux, 1998; Walpita, 2004; Walpita and Peters, 2007). The exact position and sequence composition of these critical regions varies among genera. CRI is present at the 3’ terminus of the genomic and antigenomic promoters, and in HPIV3, CRI consists of the first 12 nucleotides of the GP and AGP. Most nucleotides in CRI are highly conserved, and when mutated, result in severe reductions in gene expression (1-3% of wild-type). The second critical element, CRII, is present 79-96 nucleotides from the 3’ terminus and, in HPIV3 and SeV, consists of a repeated 3’[CNNNNN]3 motif. The critical nature of this sequence motif has been confirmed in the HPIV3 GP, but not in the HPIV3 AGP. In this study, we have confirmed the importance of CRII in the HPIV3 AGP.

The conserved and mutation sensitive nature of the nucleotide sequences within CRI and CRII suggests that both of these regions play crucial roles in directing genome replication. The role(s) of CRI and CRII in RNA replication of these viruses is not defined, though some roles have been proposed based on their positions within the replication promoter. It is very likely that CRI (and perhaps CRII) is a recognition site for the RDRP. CRI or CRII could also serve as recognition sites for cellular factors.
involved in replication, or as encapsidation signals for the nascent genomic or antigenomic RNA. Mutational analysis of the SV5 GP has also suggested a role for CRI in suppressing the host cell immune response to paramyxovirus infection (Manuse and Parks, 2009).

Another factor affecting replication within the *Paramyxovirinae* is the rule of six. The rule of six is a requirement that the total nucleotide length of a genome be a multiple of six for the genome to serve as a functional template for replication. This rule appears to apply to all members of the *Paramyxovirinae* (Calain and Roux, 1993; Durbin et al., 1997; Halpin et al., 2004; Murphy and Parks, 1997; Rager et al., 2002), but was initially developed based on two findings with SeV. First, examination of the helical SeV N-RNA by electron microscopy led to an estimate that individual N proteins encapsidate six nucleotides each (Egelman et al., 1989). Second, any non-hexameric disruption of the genome length (by insertion or deletion) led to disruption of replication (Calain and Roux, 1993). Since the RDRP recognizes the 3’ ends of the GP and AGP in the context of bound N protein, precise encapsidation of the 3’ ends is thought to be essential for replication initiation. Non-hexameric insertions or deletions within the genomic sequence would disrupt the positioning of N proteins on CRI and/or CRII, so any misalignment may lead to the N-RNA not being recognized as a suitable template for genome replication.

It should also be noted that the spacing between CRI and CRII is important. Insertions or deletions that alter the natural spacing result in significant reductions in replication (Murphy, Ito, and Parks, 1998; Tapparel, Maurice, and Roux, 1998). Combining the three-dimensional structure of the N-RNA and the rule of six may explain
this requirement. Thirteen N protein subunits are estimated to be contained in each turn of the helical SeV N-RNA complex (Egelman et al., 1989), placing CRI (in hexamers 1 and 2) and CRII (in hexamers 14, 15, and 16) on the same face of the helix. The critical nature of these sequences combined with their suspected positions on the N-RNA complex suggest that CRI, CRII or both of these regions may serve as recognition sites for the RDRP. Still, it remains possible that CRI and/or CRII could also serve as binding sites for cellular factors involved in replication.

These conserved elements may also serve as encapsidation signals in the 5’ ends of the nascent RNAs produced during genome replication. Analysis of the RSV GP revealed that mutation of individual nucleotides (at positions 1, 2, 6 and 7) within nucleotides 1-11 (analogous to CRI) affected encapsidation of the antigenomic RNA produced during replication, as RNAs derived from these mutants were susceptible to nuclease digestion (Fearns, Peeples, and Collins, 2002). RSV does not contain an element analogous to CRII within either of its replication promoters. However, the hexameric nature of the conserved sequences within CRII in the Paramyxovirinae, in combination with the rule of 6 and the requirement for proper spacing between CRI and CRII, suggests that CRII could also function as a nucleation site for the N protein, either alone or with CRI.

One important issue regarding genome replication in the Paramyxovirinae arises from observed differences in the replication efficiencies of the GP and the AGP, with the AGP being the stronger promoter. The placement of CRI and CRII is identical in the GP and the AGP of a given virus, and in HPIV3, the CRI sequence and the conserved C residues of CRII are identical in the GP and the AGP. Thus, sequences outside of the two
critical promoter elements must influence promoter strength and may stem from the presence of additional cis-acting promoter elements (Hoffman et al., 2006; Keller, Murphy, and Parks, 2001; Tapparel and Roux, 1996; Walpita and Peters, 2007). These regions, unlike CRI and CRII, appear to enhance RNA replication, but are not absolutely required for RNA synthesis. One such cis-element spans nucleotides 13-28 of the HPIV3 GP (Hoffman et al., 2006). This element enhances genome replication, but is distinct from CRI, as point mutations within the GP 13-28 element do not significantly impact genome replication. A significant decrease in replication was seen only when eight or more nucleotides were mutated within the element. The only other cis-element shown to contribute to genome replication of the Paramyxovirinae was found in the SV5 AGP in nucleotides 51-66 (Keller, Murphy, and Parks, 2001).

