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CHARACTERIZING THE ROLE OF N TERMINUS OF INFLUENZA A NUCLEOPROTEIN FOR LOCATION AND VIRAL RNP ACTIVITY

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CHARACTERIZING THE ROLE OF N-TERMINUS OF INFLUENZA A NUCLEOPROTEIN FOR LOCATION AND VIRAL RNP ACTIVITY

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

by

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ABSTRACT

The influenza viral ribonucleoprotein complexes (vRNPs) are responsible for viral RNA synthesis. Each vRNP is comprised of one vRNA segment, the viral RNA dependent RNA polymerase complex (RdRP), and multiple copies of nucleoprotein (NP). NP serves as scaffold in formation of vRNPs, but also regulates vRNP activity. The N-terminus of NP contains a nonconventional nuclear localization signal (NLS1) essential for initial vRNP nuclear import, but also interacts with host RNA helicases to enhance viral RNA replication in the nucleus. NP contains at least one additional NLS sequence, with bioinformatics revealing a third NLS in some NP proteins.

Published yeast-two hybrid results indicate that the first 20 amino acids of NP can sufficiently bind with cellular protein UAP56. Suggesting the interaction of NP-UAP56 can be a major mechanism of how NP involve in viral replication. Thus, to examine the role of the N-terminus of NP aside from its vRNP nuclear localization activity N-terminal 20 amino acid deletion mutants with or without the addition of the conventional NLS from SV-40 T-antigen were constructed, termed del20NLS-NP and del20-NP. Nuclear localization of vRNPs with these constructs was assessed by GFP expression and western blotting. All these constructs exhibit nuclear localization, consistent with NLS1 being utilized for vRNP localization but not NP localization and vRNP formation in the nucleus. Furthermore, qPCR results demonstrated decreased vRNA synthesis activity, exacerbated as the vRNA template is lengthened in both plasmids, consistent

with a lack of interaction with host RNA helicases. Interestingly, del20-NP vRNP activity is less severe than del20NLS-NP, suggesting perturbations of the N-terminus disrupt vRNP activity. To narrow down the region responsible for vRNA expression defect, del10-NP was constructed. GFP expression displayed similar activity between del10-NP and WT-NP with del20-NP showing a severe defection, suggesting NP amino acids 11-20 might be the major region responsible for the vRNA synthesis defect. However, sucrose density gradient results do not support the published interaction between NP and UAP56 in 293T cells. These results support the N-terminal region, potentially amino acids 11-20 of NP, is playing the important role in efficient viral gene expression during virus replication especially as vRNA template lengthen, and that the NLS1 of NP is not essential for NP/vRNP nuclear localization in our reconstituted vRNP assay.

ACKNOWLEDGEMENTS

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CHAPTER ONE

BACKGROUND AND SIGNIFICANCE

The Impact of Influenza

Commonly known as the flu, Influenza is a contagious respiratory disease caused by influenza viruses which creates seasonal health issues and even several pandemics. According to the Center of disease control (CDC), seasonal flu-related hospitalizations since 2010 ranged from 140,000 to 710,000, while flu-related deaths are estimated to have ranged from 12,000 to 56,000 in United States. Further, three times during the 20th century the world experienced Influenza pandemics: 1918 Spanish flu (H1N1), 1957 Asian Flu (H2N2), 1968 Hong Kong Flu (H3N2); and already once in the 21st century in 2009 with the swine flu (novel H1N1) (CDC). Influenza pandemics cause much higher number of deaths than seasonal influenza, with the number of deaths of the 1918 Spanish flu (H1N1) estimated to be at least 50 million worldwide with about 675,000 occurring in the United States. The 1957 Asian flu caused around 1.5 to 2 million worldwide and 70,000 deaths in the US, the 1968 Hong Kong flu resulted in 1 million worldwide and 34,000 deaths in the US, and the 2009 swine flu has reported with around 18,000 worldwide and 4,000 deaths in the US. All these pandemics were caused by Influenza virus type A.

There are several reasons why Influenza epidemics can be unmanageable and result in pandemics. First, the incubation period is extremely short (1-4 days). Second, virus particles are spread through droplets of sneezing or coughing easily, just one droplet can contain 100,000 to 1,000,000 virus particles. Next, since the initial flu symptoms are usually not severe, lots of infected individuals tend to continue their normal activities which help spread the virus. Influenza is spread by tiny droplets made when the virus carrier coughs, and can be passed on without the carrier even having any symptoms. Further, Influenza can be hard to diagnose without a laboratory exam as several diseases share the same symptoms. People with influenza infection can have symptoms such as fever, cough, sore throat, runny or stuffy nose, muscle or body aches, headaches, fatigue, and rarely some people will have vomiting and diarrhea (CDC). These symptoms are similar to the common cold, but the common cold is a much milder disease that can be caused by one of over 100 different types of rhinoviruses and is not lethal. Most patients can recover from influenza infection in several days to less than two weeks (CDC). However, to young children, elderly, and people who have immune deficiencies or disease (like AIDS), there is a higher chance for complications such as a secondary bacterial pneumonia which can result in death (WHO).

The Influenza A Virion

Influenza virus is a member of the Orthomyxoviridae family. There are three types of influenza viruses that infect people, type A, type B, and type C (CDC). They are classified according to antigenic differences in their nucleoprotein (NP) and matrix protein (M1), which are major structural components of their virions. All of them can cause similar symptoms in human but not confer immunity to one another. Among these three types, Influenza A viruses are responsible for all known pandemics in 20th and are the focus of this research.

Influenza A viruses can be further classified to different subtypes based on the antigen type of hemagglutinin (HA) and neuraminidase (NA). HA is a protein that mediates binding and entry of the viral genome into the target cell, while NA is involved in the release of progeny virions from infected cells (Suzuki Y., 2005). Both HA and NA occur as a variety of subtypes, presently there are 18 different known HA antigens and 11 different known NA antigens (CDC). The result is that there are many strains of influenza A virus, however, only H1, 2, and 3, and N1 and 2 are commonly found in human (Lynch JP et al., 2007). This research utilizes the Udorn Influenza A H3N2 lab strain virus to investigate the role of the N-terminus of Influenza Nucleoprotein (NP) in viral replication.

The core of the influenza A virion consists of structures called viral ribonucleoprotein complexes (vRNPs) which are responsible for viral transcription and replication. Each vRNP is comprised of one vRNA segment, the viral RNA dependent RNA polymerase complex (RdRP), and multiple copies of

nucleoprotein (NP). RdRP is the composed of three subunits, one acidic (PA) and two basic (PB1 and PB2) which transcribe and replicate viral RNA in the nucleus. The vRNPs are packaged with M1 (matrix protein) and further surrounded by a host-derived lipid bilayer envelope containing three transmembrane viral proteins: HA, NA, and M2, resulting in progeny virions budding from the plasma membrane of infected cells (Figure 1). Virions contain a distinct arrangement of the eight vRNPs required for functional virus (Oxford and Hockley., 1987; Noda et al., 2006). The HA and NA proteins are external and raft anchored on the surface. Four M2 proteins form an ion channel which is internal and loosely attach with lipid rafts. The M2 ion channel is required to alter endosome pH and facilitate fusion of the virion with the endosome (Nayak et al., 2009).

Influenza A viruses are enveloped, negative-sense, single-stranded, RNA viruses whose genome contains eight segments coding for at least 10 unique proteins. Some RNA segment encodes multiple proteins using different reading frame from same segment (Figure 2). The term negative-sense RNA implies that the RNA genome cannot be translated into protein directly, it must first be transcribed to positive-sense RNA before it can be translated into protein products. Influenza transcription occurs in the nucleus so that host-splicing machinery can be utilized. Other encoded well defined viral proteins include NS1, and NS2, (Nonstructural proteins, NS2 also known as NEP). Worth mentioning, 7 more novel proteins: PB1-F2 (Chen et al., 2001), PB1-N40 (Wise HM et al.,

2011), PA-X (Jagger BW et al., 2012), PA-N155, PA-N182 (Muramoto Y et al., 2013), M42 (Shih SR et al., 1998), and NS3 (Selman M et al., 2012) were discovered over the past 20 years though they are less well characterized and may not be expressed in all viral genotypes.

The Influenza A Virus Life Cycle

The Attachment and Entry to the Host Cell

Influenza A viruses specifically target the ciliated columnar epithelial cells lining the sinuses and airways. It is believed that NA has ability to break down respiratory tract secretions, making access to respiratory cells easier (Bouvier and Palese., 2008). The virion attaches to N-acetylneuraminic acid (sialic-acid) containing receptors at the cell surface. Once attachment is complete, the virus-receptor complex is formed and taken into cells via receptor-mediated endocytosis, and the virus enters the host cell in an endosome. The endosomal low pH environment induces a conformational change in HA, exposing a fusion peptide and triggering the fusion of the viral and endosomal membranes (Hunag Q et al., 2003). The acidic environment also allows the M2 ion channel to be opened, which leads to the release of vRNPs into the host cell's cytoplasm (Pinto LH et al., 1992). Influenza viral transcription and replication occurs in the nucleus and Nuclear localization signals (NLSs) found on vRNP proteins bind to cellular nuclear import machinery to allow vRNP nuclear localization (Figure 3).

HA forms spikes on the viral lipid membrane which interact with the host cell through sialic acid residues on the cell surface. There are two linkages are found between sialic acids and the carbohydrates they are bound to in glycoprotein, α2-3 and α2-6. Different HA subtypes prefer different linkages. For example, influenza viruses from humans prefer the α2-6 linkages; because α2-6 linkages are found frequently in the upper respiratory tract while α2-3 linkages are mostly at the lower respiratory tract, making these cells a more difficult target for infection. Conversely, avian influenza viruses prefer α2-3 linkages, and since these linkages are rare within a human's upper respiratory tract, it is rare for humans to get infected with avian influenza. Swine are susceptible to viruses of human as well as avian as they have cells with both α2-3 and α2-6 linkages in the upper respiratory tract (Skehel JJ et al., 2000). This explains why swine is considered a mixing vessel for avian and human influenza viruses. An example of this is the 2009 triple reassortment novel H1N1 virus.

Transcription of the Viral Genome

Viral transcription begins once the vRNP enter the nucleus. The viral RdRP interacts with active host RNA polymerase II to steal the 5'cap from nascent host pre-mRNAs and use this to prime viral transcription. The PB2 subunit within the RdRP recognizes and binds the 5' cap structure, and the PA subunit has endonuclease activity that cleaves the cellular mRNAs to obtain a cellular capped RNA fragment that used by the viral RdRP to prime viral transcription (Li ML et al., 2001) in a process is called cap-snatching. The

polyadenylation of the viral mRNAs occurs via a stuttering mechanism wherein the viral RdRP moves back and forth over a stretch of Uracil residues to form a poly-A tail. After obtaining a 5' cap and poly-adenylation, the viral mRNAs visually look the same as host mRNAs (Figure 4).

Splicing and Translation of Viral Protein

Segments 2, 3, 7, and 8 of influenza virus can encode multiple proteins through both alternate splicing and alternate translation mechanisms (Figure 2). Splicing is an important process in Influenza life cycle. Each influenza virion contains 8 RNA segments and can encodes and that for 10 different proteins. Segment 7 encodes the matrix protein M1, ion channel proteins M2 and M42; segment 8 encodes the nonstructural protein NS1, nuclear export protein NS2(NEP), and NS3 by alternative mRNA splicing M2 and NEP and NS3 are spliced products and are usually found in a much lower amount compare with NS1 and M1 (Amorim MJ et al., 2006). Influenza virus uses the host cells' splicing machinery to express these proteins (Engelhardt OG et al., 2006). The virus not only hijack the cellular splicing machinery, it also inhibits the host cell from using its own splicing machinery for the processing of host cell mRNAs. Viral nucleoprotein (NP) is also reported to interact with UAP56 (Momose F et al., 2006), which is a cellular splicing factor and a member of DEAD Box family of RNA helicases (Shen et al, 2007). However, when UAP56 is downregulated using siRNA, no change in ratio of spliced mRNAs to their respective intron containing mRNAs is observed (unpublished Newcomb lab; Nagata et al., 2008;

Read and Digard., 2010). Therefore, UAP56 and the reported UAP56-NP interaction does not appear to be involved in viral mRNA splicing.

Influenza virus also employs alternate translation to make the most of its small genome. Segment 2 encodes the polymerase basic (PB) proteins PB1, PB1-F2, and PB1-N40 by using alternative translation initiation sites (Wise HM et al., 2011; Muramoto Y et al., 2013) and segment 3 encodes the polymerase acidic (PA) proteins PA and PA-X by a ribosomal frameshift (Jagger BW et al., 2012), as well as two additional N-terminally truncated forms, PA-N155 and PA-N182 (Muramoto Y et al., 2013), by using alternative translation initiation sites.