To be able to compare the AGP to the previously characterized HPIV3 GP, and to determine if elements similar to the 51-66 element of SV5 existed in HPIV3, we analyzed nucleotides 13-78 of the HPIV3 AGP. Since the sequences of the HPIV3 GP and AGP are similar, the AGP was thought to contain an element analogous to the 13-28 element of the GP. In previous work, the region between CRI and CRII of the AGP, nucleotides 13-78, was shown to be insensitive to point mutations (Hoffman and Banerjee, 2000) and was therefore considered to have a less critical role in directing RNA replication than the two conserved promoter elements. The 13-78 region of the AGP is characterized more precisely in this study. In addition to confirming the presence of CRII in the HPIV3 AGP, we have also identified an additional cis-element in nucleotides 13-39.
Materials and Methods

**Plasmid construction.** Construction of the HPIV3 minireplicon pHPIV3 MG(-) has been described previously (Hoffman and Banerjee, 2000). The T7 RNA polymerase transcript produced from pHPIV3 MG(-) contains 97 nucleotides from the 5’ terminus of the genome, a negative-sense copy of the luciferase gene, 91 nucleotides from the 3’ terminus of the genome, and the antigenomic hepatitis delta virus ribozyme gene. To facilitate detection of viral replication products, a construct in which the N gene start signal was disrupted in pHPIV3 MG(-) (Hoffman and Banerjee, 2000), termed pHPIV3 MG(-) KONGS (Knock Out N Gene Start), served as the parental vector for creating mutations in the AGP. Mutations in pHPIV3 MG(-) KONGS were created by megaprimer PCR-directed mutagenesis (Sarkar and Sommer, 1990). Megaprimer PCR products containing mutations were digested with EcoRI and HindIII and ligated into the same sites of pHPIV3 MG(-) KONGS. All mutations were confirmed by sequencing.

**Transfection.** HeLa cell monolayers were grown in 6-well plates to 90% confluence and infected with the recombinant vaccinia virus vTF7-3, which expresses T7 RNA polymerase, at a multiplicity of infection of 5. After 1 h at 35°C, the minireplicon and support plasmids encoding HPIV3 N, P, and L genes were transfected into the infected HeLa cells using Lipofectamine LTX with Plus reagent (Invitrogen) according to the manufacturer’s instructions, with the following exception: 2.5 µl of Lipofectamine LTX and 2.5 µl Plus reagent per well were used per transfection. The plasmid amounts per transfection were: 1.2 µg of pHPIV3 MG(-) KONGS, 0.2 µg of pHPIV3 N, 0.2 µg pHPIV3 P, and 0.195 µg of pHPIV3 L. At 24 hours post-transfection, the transfection
medium was removed and replaced with 1.4 ml of Opti-MEM (Invitrogen) containing actinomycin D (2 µg/ml) and 5% FBS.

**RNA isolation.** Replication-specific RNA products from transfected HeLa cell lysates were treated with micrococcal nuclease (NEB) at 48 hours post-transfection as described previously (Durbin et al., 1997). The nuclease-resistant RNA was then extracted via TRIzol (Invitrogen).

**Primer extension analysis.** Genomic RNA was detected using a [γ-32P]ATP-labeled oligomer (5’-AGAGATCCTCATAAAGGCCAAG-3’) that primes at nucleotide 33 of the luciferase gene and is extended 123 nucleotides to the 5’ end of the genomic RNA. Antigenomic RNA was detected using an end-labeled oligomer (5’-TTCTTTATGTTTTTGGCGTC-3’) that primes at nucleotide 1656 of the luciferase gene and is extended 105 nucleotides to the 5’ end of the antigenomic RNA. These primers were used with 20% of the nuclease-treated extracts in standard reverse transcription reactions with Moloney murine leukemia virus reverse transcriptase (New England Biolabs) at 44°C for 45 min. The extension products were separated on a 6% acrylamide-7 M urea gel and analyzed on a Storm Model 860 PhosphorImager using ImageQuant software.

**Results**

**Analysis of HPIV3 AGP nucleotides 79, 85, and 91 in RNA replication.** To analyze their role in genome replication, AGP nucleotides 79, 85, and 91, predicted to be critical nucleotides in CRII, were individually mutated in HPIV3 minireplicon plasmids (Fig. 7). A previously described minireplicon, MG(-) KONGS, in which the N gene start signal is disrupted and transcription is eliminated (Hoffman and Banerjee, 2000), was
used as the parental plasmid for introducing mutations into the AGP for two reasons. First, minigenome replication in MG(-) KONGS is slightly enhanced in this construct, facilitating detection of RNA replication products. Second, because some of the AGP mutations would overlap the L gene stop signal (present at nucleotides 45-56 of the AGP), we wanted to avoid using the wild-type minigenome in which some mutations could affect both transcription termination and replication efficiency. Minireplicon mutants were then transfected (along with support plasmids encoding the N, P, and L proteins) into HeLa cells infected with a recombinant vaccinia virus encoding T7 RNA polymerase. After 48 h, cell lysates were treated with micrococcal nuclease to reduce the background of unencapsidated RNA for analysis of replication products. Undigested RNA was subsequently extracted from the nuclease-treated lysates for analysis by primer extension. Replication efficiency for a given mutant was expressed as a ratio of genome to antigenome synthesis (termed AGP activity) to standardize levels of genomic RNA synthesis to the amount of template antigenomic RNA produced by the mutant.