Replication of Viral Genome

Influenza viral genome is made with negative sense RNA, for the genome to be replicated it needs to be converted into positive sense RNA first, which then serves as templates to produce genomic viral RNAs. Replication begins after the viral protein synthesis and accumulation of viral proteins. Replication does not require a primer, the RdRP initiates viral RNA synthesis de novo. The eight negative sense RNA segments are used to generate eight complimentary positive sense RNA (cRNA) which have a 5’ tri-phosphate end and no 3’ poly-A tail. The cRNAs is encapsulated by free NP and bound at the ends by viral polymerase proteins. The viral RdRP uses cRNA as template to make many vRNAs, which are also encapsulated by free NP and bound by the viral RdRP to form vRNPs. The new vRNPs are be used to either assemble new virions (Figure 3) or used to amplify viral gene expression (Figure 4).

Virion Assembly and Release

Chromosomal Maintenance 1 (CRM1) is the major mammalian export protein that facilitates the transport of large marcromolecules (like RNA and protein) across the nuclear membrane to the cytoplasm. Influenza viral protein M1 binds with negative sense vRNPs and NEP, NEP then binds to CRM1 and forms a “daisy-chain” complex. Through this complex, the vRNPs are exported out of the nucleus (Figure 3).

Once the vRNPs have left the nucleus, they start traveling to the cell membrane where assembly and budding occur. The final step of closing and budding off of the viral particle is triggered by an accumulation of vRNP bound M1 (Bouvier and Palese., 2008). For a virion to be infectious, it must contain all 8 vRNA segments. Packaging signals near the ends of the virus RNAs ensure that only one copy of each genome segment is selected for a new virion. M1 interacts with viral membrane glycoproteins (HA and NA) to assemble virions. After budding a virion will attach to cell receptors through HA until NA destroys the receptors to release the new progeny virions from the plasma membrane (Figure 3). Additionally, NA will remove sialic acid residues from the virions to prevent the new progeny virions to bind with each other and enhance infectivity (Bouvier and Palese., 2008).

Antigenic Variation

One major reason Influenza is hard to control is the rapid genetic mutation rate resulting in genetic variation. The viruses are constantly changing in two different methods, antigenic drift and shift. Antigenic drift are small genetic changes which happen continually over time during influenza genome replication because of the lack of proofreading abilities of the viral RdRP. These mutations usually produce viruses that are closely related and share the same antigenic properties and immune systems which had been exposed to the similar virus can typically recognize it and respond. However, if these small genetic changes keep accumulating over time, the changes can eventually be great enough to cause antigenic difference. In this case, the body's immune system will not be able to recognize those altered viruses. Influenza antigenic drift on HA and NA are the reason neutralizing antibodies targeting Influenza are HA and NA are subtype specific, the antigenic changes account for the different subtypes.

Antigenic shift is an abrupt, major change in the influenza A viruses, resulting in an entirely new HA subtype and/or NA subtype being introduced into human population. When a cell is infected with two different influenza viruses, the RNAs of both viruses are copied in the nucleus. When new virus particles are assembled and budding, the 8 RNA segments from each virus may package in new combinations to form novel virus. This process is called genetic reassortment (Figure 5). One evolutionary important example of reassortment is

the exchange of RNA segments between mammalian and avian influenza viruses which can give rise to pandemic influenza.

Treatments and Vaccination for Influenza

The two main methods of controlling Influenza infection are vaccination and antiviral treatments. Vaccination can prevent Influenza infection by activation of individuals' immune system to produce antibodies against specific NA/HA subtypes that are predicted to be in circulation during the year of certain areas. Immunization is usually achieved using inactive virus vaccine or, less commonly, live attenuated virus vaccine. Inactivated virus vaccines involve the subcutaneous injection of a killed or inactivated virus; live attenuated virus vaccines are live viruses that encode viral proteins altered to diminish their pathogenic abilities yet still elicit immune response and are given using a nose spray inoculation. Vaccine must be produced prior to the influenza viral season and is engineered to target predominant circulating strains. However, the high diversity of rapid evolution of Influenza A viral subtypes makes vaccination treatment difficult, underlining the importance of antivirals. However, influenza genes encoding resistance to existing antiviral treatments have been reported, highlighting the need for new classes of antivirals (for review, Davis et. al. 2014).

The first class of antiviral treatments include amantadine and rimantandine, which inhibit the influenza M2 ion channel. Resistance to M2 ion-

channel inhibitors can be acquired via a single amino acid substitution in the transmembrane region of the M2 protein (Holsinger et al, 1994). According to the Center of Disease Control, 100% of H3N2 influenza viruses circulating in 2009-2010 and 99.8% of 2009 pandemic H1N1 were resistant to M2 inhibitors. These data indicate that M2 ion channel inhibitors are no longer efficacious. The current class of antivirals include zanamivir and oseltamivir, which inhibit activities of influenza neuraminidase. Neuraminidase is one of the viral proteins that can be found on the surface of influenza viruses that enables the virus to be released from the host cell. Still, multiple single-amino-acid changes in neuraminidase can result in resistance and emerge during treatment (Kiso et al, 2004). The 2009 pandemic H1N1 contains resistance to neuraminidase inhibitors in addition to M2 ion-channel inhibitors (van der Vries et al, 2011) and it is likely only a matter of time before resistance to neuraminidase inhibitors rises to the level of resistance to the M2 inhibitors. Still, there are a number of new NA inhibitors in development, including CS-8958 from Daiichi Sankyo Co, A-322278 from Abbott Laboratories, and Peramivir from BioCryst Pharmaceutical.

Other Influenza antivirals in development include T-705, from Toyama Chemical, an oral RNA polymerase inhibitor; DAS181/Fludase from Nexibio, which incorporates a sialidase that removes sialic acids from mucosal membranes thereby preventing viral attachment via HA glycoprotein. Most of these Influenza inhibitors are either under phase II or III trail development (Yamashita et al., 2008; Baz M et al., 2008). Most exciting, a new antiviral

targeting the PA endonuclease cap-snatching activity was recently approved for use in Japan. While all these antiviral studies are promising, the fact that there is lack of novel effective influenza antiviral treatments, and that treatment use selects for resistance, indicates an urgent need to discover new molecular targets that can be exploited to produce novel antiviral therapies. In this study, I aim to exploit biochemical interactions important for successful viral RNA synthesis in order to contribute toward the goal of developing innovative treatments.

Influenza A Nucleoprotein

Influenza A virus contains 8 genomic segments, the gene segment 5 of influenza A virus encodes a 498 amino acids (aa) long protein called Nucleoprotein (NP) which is a multifunctional protein with critical roles during various stages of the viral life cycle. Phylogenetic analysis of virus strains isolated from different hosts reveals that the NP gene is relatively well conserved among influenza A, with a maximum amino acid difference of less than 11% (Shu *et al.*, 1993). Given NPs conservation, NP may be less prone to tolerate mutation and evolve resistance, making NP interactions compelling antiviral targets (Davis AM et al., 2014).

Nucleoprotein (NP), a 56kDa protein, is rich in arginine, glycine and serine residues and has a net positive charge at neutral pH. NP is modified by

phosphorylation (Privalsky and Penhoet, 1978). An electron microscope (EM) model of a recombinant influenza virus RNP revealed that the molecule has an elongated, curved, banana-like shape, perhaps comprising two domains (Martin-Benito et al., 2001). The crystal structure of NP reveals a head and body domain with an RNA binding pocket (Ye). The primary function of NP protein is to encapsulate the viral genome and form homo-oligomers to maintain ribonucleoprotein complex (RNP) structure (Prokudina Kantorovich EN et al., 1996). NP is more than a structural component of the vRNP and is essential for viral RNA replication, acting as a cofactor to coat the newly synthesized viral complementary RNA (Shapiro GI et al., 1988). However, NP also plays other important roles such as the importing of vRNP into nucleus, the exporting of vRNP back to cytoplasm, and finally preventing their reentry into nucleus (Palese P et al., 2007; Neumann G et al., 1997; Ozawa M et al., 2007; Elton D et al., 2001).

The nuclear import of NP is regulated by Nuclear localization signals (NLS), which are peptide signals that allow larger molecules such as protein and protein complexes to be actively transported into the nucleus. Influenza vRNPs are larger than 50kDa, so at least one component with a NLS is needed. It has been reported that all vRNP proteins (NP, PB1, PB2, and PA) contain at least one NLS (Cros et al., 2005, Bullido et al., 2000, Neuman et al., 1997, Wang et al., 1997, Akkina et al., 1987, Jones et al., 1986, Nieto et al., 1994). The N-terminus of NP contains a nonconventional nuclear localization signal (NLS1) located at amino

acids 3 through 13 (NLS1) essential for initial vRNP nuclear import. NP contains at least one additional NLS sequence which is a classical NLS (cNLS) located at amino acids 198 through 216 aa (NLS2) (Wu WW et al., 2007; Ozawa M et al., 2007; Elton D et al., 2001; Cros et al., 2005). In addition, bioinformatics revealed a third NLS located between the two NLSs in some NP proteins (Krishna et al, 2008). Although several studies have concluded NLS1 is essential for NP nuclear import (Cros et al, 2005; Wu and Panté, 2007), there is still debate regarding the relative importance of each NLS for NP nuclear localization (Ozawa et al, 2007).

Influenza viral RNA replication has two steps, first cRNA is synthesized using vRNA and then the cRNA serves as template to produce more vRNA. Viral RNA replication leads to increased vRNA templates available for transcription, amplifying production of viral mRNAs. NP has an active role in viral replication activity as it is responsible for anti-termination at the polyA addition site during vRNA to cRNA replication (Beaton et al, 1986). NP and the RdRP stabilize nascent cRNA and vRNA replication products (Vreede et al, 2004), and although NP is not essential for RNA replication on short templates (Lee et al, 2002), NP is required for synthesis of template sized RNAs (Beaton et al, 1986), and NP and RdRP interaction enhances unprimed RNA synthesis *in vitro* (Newcomb et al, 2009). Thus, aside from the role of coating the RNA template, NP is elaborately involved in promoting viral RNA replication and viral gene expression.

NP interacts with many of viral and host proteins at various times during infection. For example, NP interacts with factors to alter vRNP location. The

NLS1 of NP interacts with importin α for importing vRNP complex into the nucleus (Hutchinson EC and Fodor E, 2012) and NP interacts with CRM1 to export vRNPs into cytoplasm (Elton D et al., 2001). NP also interacts with factors to facilitate viral RNA expression. NP interaction with MCM serves as scaffold between nascent RNA chains and the viral polymerase (Kawaguchi A and Nagata K, 2007), while NP interaction with Tat-SF1 and UAP56 are thought to serve as a chaperone for NP (Naito T et al., 2007; Kawaguchi et al., 2011).

UAP56 is a 48-kDa polypeptide cellular splicing factor belonging to the DEAD-box family of RNA dependent ATPases and RNA helicases (Shen et al, 2007), and is involved in cellular mRNA remodeling during mRNA processing and nuclear export (Fleckner et al, 1997). Furthermore, UAP56 appears to be involved in most of the mRNAs nucleus export and their proper translocation in the cytoplasm (Meignin et al, 2008). UAP56 is proposed to function as a chaperone to promote free NP binding to nascent viral RNA replication products resulting in enhanced viral RNA synthesis (Kawaguchi et al., 2011). UAP56 can interact with NPs of avian and human influenza A viruses. However, NP may rely more heavily on one for viral RNA replication in the host cell. In our lab, we hypothesize the unique roles in influenza infection for this host factor as UAP56 knockdown resulted in accumulation of dsRNAs (Wisskirchen et al., 2011). The N-terminus of NP not only contains the essential NLS1, but was also implicated in interaction with UAP56, demonstrated through yeast two-hybrid analysis with partial NP proteins, revealing amino acids 1-20 are sufficient for interaction with

UAP56 (Momose et al., 2001). This interaction is reported to promote free NP binding to nascent viral RNA replication products, resulting in enhanced viral RNA synthesis *in vitro* (Kawaguchi et al., 2011). In agreement with this, the N-terminus of NP is required for viral ribonucleoprotein formation with long templates *in vivo* (Sanchez et al., 2014).

In this research, my goal is to understand the role of N-terminus of NP in viral RNA synthesis aside from its role in nuclear localization as the first 20 amino acids have been reported binding with host factor UAP56 while the region is also overlapping with one of NP's non-conventional NLS signal.

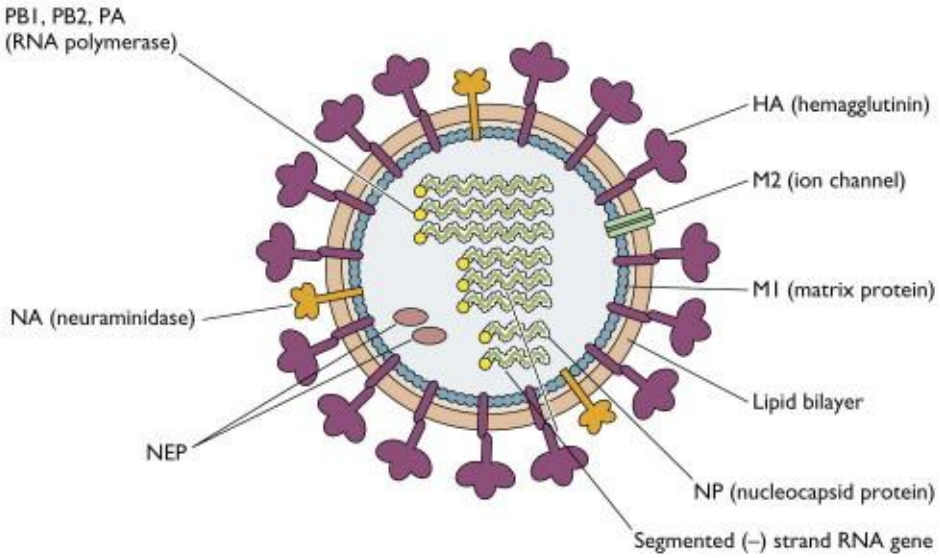


Figure 1. Influenza A Virion

Roughly spherical, Influenza A virion consists of 8 vRNP segments, surrounded by a matrix of M1 proteins, and a host derived lipid bilayer envelope which contains three transmembrane proteins (M2, HA, and NA).

Credit: Racaniello V (2009). Influenza Viral RNA synthesis. *Virology Blog – About Viruses and Viral Disease*.
< http://www.virology.ws/2009/04/30/structure-of-influenza-virus/>

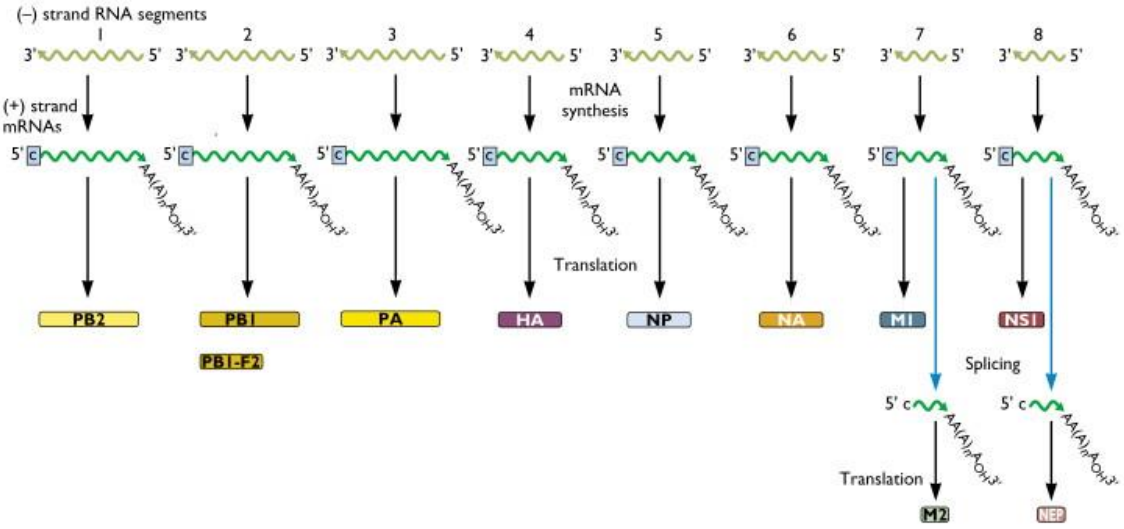


Figure 2. Some Influenza RNA Segments Encodes Multiple Proteins

There are eight segments of viral RNA within the Influenza A virion. They are responsible to code for at least 10 unique viral protein. This figure contains an 11th novel viral protein PB1-F2, which had been reported may has functions to kill host immune cells responding to influenza virus infection (Chen et al., 2001). In this image segment 2 undergoes frameshift during translation and segments 7 and 8 undergo alternate splicing.

Credit: Racaniello V (2009). Influenza Viral RNA synthesis. *Virology Blog – About Viruses and Viral Disease.*
< http://www.virology.ws/2009/05/01/influenza-virus-rna-genome/>

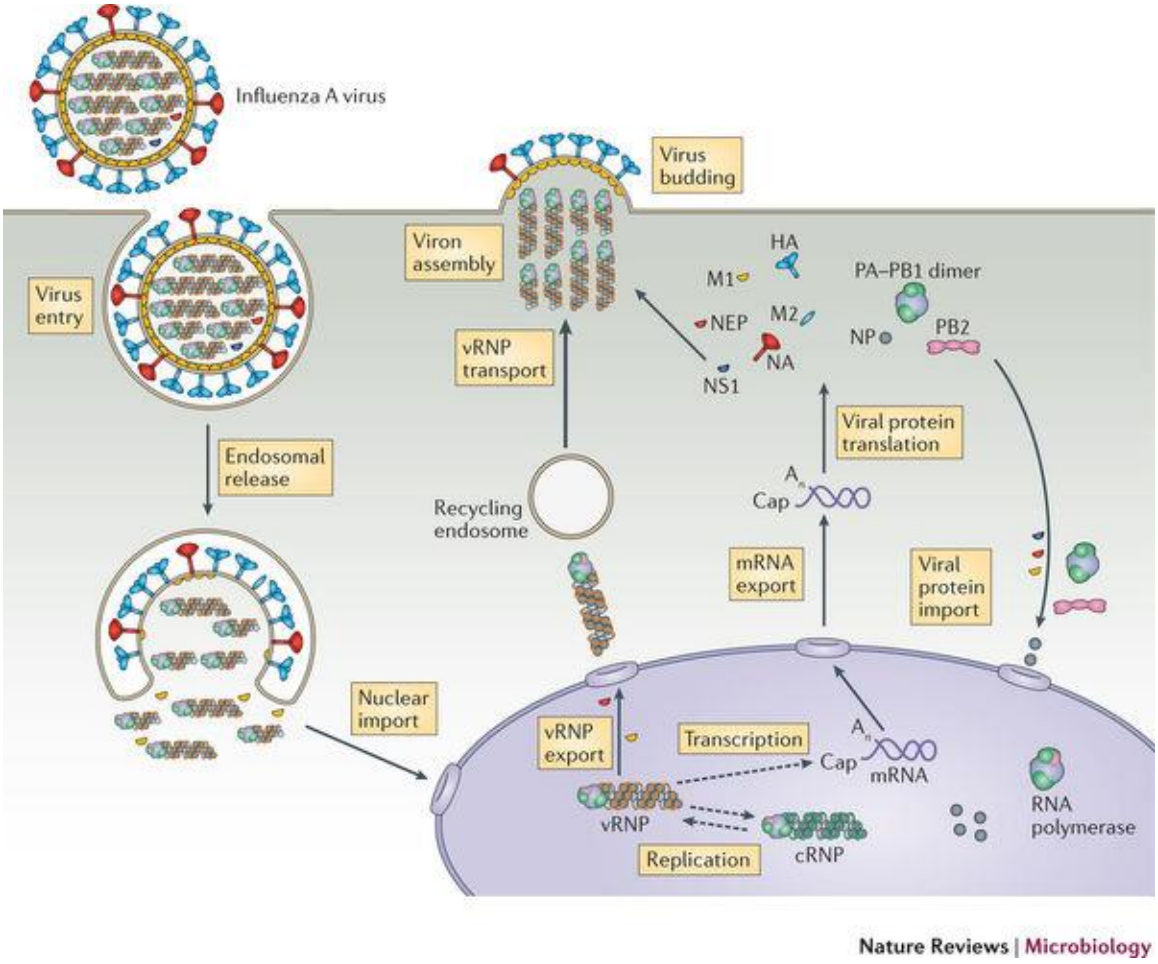


Figure 3. Influenza A Life Cycle

Influenza A virus binds to the host cell surface with HA and the virion is transported into the cell in an endocytic vesicle via receptor mediated endocytosis. The low pH in the endosome activates a conformational change in HA and opens the M2 ion channel, allowing the release of vRNPs into the cytoplasm. The vRNPs then enter the nucleus with the assist of NLSs. Viral transcription and replication occur in the nucleus. Newly replicated vRNPs travel back to the cytoplasm to initiate the virion assembly process. The vRNPs assemble with the viral glycoproteins at the cell membrane where the virions will assemble and bud.

Credit: Samji T (2009). *Influenza A: Understand the Viral Life Cycle*. Yale J Biol Med. 82(4): 153-159

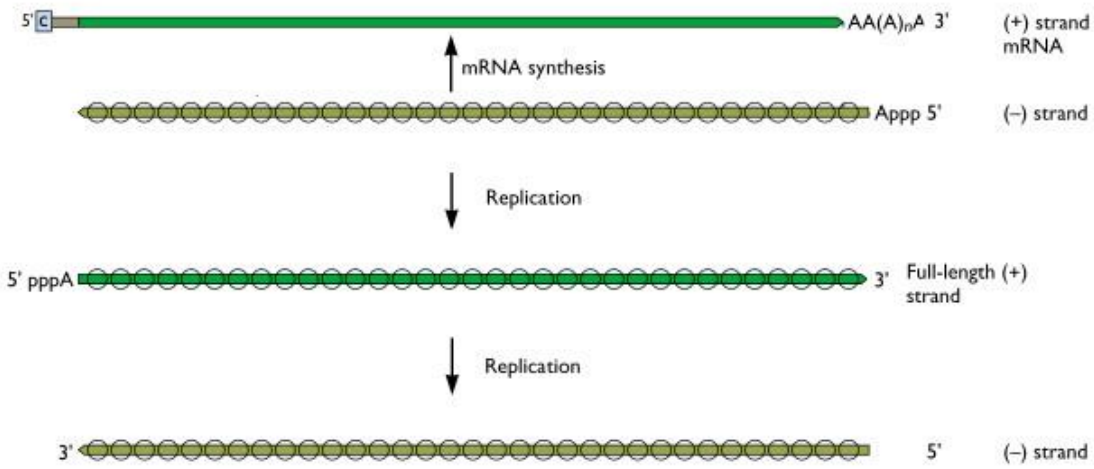


Figure 4. Influenza A Transcription and Replication

Transcription results in a positive sense mRNA that has 5’ cap and host sequences and terminates early using stuttering to generate a poly A tail. During replication, the viral RNA polymerase generates a complementary RNA (cRNA) replication intermediate, which is a full-length complement of the vRNA that serves as a template for the synthesis of new copies of vRNA.

Credit: Racaniello V (2009). Influenza Viral RNA synthesis. *Virology Blog – About Viruses and Viral Disease*.
< http://www.virology.ws/2009/05/08/influenza-viral-rna-synthesis/ >

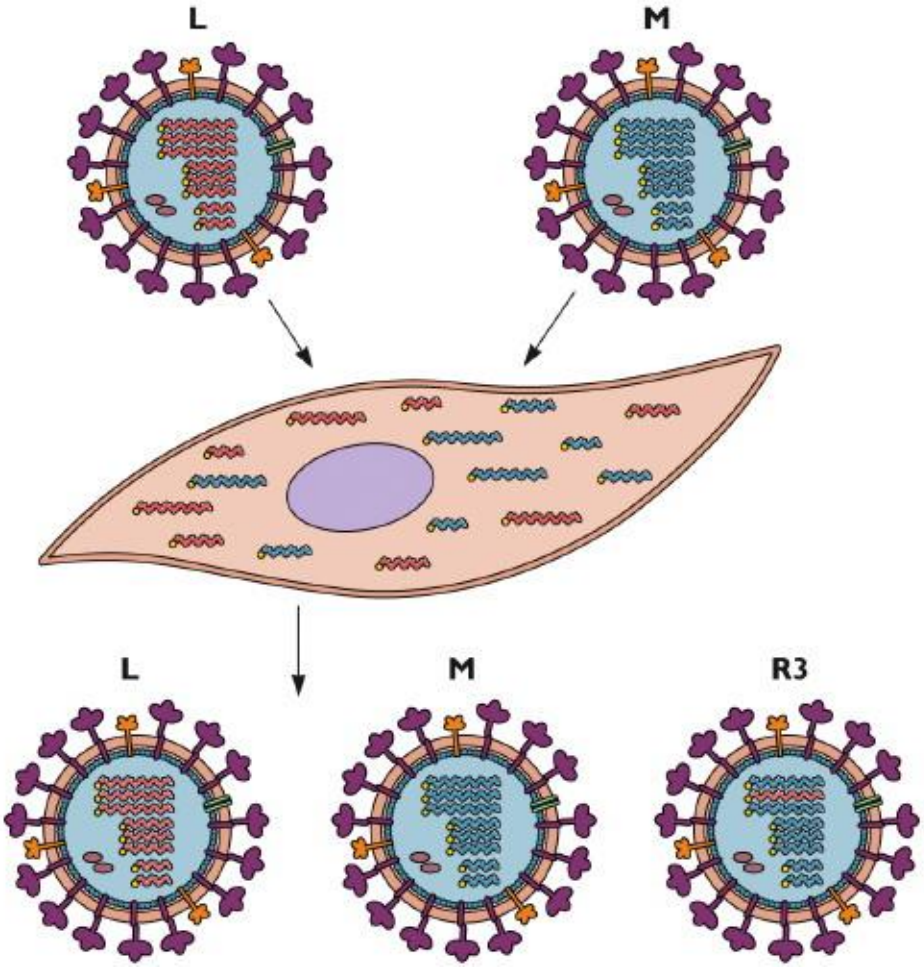


Figure 5. Reassortment of the Influenza Virus Genome

When a cell is infected with two different influenza viruses, the RNAs of both viruses are copied in the nucleus. When new virus particles are assembled at the plasma membrane, each of the 8 RNA segments may originate from either infecting virus. The progeny that inherit RNAs from both parents are called reassortants. This process is illustrated in the diagram above, which shows a cell that is co-infected with two influenza viruses L and M. The infected cell produces both parental viruses as well as a reassortant R3 which inherits one RNA segment from strain L and the remainder from strain M. Many different combinations of reassortants are possible.