Surprisingly, mutation of AGP nucleotide 79 resulted in only a slight reduction in replication compared to WT (Fig. 7, lane 3). This contrasts with the analysis of CRII in the HPIV3 GP, in which mutation of GP nucleotide 79 in a transcription-competent HPIV3 minireplicon resulted in reporter gene expression at 25% of wild-type levels (Hoffman and Banerjee, 2000). Mutation of AGP nucleotides 85 and 91 resulted in severe reductions in RNA synthesis, suggesting that these nucleotides are more critical for genome replication. These results are consistent with previous analysis of nucleotides 85 and 91 of the HPIV3 GP and confirm the presence of a functional CRII in the AGP of HPIV3.
Fig. 7. Analysis of mutations of nucleotides 79, 85, and 91 in the antigenomic promoter of HPIV3. Micrococcal nuclease-treated RNA extracts from cells transfected with the indicated minigenomes were analyzed by primer extension. Genomic RNA was detected with a positive-sense primer, and antigenomic RNA was detected with a negative-sense primer. Averages were based on three or more experiments with each mutant. Lanes marked –L indicates that the L plasmid was omitted in transfections with the parental MG(-) KONGS minigenome. (A) Sequences of mutations made in bases 79, 85 and 91 compared to wild-type MG(-) KONGS. (B) Representative primer extension experiment of mutations made in bases 79, 85, and 91. SD, standard deviation.

**Analysis of antigenomic promoter nucleotides 13-78 in replication.** To clarify the role of AGP nucleotides 13-78 in replication, a series of mutations which collectively scan the AGP 13-78 region were introduced into MG(-) KONGS (Fig. 8). In these mutants, targeted sequences were scrambled to preserve the original base composition. Mutation of AGP nucleotides 13-28 and 29-39 resulted in significantly reduced RNA replication, suggesting the presence of a cis-element in this region. Mutation of
nucleotides 40-50 and 51-66 did not significantly affect replication, while mutation of nucleotides 67-78 had a relatively minor effect on replication.

Nucleotides 13-39 of the HPIV3 AGP were not previously recognized as involved in directing the synthesis of genomic RNA (Hoffman and Banerjee, 2000). To more precisely characterize this region and be able to compare the role of this region in RNA replication with results from previous analysis of the HPIV3 GP, we made additional 4-base mutations spanning this region (Fig. 9). RNA synthesis was significantly reduced in mutants in which nucleotides 13-16, 17-20, and 29-32 were altered. These findings contrast with the results of previous analysis of the GP 13-28 element, in which no 4-nucleotide mutation showed a significant deleterious effect (Hoffman et al., 2006). It was only when eight or more nucleotides were altered when a substantial decrease in RNA synthesis was observed (Hoffman et al., 2006). Thus, it appears that the AGP 13-39 region is more sensitive to mutation than the GP 13-28 region. Also, mutations within this region tend not to result in decreased synthesis of genomic RNA, but cause a shift in the balance of genomic to antigenomic RNA synthesis. Interestingly, two mutants, 21-24 and 25-28, seemed to slightly (~2-fold) enhance AGP activity.
Fig. 8. Scanning mutagenesis of nucleotides 13-78 of the antigenomic promoter of HPIV3. Micrococcal nuclease-treated RNA extracts from cells transfected with the indicated minigenomes were analyzed by primer extension. Genomic RNA was detected with a positive-sense primer, and antigenomic RNA was detected with a negative-sense primer. Averages were based on three or more experiments with each mutant. Lanes marked –L indicates that the L plasmid was omitted in transfections with the parental MG(-) KONGS minigenome. (A) Sequences of scanning mutations in the 13-78 region compared to wild-type MG(-) KONGS. (B) Representative primer extension experiment of mutations in the 13-78 region. SD, standard deviation.
**Fig. 9.** Defining important bases within nucleotides 13-40 of the antigenomic promoter of HPIV3 in RNA replication. Micrococcal nuclease-treated RNA extracts from cells transfected with the indicated minigenomes were analyzed by primer extension. Genomic RNA was detected with a positive-sense primer, and antigenomic RNA was detected with a negative-sense primer. Averages were based on three or more experiments with each mutant. Lanes marked –L indicates that the L plasmid was omitted in transfections with the parental MG(-) KONGS minigenome. (A) Sequences of scanning mutations in the 13-40 region compared to wild-type MG(-) KONGS. (B) Representative primer extension experiment of mutations made in the 13-40 region. SD, standard deviation.

Author’s note: Due to the unacceptably high standard deviations obtained in the analysis of this region, additional replicates of this series of mutants will be performed prior to submission of this manuscript for journal publication.