Credit: Racaniello V (2009). Influenza Viral RNA synthesis. *Virology Blog – About Viruses and Viral Disease*.
< http://www.virology.ws/2009/06/29/reassortment-of-the-influenza-virus-genome/>

CHAPTER TWO

LOCALIZATION OF N-TERMINAL NP MUTANTS

Background

Given NP contains at least two nuclear localization signals (NLSs), there is still debate regarding the relative importance of each for NP nuclear localization during viral infection (Cros et al., 2005; Wu and Panté, 2007; Ozawa et al., 2007). To understand the role of N-terminus of NP on Influenza viral replication I first set out to understand the role of the N-terminus on NP nuclear localization, since NLS1 is within the N-terminus.

I used two different methods to analyze NP location, cellular fractionation and fluorescent microscopy. For both I constructed N-terminal 20 amino acid deletion mutants with or without the addition of the conventional NLS from SV-40 T-antigen, termed del20NLS-NP and del20-NP. In addition, I also constructed a WT-NLS NP (WT means Wild type) to study the impact of the extra conventional NLS to NP's localization activity (Figure 6). For cellular fractionation NP mutants were constructed with a C-terminal FLAG epitope, while for fluorescent microscopy the NP mutants were fused at the C-terminus to GFP.

Results

For cellular fractionation, NP mutants were transfected into 293T Human embryonic kidney cells. At ~48 hours post transfection proteins of cytoplasmic and nuclear fractions were isolated separated by SDS-PAGE gel and probed using western blotting technique to assess NP and mutant NP protein expression and location. Anti-FLAG antibody detects the C-terminal epitope tag of both WT NP and NP mutants. Anti-Hsp90 was used to detect Hsp90, which is a protein located in the cytoplasm and demonstrates only slight contamination into the nuclear fraction and uniform protein loading. The anti-Flag antibody confirmed that all the mutants: WTNLS-NP, del20-NP, and del20NLS-NP were present in both the nucleus and cytoplasm, similar as WT-NP (Figure 8). While we find that del20-NP and del20NLS-NP demonstrate relatively weaker protein expression compare with WT-NP and WTNLS-NP, there is significant NP expression in all mutants to characterize NP function in reconstituted vRNPs (Davis et al., 2017).

For fluorescent microscopy, NP and NP mutant fusion with GFP allow visualization of NP location in the host cells. The plasmids constructed and used here were named WT-NP-GFP, WT-NLS-NP-GFP, del20NP-GFP, and del20-NLS-NP-GFP. Plasmids were transfected into A549 human lung adenocarcinoma epithelial cells. SouthernBiotechTM DAPI-Fluoromount-GTM Clear Mounting Media was used to stain the cell nucleus. The GFP/protein and DAPI/nucleus were then observed using a EVOS^{TM} FL fluorescent microscope to access NP and NP mutant localization. Plasmid eGFP alone was also

transfected to monitor transfection efficiency and eGFP location. At 48 hours' post transfection, the cells were fixed with formaldehyde and the nucleus stained with DAPI mounting media. The fusion proteins were observed through fluorescence microscopy. The result is consistent with cellular fractionation in that all the NP mutants are expressed inside the nucleus (Figure 9). Interestingly, from the fluorescence microscopy image, we see punctate nuclear staining in all samples containing an N-terminal NLS, either from SV40 or NLS1 of NP. Punctate staining is less visible with the del20-NP-GFP; although the protein still expressed inside the nucleus, it is relatively spread out compared with other NP-GFP fusion proteins (Figure 9). This may indicate that NLS1 or the additional NLS from the SV40-antigen could provide specific trafficking activity that can import and 'accumulate' the proteins in certain area of nucleus.

In summary, cellular fractionation and fluorescence techniques (Figure 8 and 14) were consistent and both indicate the N-terminal NP mutants with or without additional NLS from SV40 can all be found in the nucleus similar to WT-NP. We conclude NLS1 is not essential for newly expressed NP nuclear localization and therefore the conventional NLS signal from SV40-antigen is not required for nuclear localization of NP in reconstituted vRNP assays

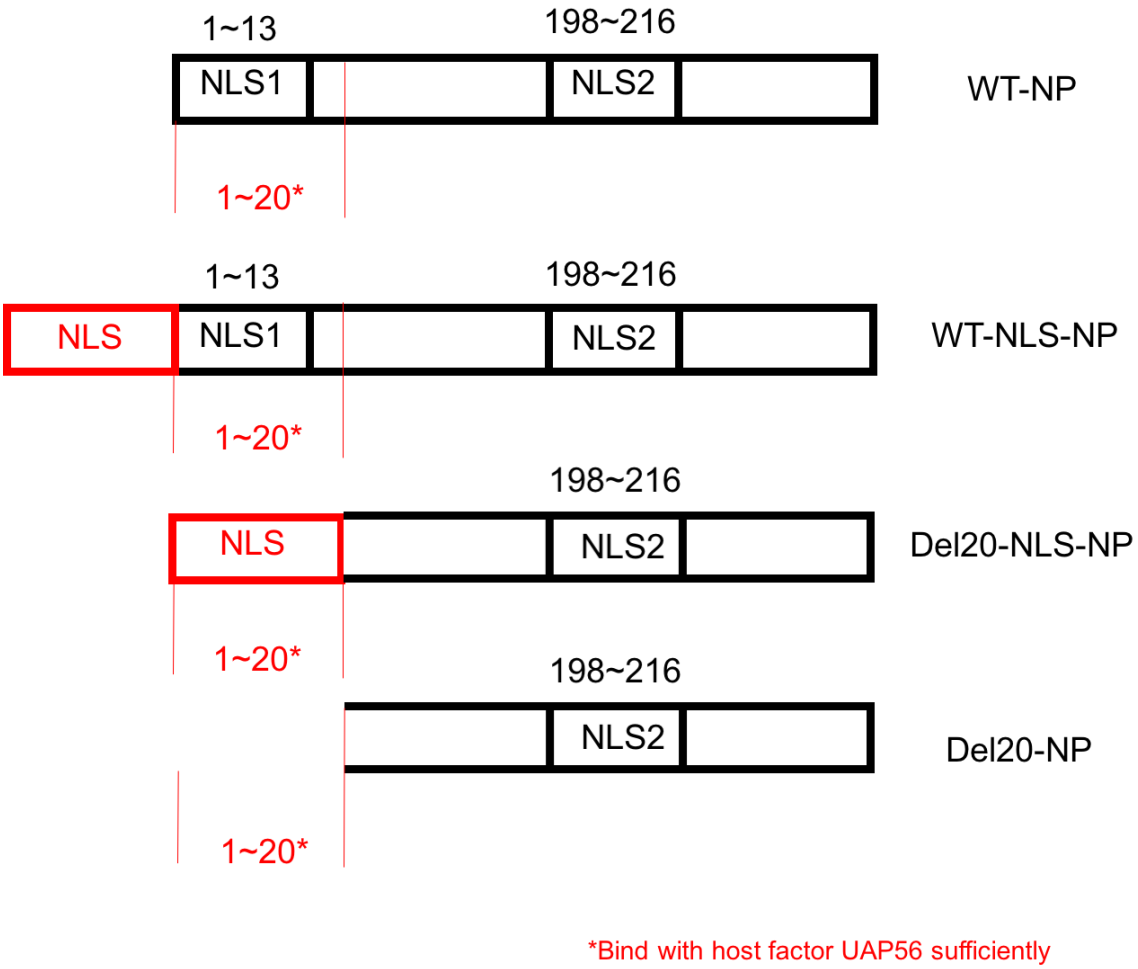


Figure 6. Nuclear Localization Signals (NLSs) on Nucleoprotein (NP) and NP Mutants Examined in this Study.

The nuclear localization signal (NLS) is an amino acid sequence that 'tags' a protein for import into the cell nucleus. NP encodes at least two NLS peptides (Cros, 2005; Wu and Panté, 2007; Ozawa, 2007). The N-terminus of NP is also known to interact with host factors UAP56. del20-NLS-NP was constructed by Abel Sanchez, I then constructed del20-NP and WTNLS-NP. The additional NLS is a well-defined conventional NLS from SV-40 T-antigen.

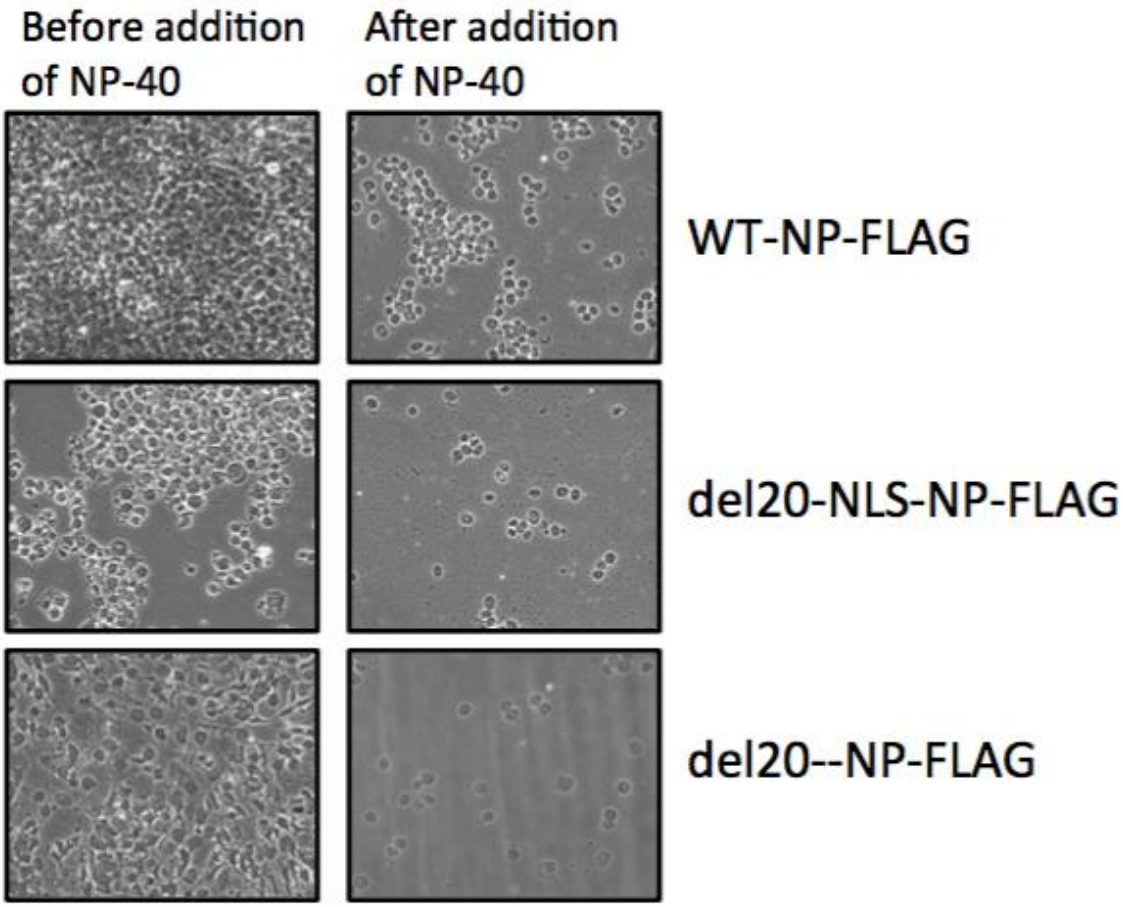


Figure 7. Microscopy Confirms Broken Cytoplasm and Intact Nuclei after Addition of NP-40.

293T expressing WT-NP, WTNLS-NP, del20NLS-NP, del20-NP were collected and fractionated with NP-40 non-ionic detergent to break open the cellular plasma membranes. Nuclei were pellet, centrifuged, then subjected to sonication for protein isolation.

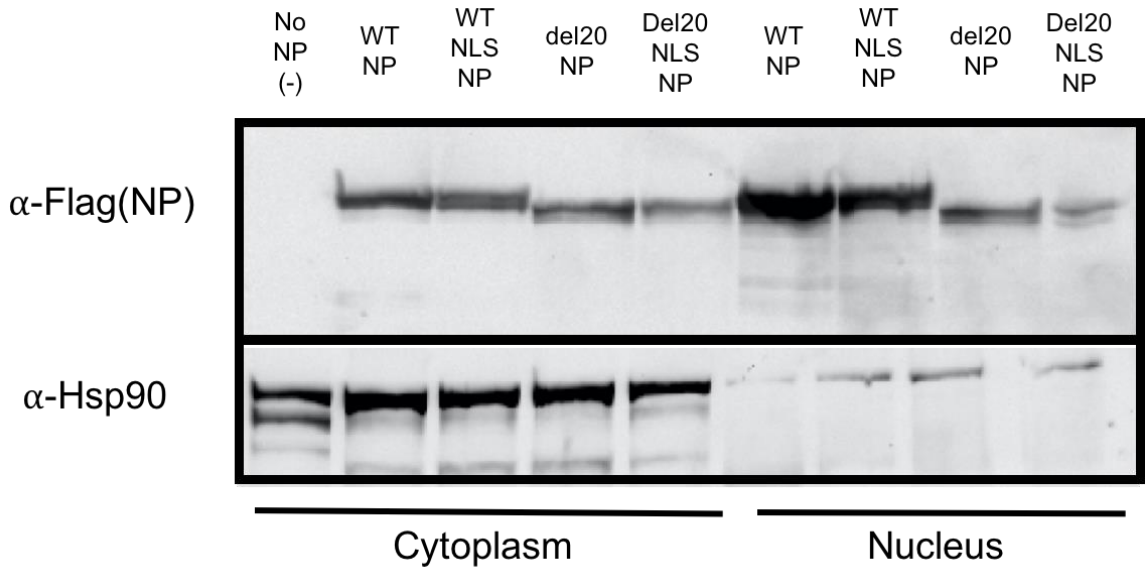


Figure 8. Mutant NPs are Expressed and Localized in the Nucleus and Cytoplasm Similar to WT-NP

The protein extracts from both the cytoplasm and nucleus were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose, and analyzed by western blot technique with anti-FLAG and anti-Hsp90. Anti-FLAG antibody detects the C-terminal epitope tag of all NP and NP mutant.

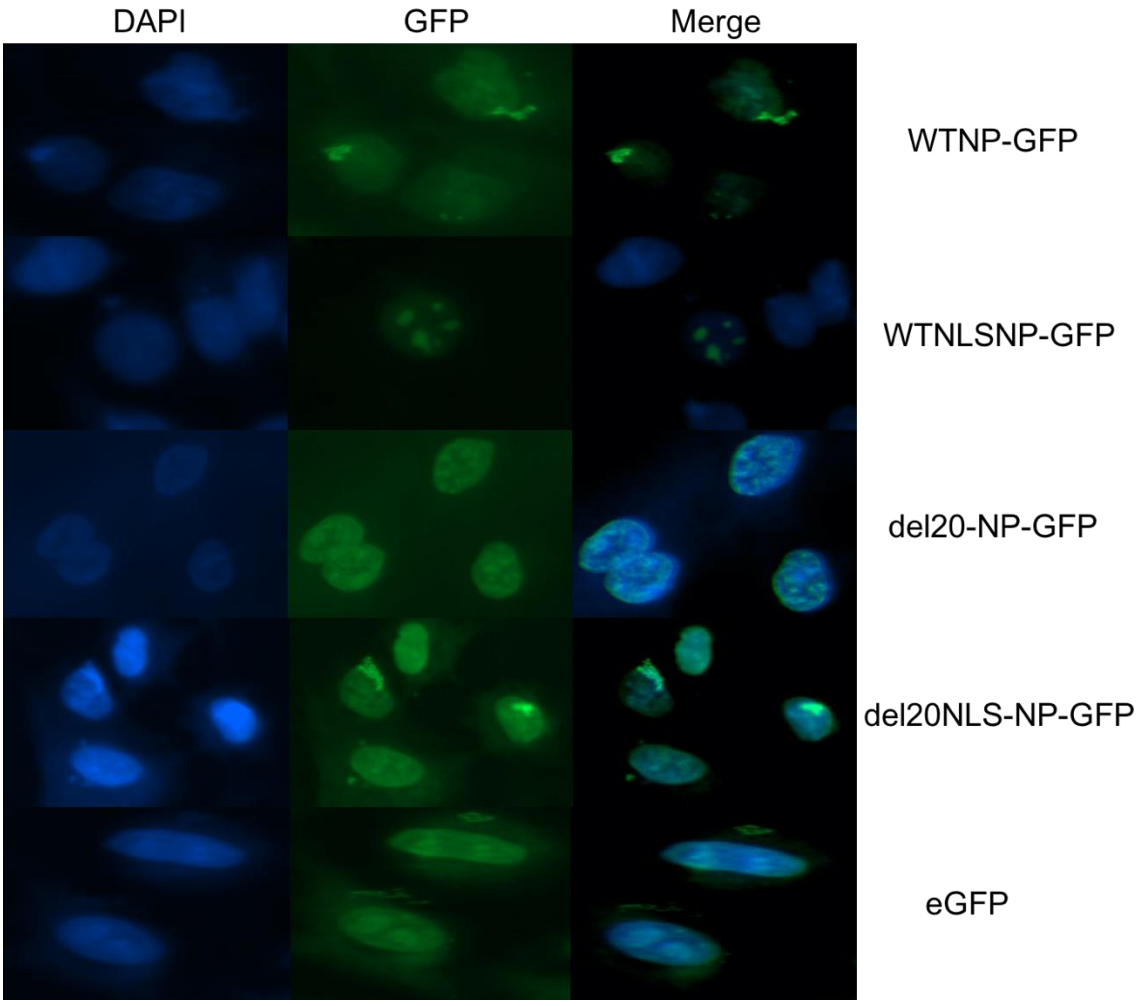


Figure 9. NP-GFP Fusion Proteins Demonstrate all NP Mutants are Located in the Nucleus

WT-NP-GFP and NP mutant-GFP proteins or eGFP were expressed in A549 cells grown on poly-L-Lysine coverslips. Cells were washed with *Dulbecco's phosphate-buffered saline (DPBS) and fixed with 2% formaldehyde. The coverslips were* mounted onto glass slides using Southern Biotech™ Dapi-Fluoromount-G™ Clear Mounting Media which stains the cell nucleus (blue). The slides were then observed using *EVOS™ FL fluorescent microscope* and images were captured with associated camera software *EVOS™ FL Color Imaging System* for GFP (green) and DAPI (blue).

CHAPTER THREE

ROLE OF INFLUENZA A NP N-TERMINUS IN VIRAL RNA SYNTHESIS

Background

The N-terminal 20 amino acids of NP is sufficient to bind host factor UAP56 (Momose et al., 2001), an RNA helicase (Shen et al, 2007) that functions as a chaperone of NP to enhance viral RNA synthesis (Momose et al, 2001; Kawaguchi et al, 2011). Further, losing the N-terminus of NP (amino acid 1-20) leads to decreased viral RNA synthesis, exacerbated by template length (Sanchez et al, 2014). Therefore, the identification of the importance of the N-terminus of NP and its role has potential to reveal new antiviral targets within NP and interacting factors.

Western blotting and NP-GFP fusion protein techniques have demonstrated that NLS1 is not required for newly made NP nuclear localization (Chapter 2). Thus, to examine the role of the N-terminus of NP in viral RNA synthesis, in addition to the mutants WTNLS-NP, del20NLS-NP, and del20-NP I also constructed an N-terminal 10 amino acid deletion mutant, termed del10-NP to further narrow down the region of NP responsible for the defect in expression from long templates (Sanchez et al., 2014). Del20-NLS-NP was used to serve as a comparison as we already knew this mutant had a severe defect. WTNLSNP, was also used to study the impact of altering the N-terminus with an additional

NLS signal on the activity of NP in viral RNA synthesis. These NP mutants were analyzed using reconstituted vRNPs.

The reconstituted vRNP assay is a transfection-based system which exploits components of the reverse genetics procedure (Neumann et al, 1999) without generating recombinant mutant viruses. Instead, the mammalian cells (in this research, 293T Human embryonic kidney cells) are transfected with plasmids to generate one type of vRNP or cRNP complexes by expressing influenza RdRP and NP proteins alongside vRNA or cRNA templates respectively (Figure 10). The vRNA template can be directly transcribed to mRNA by the influenza RdRP, while the cRNA template must first be replicated to vRNA for transcription to occur. Transcription is assessed indirectly by detection of the subsequently translated protein through immunoblot and directly by reverse transcription PCR. The reconstituted vRNP assay was set up with RdRP (PB1, PB2, and PA), the vRNA template as either Flag-M vRNA or GFP-M vRNA and WTNP, no NP, or NP mutants WT-NLS-NP, del20NLS-NP, del20-NP and del10-NP. Cells pellets were collected for either protein or RNA isolation. Western blot technique was used to analyze viral protein expression and Reverse-transcriptase (RT) with quantitative polymerase chain reaction (qPCR) were used to analyzed viral RNA expression.

Results

To study the expression of vRNP, plasmids to express reconstituted vRNP with either a short template Flag-M vRNA (7 amino acid tag) or a long template GFP-M vRNA (238 amino acid tag) were transfected into 293t cells. The cells were collected 48 hours' post-transfection and the total protein or RNA was isolated. Total protein was separated by SDS-PAGE and Western Blot with Anti-Flag to detect the viral NP and M-FLAG expression. Anti-tubulin was used as a loading control. The result show the N-terminus of NP is not essential for FLAG-M expression in reconstituted vRNPs as the del20-NP and del20NLS-NP both show some viral protein (M) expression but is essential for longer GFP-M expression (Figure 11 and 12). Interestingly, addition of SV-40 NLS at the N-terminus results in a more severe defect than del20 alone, especially for the long vRNA template (GFP-M vRNA) (Figure 12). These results indicate the N-terminus of NP is important for gene expression from long templates and deletion of the N-terminal 20 amino acids and/or perturbation of the N-terminus results in severe gene expression defect with long templates. This is consistent with the fact that encoding a FLAG tag at the N-terminus of NP results in a non-functional vRNPs, hence why our NP proteins contain a C-terminal tag (unpublished data from Fady Boutros, Newcomb lab).

To directly assess the mRNA synthesis activity of NP and NP mutants, total RNA was also analyzed. RNA was separated on a 1% agarose bleach gel to check integrity and confirm RNA concentration consistency by analysis of rRNA

(Figure 13). The samples were then treated with DNase and reverse-transcribed (RT) using oligo dT to obtain cDNA of all polyadenylated RNAs, including viral mRNAs, which then were analyzed using M specific primers and qPCR. qPCR with primers that targeted PA expression is used to normalize expression as PA is the limiting factor for vRNP expression during transfection. The results show M RNA expression is decreased in all mutants tested and with both templates indicating all NP mutants exhibit a severe defect in viral gene synthesis (Figure 14). Consistent with the indirect protein results, the RNA defect was more severe with the GFP-M template, although a significant defect was observed with the FLAG-M template as well, even with del20NP, which showed only slight M protein defect in Western analysis (Figure 11). Duplicate trials with Flag-M vRNA template and analysis by relative qPCR demonstrate 32% and 23% activity with WT-NLS-NP, 15% and 7% with del20-NP, and 1.7% and 2% with del20-NLS-NP, compared to WT-NP set at 100% (Figure 14). With GFP-M vRNA template (one trial) a more severe defect is seen, at only 2.6 % expression with WTNLS-NP, 0.8% with del20NP, and 1.6% with del20NLS-NP (Figure 14). There is less activity with the longer template (GFP-M vRNA) than the shorter template (Flag-M vRNA), consistent with the report of del20NLS-NP defect exacerbated by increasing vRNA template length (Sanchez et al., 2014). Our results reveal that addition of the NLS at the N-terminus contributes to the defect, comparing WT-NP with WTNLS-NP (100% vs 32% and 23% with FLAG-M template and 2.6% with GFP-M template). We conclude the N-terminus of NP (amino acid 1-20)

plays an important role in viral RNA expression and deletion or perturbation will inhibit viral gene expression.

To narrow down the amino acids within the N-terminus of NP responsible for the defect of vRNA synthesis. Plasmid del10NP was constructed and analyzed for vRNP function by assessing GFP expression using the reconstituted vRNP system with vRNA template GFP-M. eGFP was used as a monitor for transfection efficiency. The result shows that del10-NP displayed similar activity as WT-NP with del20-NP showing more severe defection (Figure 15). This suggests the region important for robust vRNA synthesis is likely located within NP amino acids11-20. However, RNA analysis of these samples is underway to confirm no defect is observed at the RNA level.

In summary, reconstituted vRNP assay was used to assess N-terminal mutants for RNA expression activity indirectly with Western Blot and fluorescence microscopy, and directly by RT- qPCR. Our data indicate that the N-terminus of NP plays an important role in vRNA synthesis activity, because deletion of the N-terminal 20 amino acids or perturbation of the N-terminus by addition of amino acids results in a defective protein, particularly for RNA expression from long templates. Further probing of the N-terminus with a deletion of the N-terminal 10 amino acids suggest that the region responsible for the defect of vRNA expression is likely located within NP amino acid 11-20 (Figure 15).