**Mapping the 5’ end of the 13-39 element.** To more precisely define the 5’ end of the AGP 13-39 element and to confirm the importance of this region in replication, a series of mutants were created that progressively mutated into the 13-39 element from the...
5’ end (Fig. 10). Mutation of AGP nucleotides 40-43, located outside the 13-39 region, did not significantly reduce RNA synthesis. Extensions of this mutant by four and eight nucleotides, covering nucleotides 36-43 and 32-43, respectively, resulted in severe reductions in RNA synthesis as expected. These results confirmed that the 5’ end of this element mapped to nucleotides 36-39. Surprisingly, an additional 4-nucleotide extension of the mutation (mutant 28-43) showed a 10-fold improvement in AGP activity, from 6% activity with the 36-43 and 32-43 mutants to 64% with the 28-43 mutant. This was puzzling given that several overlapping mutants (32-43, 36-43, 13-28, 29-39, and 29-32) showed low AGP activity. It is worth reminding, however, that mutants 21-24 and 25-28 resulted in enhanced AGP activity (Fig. 9).

There were several potential explanations for the increased replication of the AGP 28-43 mutant relative to the other mutants. It was possible that the 28-43 mutant, by chance, created a replication-promoting stimulatory sequence. It was also more likely that the 28-43 mutant disrupted a WT sequence that repressed AGP activity. To discern these possibilities, we focused our analysis on bases 28-31 as this was the only region altered when comparing the 28-43 mutant with high AGP activity to the 32-43 mutant with poor AGP activity.
Fig. 10. Mapping of the 5’ end of a cis-element located in nucleotides 13-39 in the antigenomic promoter of HPIV3. Micrococcal nuclease-treated RNA extracts from cells transfected with the indicated minigenomes were analyzed by primer extension. Genomic RNA was detected with a positive-sense primer, and antigenomic RNA was detected with a negative-sense primer. Averages were based on three or more experiments with each mutant. Lanes marked –L indicates that the L plasmid was omitted in transfections with the parental MG(-) KONGS minigenome. (A) Sequences of mutations made in bases 28-43 compared to wild-type MG(-) KONGS. Wild-type sequence is underlined. (B) Representative primer extension experiment of mutations made in bases 28-43. SD, standard deviation.

We began by swapping sequences between the 28-43 (high AGP activity) and the 29-32 (low AGP activity) mutants. It should be noted that the sequence changes in the 29-32 mutant (Fig 9) differed from those in the 28-43 mutant. Thus, if the sequence of bases 29-32 were responsible for the high AGP activity of the 28-43 mutant and the low
AGP activity in the 29-32 mutant, swapping those sequences should result in corresponding activity changes. The new 29-32 mutant (29-32 #2), in which the 29-32 mutation matched the sequence changes found in 28-43, did replicate better than the original 29-32 mutant (Fig 11). However, it did not replicate better that WT, as would be expected if the 29-32 sequence from 28-43 had a stimulatory effect. Insertion of the 29-32 sequence from the original 29-32 mutant into the 28-43 mutant did not reduce the high AGP activity of the 28-43 mutant. At this point we were surprised by these results and wanted to confirm the phenotype of the original 29-32 and 28-43 mutants. Thus, we created additional 29-32 and 28-43 mutants (29-32 #3 and 28-43 #3). Each of these mutants had AGP activities similar to that of the original 29-32 and 28-43 mutants (Fig. 11), confirming the phenotypes of the original mutants.
**Fig. 11.** Detailed mutagenic analysis of nucleotides 28-43 in the antigenomic promoter of HPIV3. Micrococal nuclease-treated RNA extracts from cells transfected with the indicated minigenomes were analyzed by primer extension. Genomic RNA was detected with a positive-sense primer, and antigenomic RNA was detected with a negative-sense primer. Averages were based on three or more experiments with each mutant. Lanes marked –L indicates that the L plasmid was omitted in transfections with the parental MG(-) KONGS minigene. (A) Sequence alignment of original MG(-) KONGS mutants spanning nucleotides 29-32 and 28-43 with wild-type MG(-) KONGS. (B) Sequences of new mutations made in bases 28-43 compared to wild-type MG(-) KONGS. Wild-type sequences are underlined. New mutants are indicated in bold. (C) Representative primer extension experiment of mutations made in bases 28-43. SD, standard deviation.
Satisfied that the phenotypic difference between the original 29-32 and 28-43 mutants was not due to a gain in activity produced by the 29-32 sequence, we turned our attention to nucleotide 28. This position was altered in the 28-43 mutants with high AGP activity, but remained WT in the 29-32 and 32-43 mutants with low AGP activity. It was possible that WT nucleotide 28, either alone or in combination with the surrounding sequence, repressed AGP activity. Thus, mutation of this sequence would disrupt the repressing signal and result in increased replication efficiency. To test this, two additional mutants were constructed, a point mutant of nucleotide 28 (A28C) and a mutant in which bases 28-32 were altered (28-32). The 28-32 mutant extended the mutagenic region of the 29-32 #2 mutant to nucleotide 28, creating the same mutation sequence as in the original 28-43 mutant. Surprisingly, mutation of nucleotide 28 alone resulted in the highest AGP activity of all the mutants analyzed in this study (Fig. 11, lane 9), strongly implicating this nucleotide as a repressor of genome replication. Addition of the A28C change to the sequence of the 29-32 #2 mutant also increased the AGP activity, in this case approximately 3-fold (Fig. 11, lane 10 compared with lane 4). Thus, it appears that mutation of nucleotide 28 is responsible for the high level of AGP activity seen in the original 28-43 mutant.