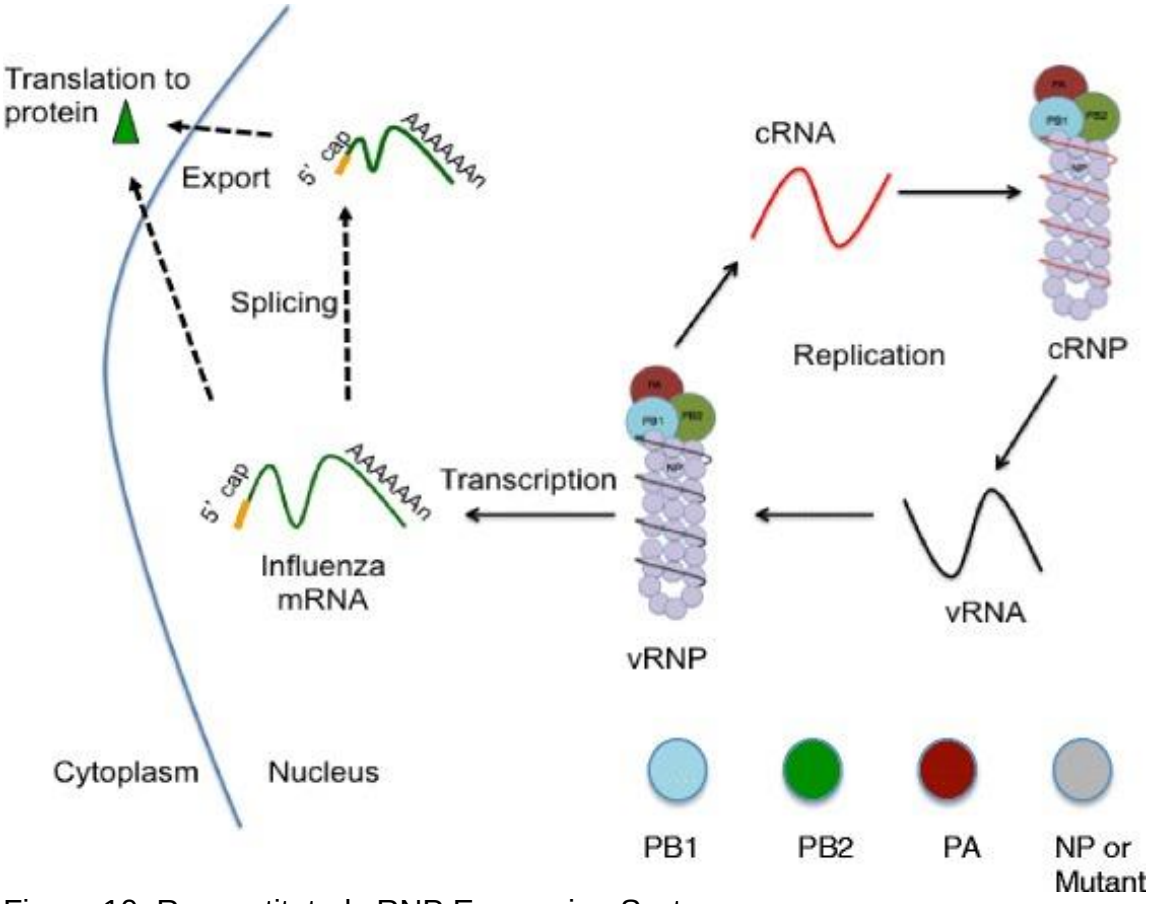


Figure 10. Reconstituted vRNP Expression System

pcDNA expression vectors of PB1, PB2, PA, and NP or mutant are transfected into 293T cells to express protein components of the vRNP. A pHH21 expression vector encoding a genomic RNA template is also transfected (either vRNA or cRNA can be expressed). Cellular RNA polymerase I drives expression of the vRNA template from pHH21. Cellular RNA polymerase II drives expression of viral mRNAs from pcDNA3 encoding plasmids.

Credit: Davis AM, Ramireez J, and Newcomb LL (2017). Identification of Influenza A nucleoprotein body domain residues essential for viral RNA expression expose antiviral target. Virol J. 14(1):22. Doi: 10. 1186/s12985-017-0694-8

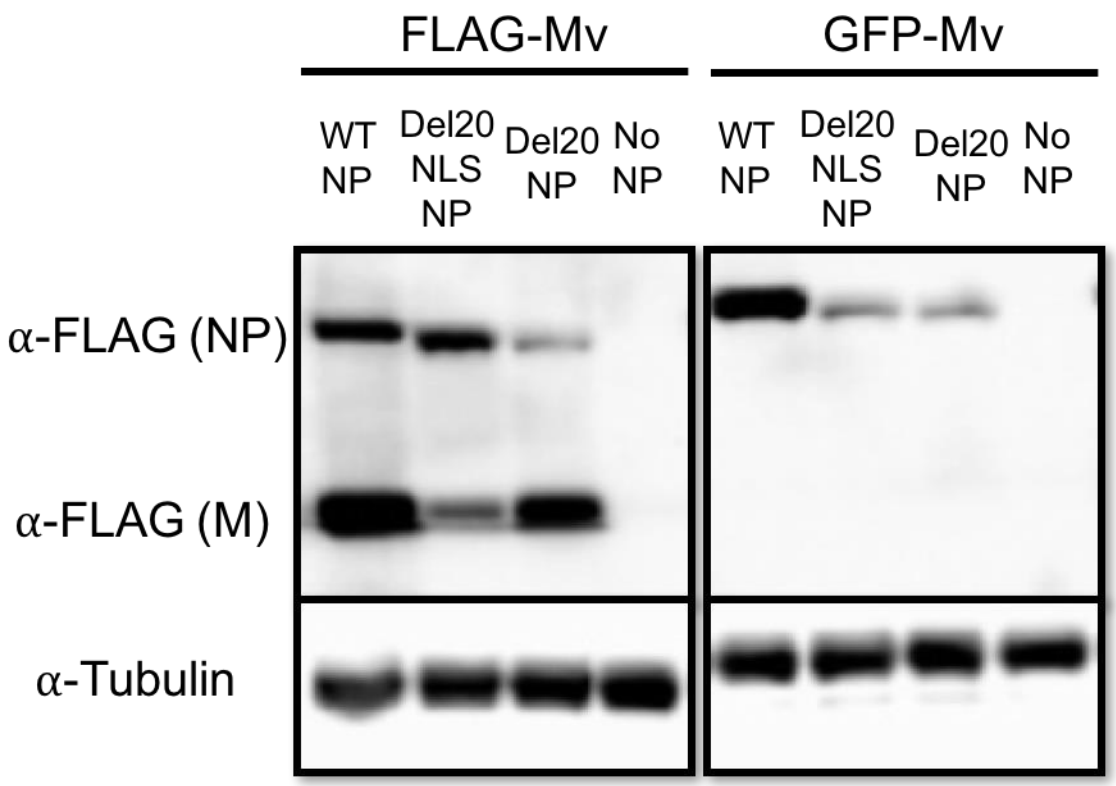


Figure 11. N-terminal NP Mutants are Expressed and Display Reduced vRNP Activity with FLAG-M vRNA Template.

293T cells were transfected with plasmids to express reconstituted vRNPs containing vRNA-M templates with either the short 7 amino acid FLAG tag or the longer 238 amino acid GFP tag encoded at the N-terminus. All NP proteins are expressed fused to a C-terminal FLAG tag for ease of detection. Total protein extracts were isolated, separated by SDS-PAGE, and transferred to nitrocellulose. Anti-tubulin was used as a loading control. Anti-FLAG antibody was used to detect FLAG-M and NP-FLAG.

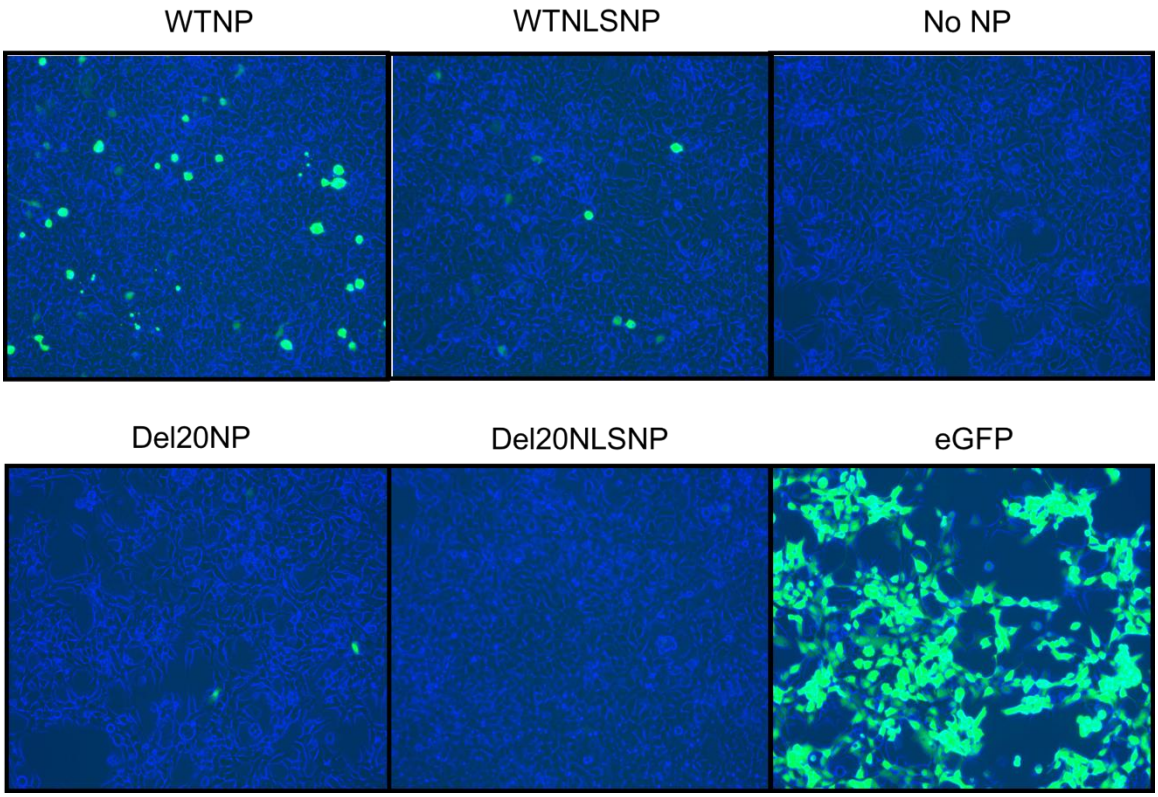


Figure 12. The N-terminus of NP is Essential for Full Reconstituted vRNP Activity from GFP-M Templates.

Plasmids to express reconstituted vRNPs (Neumann, 1999) were transfected into 293T cells, the vRNP complex containing influenza RdRP and GFP-M vRNA templates with either WTNP, WTNLSNP, del20-NP or del20NLS-NP. eGFP was transfected alone to monitor transfection efficiency. Cells were analyzed with the UV fluorescent microscopy and digital images were captured using Nikon Fluorescent inverted microscope and photo software.

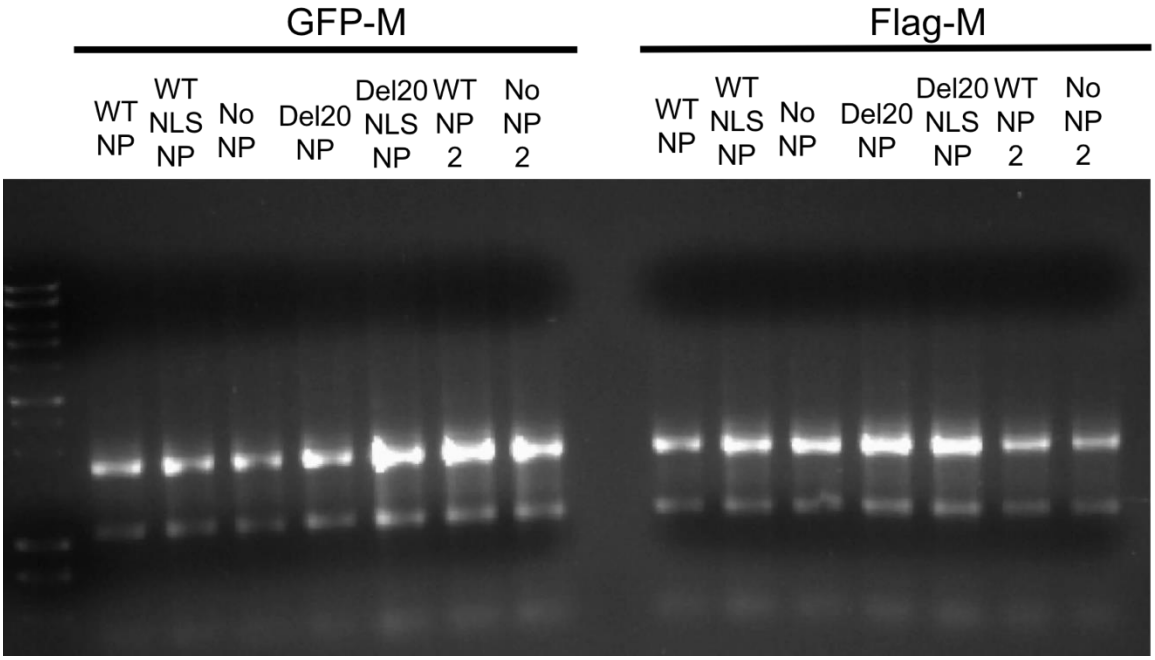
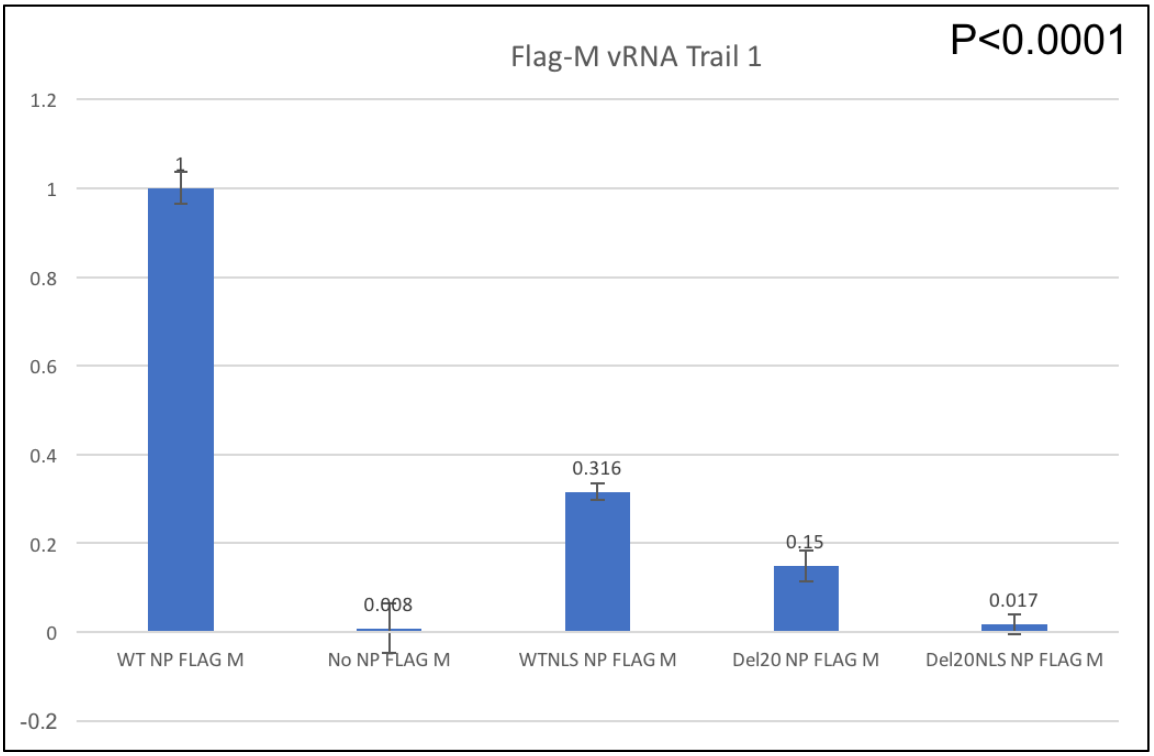
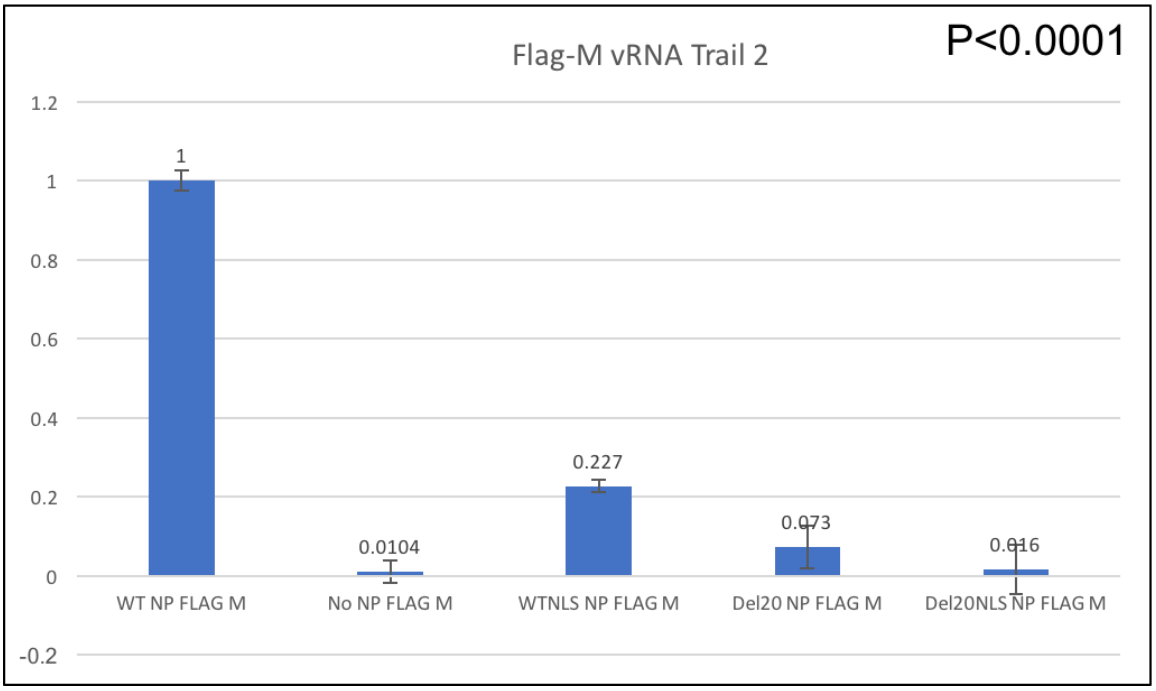


Figure 13. RNA Separated on 1% Agarose Bleach Gel to Confirm Integrity and Equal Concentration of RNA Samples.

Total RNA was isolated using Trizol according to the manufacturer's protocol. RNA was quantified and evaluated for purity by taking OD_{260} and OD_{280} readings in duplicate on the NanoDrop ND1000 Nanospectrophotometer. RNA samples were then resolved on a 1% agarose bleach gel to observe RNA integrity by intact rRNA. Shown is a representative experiment with additional WT-NP and No NP RNA samples.


Flag-M vRNA Trail 1
P<0.0001
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
1
0.008
0.316
0.15
0.017
WT NP FLAG M
No NP FLAG M
WTNLS NP FLAG M
Del20 NP FLAG M
Del20NLS NP FLAG M


Flag-M vRNA Trail 2
P<0.0001
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
1
0.0104
0.227
0.073
0.016
WT NP FLAG M
No NP FLAG M
WTNLS NP FLAG M
Del20 NP FLAG M
Del20NLS NP FLAG M

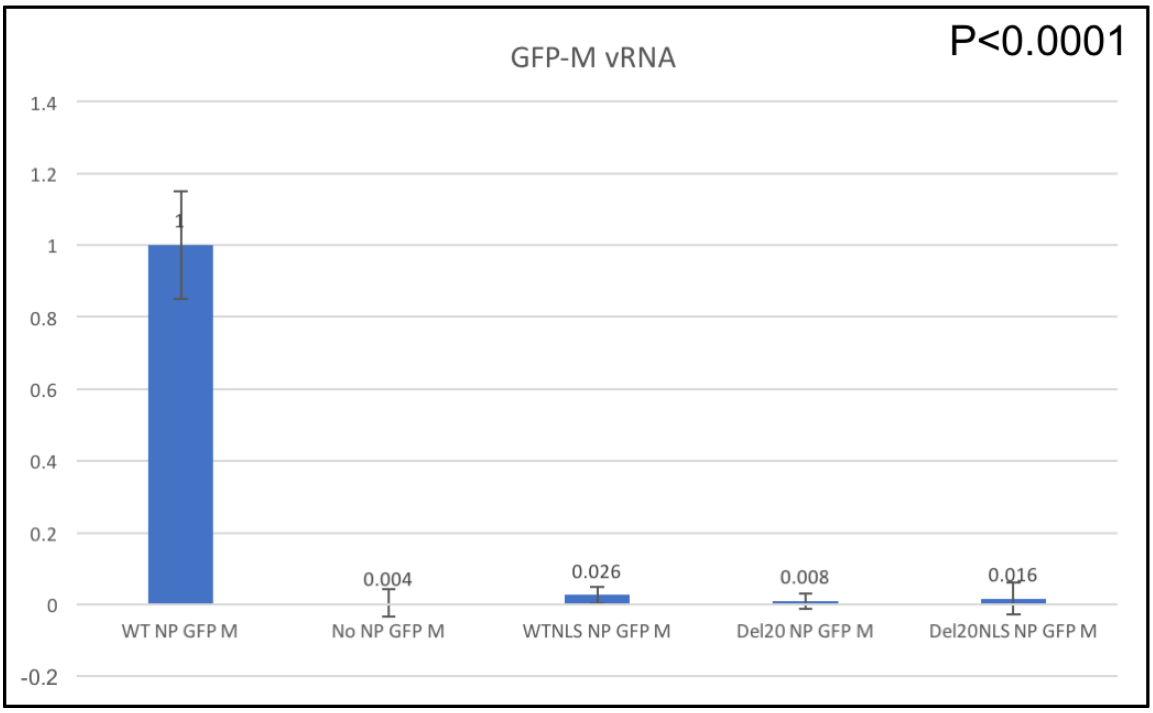


Figure 14. qPCR Data of M Expression of Duplicate Trails of Flag-M and Single Trail of GFP-M in WT-NP and NP Mutant Reconstituted vRNPs in 293T Cells.

293T cells were transfected in triplicate with plasmids to express reconstituted vRNPs with either Flag-M vRNA or GFP-M vRNA template and WT-NP or NP mutants as indicated. RNA was isolated 48 hours' post transfection. 1000ng of total RNA was treated with DNase before being reverse transcribed with oligo dT primer (mRNA) and analyzed through quantitative PCR with primers targeting the M gene. PA gene expression was used for normalization. Results were obtained from quadruplicate qPCR of duplicate trials of Flag-M vRNA and initial trail of GFP-M vRNA. Significances were evaluated through t-test by comparing with WT-NP; all samples showed *p*-values <0.0001.

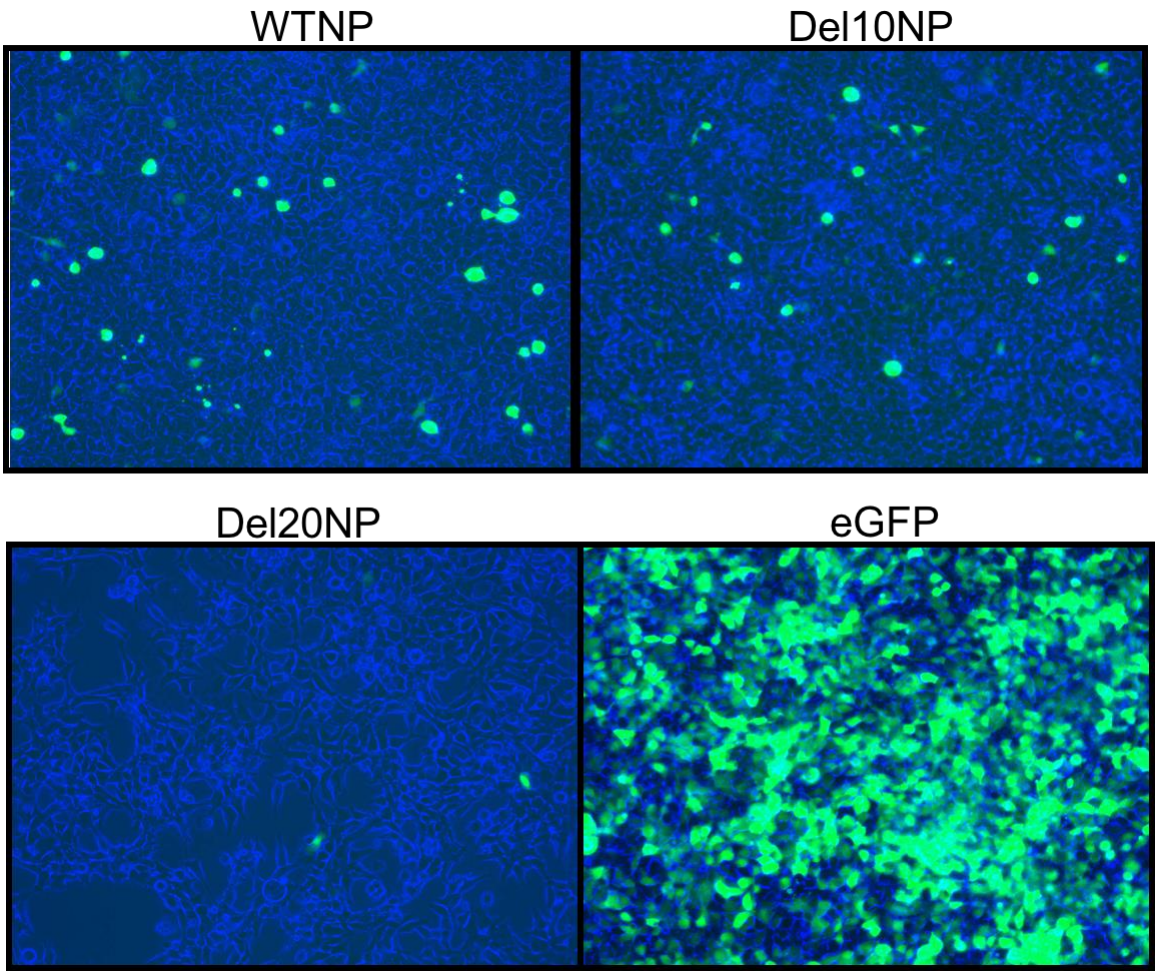


Figure 15. The Del10NP Mutant Exhibits Similar GFP Expression to WTNP in Reconstituted vRNPs.