Discussion

**Conserved Region II exists in the HPIV3 antigenomic promoter.** Conserved Region II, one of two critical elements present in the HPIV3 GP, is an internal element consisting of three hexameric subunits in which the first nucleotide must be a C residue. Sequence analysis of the AGP revealed a CRII element, but no mutational analysis had been performed to confirm the presence of a functional CRII within the AGP. In this
study, point mutants of AGP nucleotides 79, 85, and 91 showed a functional CRII exists in the AGP.

The replication phenotypes observed in our characterization of the HPIV3 AGP CRII are also similar to the phenotypes seen in previous analysis of the HPIV3 GP CRII (Hoffman and Banerjee, 2000) in that this nucleotide is less critical for replication than nucleotides 85 or 91. However, AGP nucleotide 79 appears to be much less critical for directing replication than GP nucleotide 79, though differences between experimental approaches used in these studies may not allow direct comparison of these results. In agreement with this observation, nucleotide selection experiments within the AGP CRII of SeV showed that some substitutions at nucleotide 79 were tolerated, but no substitutions were tolerated at positions 85 or 91 (Tapparel, Maurice, and Roux, 1998). Thus, it appears that HPIV3 AGP nucleotide 79 of CRII is less sensitive to mutation than nucleotides 85 and 91.

The HPIV3 AGP 13-39 element. In the HPIV3 AGP, nucleotides 13-78 constitute the region between CRI and CRII. The role of this region in directing RNA replication had not been studied in detail previously, but was expected to be similar to the thoroughly-characterized HPIV3 GP, in which a cis-element comprising nucleotides 13-28 (from the 3’ terminus) is important for GP activity. Indeed, analysis of the AGP 13-78 region revealed a possibly analogous promoter element spanning nucleotides 13-39 as important for AGP activity. Like the GP 13-28 region, the AGP 13-39 element appears to be distinct from CRI, as previously analyzed point mutations within the region did not severely disrupt replication (Hoffman and Banerjee, 2000). Thus, both regions are thought to enhance, but are not critical for, RNA replication.
While the GP 13-28 and AGP 13-39 elements appear to possess similar functions, these elements differ in their length and apparent complexity. The extensive mutagenic analysis of both HPIV3 replication promoters in previous work (Hoffman and Banerjee, 2000; Hoffman et al., 2006) and in the current study now allows for direct comparison between both of these regions. The AGP 13-39 element, in addition to being larger in size, is also more sensitive to mutation, as changes of four nucleotides within this region were sufficient to disrupt replication (Fig. 9). Changes of eight or more nucleotides within the GP 13-28 element were necessary to observe a similar effect on replication (Hoffman et al., 2006). These differences may relate to the increased efficiency of the AGP in directing RNA replication.

The AGP 13-39 element, while promoting replication overall, also appears to differ from the 13-28 element of the HPIV3 GP, and CRI and CRII, by containing sequences that both stimulate and repress replication. The role of nucleotide 28 (and possibly the 21-27 region) in suppressing RNA synthesis is supported by the observation that in every mutant in which nucleotide 28 is altered (A28C, 28-32, 25-28), AGP activity is altered. These results establish that while AGP nucleotides 13-39 appear to enhance RNA replication, AGP nucleotides 21-28 appear to suppress this process. A sequence that represses genome replication has also been identified in the SV5 GP in nucleotides 20-50 (Keller and Parks, 2003), just outside of the GP CRI of SV5, which spans nucleotides 1-19 (Murphy, Ito, and Parks, 1998). The role the GP 20-50 region plays in suppressing replication has not been determined, though this region is thought to interfere with encapsidation of nascent RNA, therefore biasing the GP towards directing viral transcription as opposed to antigenomic RNA synthesis. Since the AGPs of
paramyxoviruses solely direct synthesis of genomic RNA, it is possible that the 21-28 sequence down-regulates the rate of progeny genome synthesis.

Sequence comparison of the AGPs of the *Respirovirus* genus reveals that the 13-39 region is not well conserved (Fig. 12). There is a relatively low amount of sequence identity (26%, 7 of 27 nucleotides). Notably, nucleotide 28 (adenine) is identical in all three viruses, perhaps pointing to some function for this position in the life cycle of HPIV3. As we don’t know the function of the 13-39 element, we can only speculate on the role of nucleotide 28 within this larger element. The presence of a suppressing sequence within the 13-39 region may regulate the overall rate of viral gene expression. Accelerated or delayed viral gene expression has been shown to increase proinflammatory cytokine synthesis in cells infected with variants of SV5 (Young, Dillon, and Parks, 2006), so poorly controlled viral gene expression may influence the cellular immune response to paramyxovirus infection. This sequence may also aid in the conservation of viral and/or cellular resources used during the viral life cycle.

Fig. 12. Genomic-sense sequence alignment of first 39 nucleotides of antigenomic promoters of members of the *Respirovirus* genus, which include human parainfluenza virus types 1 and 3 [HPIV1 (RefSeq: NC_003461) and HPIV3 (GenBank: Z11575.1) respectively], and Sendai virus (RefSeq: NC_001552.1). Capitalized sequences in the consensus line (bold) indicate identical sequences; R or Y indicate conservative substitutions (Y=purine, R=pyrimidine). Dashes represent sequences not conserved within the genus.
The only other *cis*-element between CRI and CRII within the *Paramyxovirinae* known to enhance RNA replication exists in nucleotides 51-66 of the AGP of SV5 (Keller, Murphy, and Parks, 2001). The AGP 51-66 element of SV5, like the HPIV3 AGP 13-39 region, is located between two promoter elements that are critical for directing RNA synthesis and appears to enhance RNA replication, though the exact function of this region is unknown. The 51-66 element does not appear to be the sole determinant of the increased promoter strength of the SV5 AGP. Substitution of the GP CRI with the non-identical AGP CRI sequence, in addition to the AGP 51-66 element, was necessary to increase the replication efficiency of the GP (Keller and Parks, 2003). The SV5 GP does not contain a sequence analogous to the HPIV3 GP 13-28 region, so the differences observed between the replication efficiencies of the HPIV3 GP and AGP are likely due to the differences between the GP 13-28 and AGP 13-39 elements.

Based on our analysis, mutations within the 13-39 AGP element appear to bias the replication cycle away from genome synthesis towards antigenome synthesis. It is possible that this observation is the result of the RDRP preferentially binding the wild-type GP (which directs antigenome synthesis) instead of the mutated AGP. Alternatively, AGP mutations could disrupt terminal complementarity with the GP and lead to decreased replication efficiency. Increased terminal complementarity of promoter sequences has been found to contribute to increased replication efficiency in vesicular stomatitis virus (Wertz et al., 1994). Alteration of terminal complementarity of the replication promoters by mutation of the AGP was explored by sequence analysis. Alignment of the 96 nucleotides of the HPIV3 GP and AGP revealed 52% terminal complementarity (55% within nucleotides 13-39). In our study, comparison of terminal
complementarity between the GP and mutant AGP sequences revealed no correlation between replication efficiency and the extent of potential base-pairing of these regions, suggesting that the primary nucleotide sequences are responsible for observed replication phenotypes. This finding also confirms results seen in previous study of SeV (Tapparel and Roux, 1996).

The role of the HPIV3 AGP 13-39 element in RNA replication is unknown. It is possible that this region serves as a recognition site for the RDRP. Alternatively, this region may serve as a nucleation signal for the N protein on the nascent RNA, or as a binding site for cellular factors involved in RNA replication. The complexity of the function of specific sequences within the AGP 13-39 region suggests that this region may play multiple roles throughout the viral life cycle.

This study, in combination with previous work, completes the mapping of the HPIV3 replication promoters. The mapping and subsequent identification of cis-elements within these regions serve as an important step in the elucidation of the detailed molecular mechanism of RNA replication in HPIV3 and other related viruses.
CHAPTER 3.

EXAMINATION OF IN VITRO PACKAGING OF T7 RNA POLYMERASE-DERIVED MINIGENOME TRANSCRIPTS

Introduction

Proper encapsidation of genomic and antigenomic RNAs is critical for efficient genome replication of the Mononegavirales because the RDRP is thought to recognize sequences in the context of bound N protein. Therefore, encapsidation irregularities encountered by the RDRP would render a given genomic or antigenomic RNA an unsuitable template for genome replication. For this reason, the processes of nascent RNA synthesis and encapsidation are believed to be coupled during genome replication, with the RDRP placing individual N proteins on the nascent RNA (Lamb and Kolakofsky, 2001). Placement of the initial N protein on the nascent RNA could involve a specific N binding sequence. Once encapsidation is started, N proteins already bound to the nascent RNA assist in binding of additional N proteins to the nascent RNA as replication proceeds (Fig. 13). There is evidence, derived from characterization of the RSV genomic promoter, that the RDRP may place N proteins onto specific sequences at the 5’ end of the nascent RNA (McGivern, Collins, and Fearns, 2005). Evidence for this comes from genomic promoter mutants of RSV from which an accumulation of short,
unencapsidated RNAs resulted. These RNAs are believed to be abortive replication attempts in which encapsidation was inefficiently initiated due to the mutated sequence within GP nucleotides 16-34.

**Fig. 13.** Encapsidation of nascent RNA produced during genome replication of mononegaviruses. Individual N protein subunits (small ovals) are placed onto the newly-synthesized 5’ end of the nascent RNA (black line) produced by the RNA-dependent RNA polymerase (RDRP). The RDRP initiates genome replication at the 3’ end of the template RNA and synthesizes RNA in the direction indicated by the large black arrow.

The mechanism by which the T7 RNA polymerase-derived minigenome transcript is encapsidated is not known, but nascent RNA synthesis and encapsidation are not believed to be coupled during T7-mediated transcription of minigenome plasmids, as the two processes are derived from unrelated viral systems. Still, in cells expressing N proteins, free N proteins do encapsidate cellular RNA. This indicates that N proteins can encapsidate RNA independently of the coupled replication/encapsulation process of viral RNA replication.

It is not known whether the encapsidation of cellular RNAs is nonspecific, or whether some sequence specificity may be involved. If there is some sequence specificity to this encapsidation, the T7-derived minigenome transcript would be expected to contain an encapsidation signal at its 5’ terminus. Therefore, mutation of
these signals could result in decreased encapsidation of the T7-derived transcript, thus leading to decreased levels of minigenome RNA. If this occurred, the decreased replication phenotype of a given AGP mutant would be due to poor encapsidation of the T7 transcript, rather than less-efficient replication of the mutant by the HPIV3 RDRP. To distinguish between a true effect on replication and an effect on T7 transcript encapsidation (Fig. 14), some of the same AGP mutations previously analyzed using pHPIV3 MG(-) KONGS were transferred into a positive-sense HPIV3 minireplicon, termed pHPIV3 MG(+) (Fig. 14).

The T7 transcript of pHPIV3 MG(+) (Fig. 14A.) produces a positive-sense minigenome (Hoffman and Banerjee, 1997), with the GP residing at the 5’ end and the AGP residing at the 3’ end. In this construct, all AGP mutations would be contained within the 3’ termini of the T7 transcript while the 5’ termini (where any encapsidation signals would be) would contain WT sequence (Fig. 14B). Therefore, if an HPIV3 encapsidation signal is used during the encapsidation of the T7-derived MG(+) RNAs, then all transcripts should be equally encapsidated. If the results obtained from mutation of the AGP in the MG(+) construct match those previously seen in MG(-), we would conclude that the results were due to an HPIV3-specific effect on replication. However, if AGP mutants within the MG(+) construct show greater AGP activity than previously seen in MG(-), then the decreased AGP activity in the MG(-) construct may have been due to defects in encapsidation of the T7 transcript.
**Fig. 14.** MG(+) versus MG(-) constructs. (A) Structure of the positive-sense HPIV3 minireplicon pHPIV3 MG(+). Transcription of the plasmid begins at the T7 promoter (T7). The transcript is positive-sense and contains two nonviral G’s followed by the terminal 52 nucleotides of the 3’ genomic terminus (3’ GT), a gene start (GS), part of the 5’ nontranslated region of the N gene (N 5’ NTR), luciferase gene, part of the 3’ nontranslated region of the L gene (L 3’ NTR), a gene end (GE), the terminal 44 nucleotides of the 5’ genomic terminus (5’ GT), the hepatitis delta ribozyme (Rz), and a T7 RNA polymerase transcription termination signal (T7Φ). The genomic promoter (GP) contains the 3’ GT, GS, and part of the N 5’ NTR. The antigenomic promoter (AGP) contains the 5’ GT, GE, and part of the L 3’ NTR. (B) Potential consequences of mutation of the AGP sequence (indicated by black X’s) on encapsidation of the minigenome RNA (black lines) by the nucleocapsid protein (small circles) in MG(-) constructs versus MG(+) constructs. Sites of RNA encapsidation are indicated by a single nucleocapsid protein followed by an arrow; sites with a “no” sign indicate poor encapsidation. RNA synthesis is initiated by the viral RNA dependent RNA polymerase (RDRP) at the 3’ ends and proceeds in the direction indicated by the white arrow. Black arrows between the RNA strands indicate amounts of RNA synthesis and point to the progeny strand; short arrows indicate low levels of RNA synthesis.
Materials and Methods

To match the vector background from the previous analysis of AGP mutations, a transcription-deficient (KONGS) derivative of pHPIV3 MG(+), which produces a positive-sense minigenome RNA, was constructed and used as the parental vector for construction and analysis of AGP mutations. AGP mutants in pHPIV3 MG(+) KONGS were constructed and subsequently analyzed according to the same procedures described for pHPIV3 MG(-) KONGS in the Materials and Methods in Chapter 2. As previously described, replication efficiency of a given AGP mutant was expressed as a ratio of genome to antigenome synthesis, termed AGP activity, to standardize genome synthesis to the amount of template antigenome produced.

Results

In this analysis, five minigenome AGP mutants previously analyzed in pHPIV3 MG(-) KONGS were individually placed into a transcription-deficient derivative of pHPIV3 MG(+), termed pHPIV3 MG(+) KONGS (Fig. 15). These mutants were then assayed for viral replication and compared to previous results. This analysis resulted in confirmation of the replication phenotype with 4 of the 5 mutants. Mutants 28-43, 29-32, 37-40, and 25-28 displayed AGP activities similar to levels observed in MG(-) KONGS. One mutant (AGP 29-39) previously shown to replicate poorly in MG(-) KONGS replicated at close to WT levels in MG(+) KONGS.
Fig. 15. Analysis of HPIV3 AGP mutations in the positive-sense minigenome pHPIV3 MG(+) KONGS to determine the effect of mutation sequences on in vitro encapsidation of minigenome RNAs produced by T7 RNA polymerase. Micrococcal nuclease-treated RNA extracts from cells transfected with the indicated minigenomes were analyzed by primer extension. Genomic RNA was detected with a positive-sense primer, and antigenomic RNA was detected with a negative-sense primer. Averages were based on three or more experiments with each mutant. Lanes marked –L indicates that the L plasmid was omitted in transfections with the wild-type minigenome (WT). (A) Sequences of mutations made in bases 25-43 compared to wild-type MG(+) KONGS. Wild-type sequence is underlined. (B) Representative primer extension experiment of mutations made in nucleotides 25-43. (C) Results obtained from previous analysis of AGP mutants in the negative-sense minigenome pHPIV3 MG(-) KONGS. SD, standard deviation.

Discussion

This analysis was conducted to examine the potential effect of AGP mutations on encapsidation of the T7 RNA polymerase-derived transcript from pHPIV3 MG(-) KONGS. The introduction of an encapsidation defect of the T7 transcript by mutation of AGP nucleotides 29-39 in pHPIV3 MG(-) KONGS cannot be ruled out at this time. Of the five mutants analyzed in the MG(+) KONGS construct, only two AGP mutants (29-
32 and 29-39) that showed defects in replication were included in this analysis. Of these two mutants, only one (29-32) replicated poorly in both constructs. The other mutant, (29-39) replicated well in the MG(+) KONGS construct. Therefore, additional work is needed to clarify the results seen in this initial study.

The analysis of additional AGP mutants in the MG(+) KONGS construct is a logical next step. Refinement of the results of this study would be facilitated by transferring other AGP mutants within nucleotides 28-43 previously analyzed in the MG(-) KONGS construct into pHPIV3 MG(+) KONGS. In particular, two mutants constructed to map the 5’ end of the 13-39 region (described in Chapter 2) would aid in resolving the discrepancies observed in the initial MG(+) KONGS analysis: AGP 36-43 (8 nucleotides) and 32-43 (12 nucleotides). Like the AGP 29-39 mutant, both of these mutants replicated poorly (7 and 5% AGP activity, respectively) in the MG(-) KONGS construct. The common features of the AGP 29-39, 36-43 and 32-43 mutants would allow for more direct comparison of replication phenotypes within the 5’ end of the 13-39 region in the MG(+) KONGS construct.

Additional mutants from the 3’ end of the 13-39 region could also be analyzed in the MG(+) KONGS construct. The five AGP mutants studied in this construct only affected sequences towards the 5’ end of the 13-39 region. Constructing the same mutants within the 3’ end of the 13-39 region in the MG(+) KONGS construct would more thoroughly establish the effect of AGP mutation on encapsidation of T7 RNA polymerase-derived transcripts from pHPIV3 MG(-) KONGS. Mutants of interest within the 3’ end would include the 13-28 mutant constructed for the initial mapping of the HPIV3 AGP (14% AGP activity), the 13-16 mutant (12% AGP activity), and the 21-24
mutant (~212% AGP activity, high standard deviation), as these mutants represent a variety of replication phenotypes within this region.

This supplemental analysis did not conclusively define the effect of AGP mutation on packaging of the T7 transcript in the MG(-) KONGS construct. Analysis of additional AGP mutants, particularly mutants showing replication defects, in pHPIV3 MG(+) KONGS is necessary to thoroughly establish how AGP mutation affects encapsidation of the T7 transcript.
CHAPTER 4.

CONCLUSIONS

Two main objectives were undertaken to conclusively define the 13-39 element of the HPIV3 AGP and characterize the role of this region in genome replication:

1. **Clarify the role of specific regions within the 13-39 element of the HPIV3 AGP in the process of genome replication.**

   The mapping of the HPIV3 AGP was completed in this study with additional characterization of AGP nucleotides 28-43. Mutation of this sequence in previous study only reduced AGP activity to approximately 60% of WT levels, which is not a significant reduction in RNA synthesis. These results contrasted with the results obtained from other mutants constructed within this region to map the AGP 13-39 region. Analysis of additional mutants constructed within the 28-43 region revealed that WT AGP nucleotide 28 represses genome replication. Mutation of WT AGP nucleotide 28 significantly increased AGP activity in all constructs containing this mutation.

   In light of these results, nucleotides 13-39 of the HPIV3 AGP appear to enhance genome replication overall, but nucleotide 28 (and possibly 21-27) appears to control the rate of RNA synthesis from the AGP. The WT AGP nucleotide 28 sequence is conserved within the *Respirovirus* genus, possibly indicating a role for this sequence within the viral life cycle. A controlled rate of RNA synthesis may allow the virus to evade the immune response of the host cell.
The completion of the mapping of the HPIV3 AGP concludes the sequence characterization of both HPIV3 replication promoters. The detailed mapping undertaken in this study and in previous work has not been completed for other viruses within the Paramyxovirinae subfamily. This work completed an important step in the elucidation of the detailed molecular mechanism of genome replication in HPIV3 and related viruses, which may lead to new targets for vaccine development.

2. **Confirm that the 13-39 element of the HPIV3 AGP is important for genome replication using an alternative replication assay system.**

The findings of this analysis were inconclusive. Analysis of AGP activity in four out of five mutants placed in the alternative construct confirmed the results obtained in previous study. However, one mutant that replicated poorly in previous study (29-39) replicated at around WT levels in the alternative construct, which could affect the interpretation of results obtained in previous study. Based on these results, a non-HPIV3 specific encapsidation defect induced by AGP mutation in the previous construct cannot be ruled out at this time. The analysis of additional mutants in the alternative construct will be necessary to rule out this potential confounding factor.
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