Cells were transfected with plasmids to express reconstituted vRNPs with GFP-M vRNA template and either WTNP, del20-NP or del10-NP. eGFP was transfected alone to monitor transfection efficiency. Cells were analyzed with the UV fluorescent microscopy and digital images were captured using Nikon Fluorescent inverted microscope and photo software.

CHAPTER FOUR

NP N-TERMINUS INTERACTION WITH UAP56 HOST FACTOR

Background

The N-terminal 1-20 amino acids of NP is sufficient to bind host factor UAP56 using yeast two-hybrid assays (Momose et al, 2001). The NP-UAP56 interaction is reported to enhance viral RNA synthesis in vitro (Kawaguchi et al, 2011). We have demonstrated the importance of the N-terminus of NP for viral ribonucleoprotein activity with long templates in vivo (Sanchez et al., 2013, current study), but have been unable to co-immunoprecipitate UAP56 with NP, even after cross-linking (data not shown, Sanchez et al). In the host cell, there are multiple DEAD box helicases with similarity to UAP56, including URH49, also found to interact with NP (Pryor et al, 2004).

There are many methods that can be used to study protein-protein interaction, including co-immunoprecipitation, co-sedimentation in a gradient, yeast two-hybrid. Here, we use the density sucrose gradient approach in a small scale as a strategy to study WTNP-UAP56 interaction. The nucleoprotein has a Flag tag on its C-terminal for Western blot detection (WT-NP-flag). The density sucrose gradient exploits different sedimentation rates of complexes of differing molecular weight in a centrifugal force field to study protein interaction (Figure

16). Through their sedimentation behavior, we can infer if they interact with each other or not (Y Jin et al., 2013).

Result

To study NP-UAP56 interaction 293T cells were transfected with NP-FLAG expressing plasmid. Total protein extracts were isolated 48 hours' post-transfection. Sucrose gradients were layered to create a gradient from 40%- 5% sucrose. After incubation at 4°C overnight the gradient is ready and the protein extract added to the top of the gradient (5%) and centrifuged for 16 hours at 50,000 RPM in the ultracentrifuge at 4°C. 300ul Fractions were collected from the top of the gradient and analyzed with SDS-Page gel and Western blot.

The result of sucrose density gradient approach shows NP located at fraction 8 and 9 while the UAP56 located at fraction 3-6. They have a significantly different in their sedimentation behavior (Figure 17). Interacting proteins should demonstrate distribution identical or overlapping distribution in the gradient centrifugation, while non-interacting proteins would not (Harlan et al., 2003). Our result, along with previous co-immunoprecipitation trials, indicates NP expressed alone in the 293T host cell has no interaction with UAP56. Therefore, we did not proceed with further efforts to prove the interaction and instead pursued characterization of additional N-terminal NP mutant del10NP. We conclude that while the N-terminus of NP is important for viral RNA elongation, consistent with a defect in the reported NP-UAP56 interaction, in 293T cells I was unable to

prove this interaction exists and cannot directly assess the N-terminal NP mutants.

Another approach that we attempt to use for protein-protein interaction studying is the tripartite split-GFP system (Figure 18). Tripartite split-GFP is a novel micro-tagging system to monitor protein-protein interactions in vivo and in vitro. Its advantage is the small sizes of the GFP10 and GFP11 tagging peptides which makes it well suited to study interactions of unstable protein complexes that are difficult to detect with larger GFP tags (Cabantous et al, 2013; Figure 18). We met obstacles while trying to construct the split-GFP plasmids as the plasmids couldn't ligated during the construction, and while the sucrose gradient result came out suggesting negative interaction between NP alone with UAP56, we did not further pursue this approach, instead focusing on characterizing N-terminus of NP in viral gene synthesis.

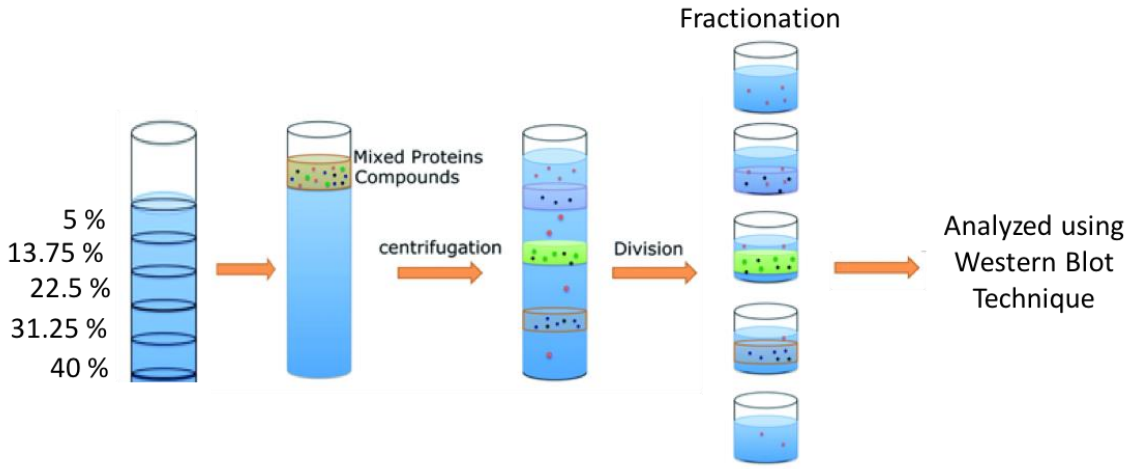


Figure 16. Sucrose Density Gradient

A demonstration graph of the small scale of sucrose density gradient centrifugation for protein isolation. A gradient of sucrose solution is prepared. Total protein from 293T cells transfected to express WT-NP-FLAG are laid on top of the sucrose gradient. The tube is centrifuged at 4 ^{o}C for 16 hours. The solution is fractionated with equal volume into 10 fractions from the top to the bottom of the tube, the proteins within each fraction are analyzed by SDS-PAGE gel and Western blot.

Credit: Modified from Y Jin, X. H. Cheng, F. Z. Yang, and L Fu (2013). Ultracentrifugation-based multi-target affinity selection mass spectrometry. RSC advance. DOI: 10.1039

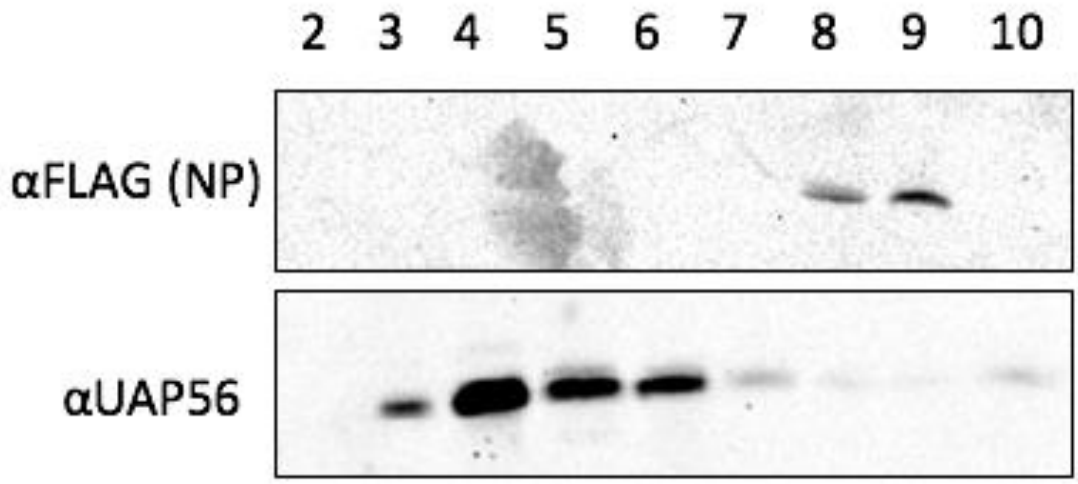


Figure 17. Sucrose Gradient Fraction of WT-NP Expressed Alone.

10 gradient fractions were collected and analyzed using SDS-Page gel and Western blot technique. Anti-Flag was used to detect WT-NP-FLAG and anti-UAP56 was used to detect the UAP56. Sucrose Gradient Fractionation reveal that WT-NP and UAP56 do not sediment together. This suggest that NP and UAP56 do not interact when NP is expressed in 293T cells.

Credit: Jennifer Gallardo, Newcomb lab

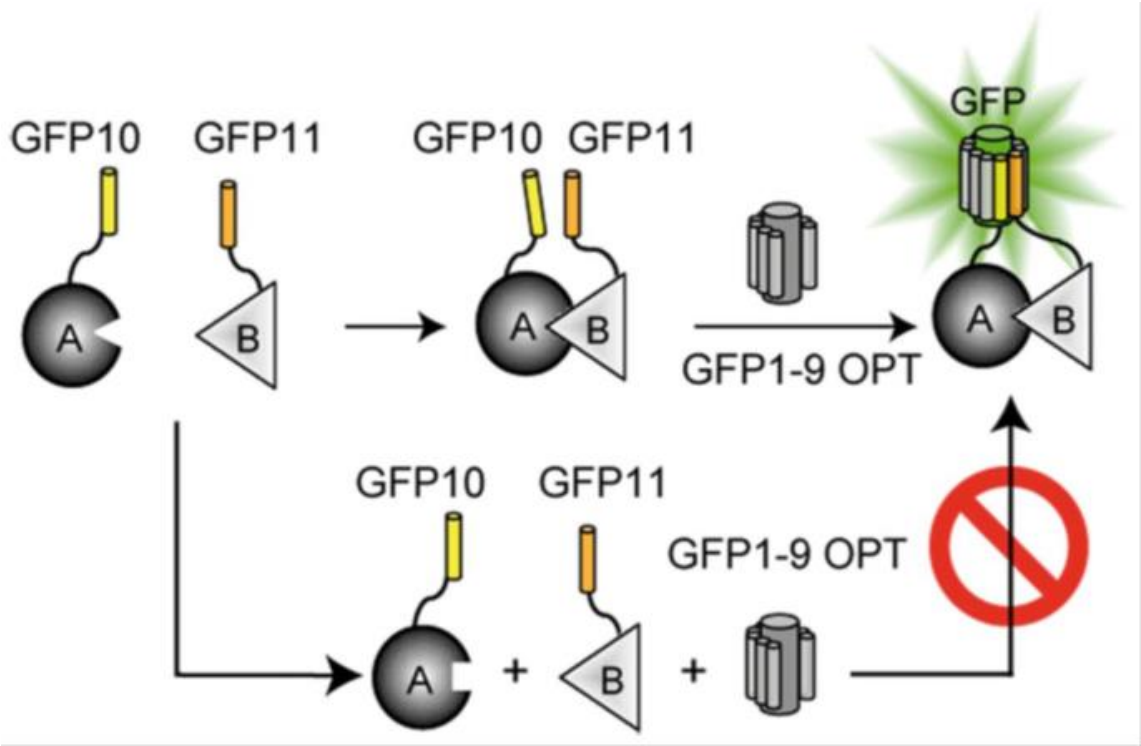


Figure 18. Tripartite Split-GFP System

The system is based on tripartite association between two twenty amino-acids long GFP tags, GFP10 and GFP11, fused to interacting protein partners, and the complementary GFP1-9 detector. When proteins to which GFP10 and GFP11 are fused to interact, GFP10 and GFP11 self-associate and recruit GFP1-9 to reconstitute a functional GFP, allowing visualization and study of the interaction between proteins (Cabantous et al, 2013).

Credit: Cabantous S, Nguyen H, Pedelacq J, Koraichi F, Chaudhary A, et al. (2013). A new protein-protein interaction sensor based on tripartite split-gfp association. Scientific Reports, 3, 2854-2862.

CHAPTER FIVE

MATERIAL AND METHODS

Cells

293T Human embryonic kidney and A549 human lung adenocarcinoma epithelial cell lines were purchased from ATCC (American tissue culture collection) and maintained in a water-jacketed incubator at 37°C with 5% CO_2 output in DMEM (Dulbecco's Modified Eagle's Medium) with 10% FBS (Fetal bovine serum). Cells maintenance and passage are performed as manufacture's protocol.

Plasmids

pcDNA plasmids WT-NP-FLAG, WT-NLS-NP-FLAG, del20-NLS-NP-FLAG, del20-NP-FLAG, del10-NP-FLAG, PA, PB1, and PB2 were used to encode Influenza A/Udorn/307/72 (H3N2) mRNA to express PA, PB1, and PB2, NP viral proteins for vRNP expression. pHH21 GFP-M vRNAs and pHH21 Flag-M vRNAs were used to express either GFP-M or Flag-M vRNP expression. The expressed GFP-M vRNA or Flag-M vRNA must be processed by viral polymerase ribonucleoprotein to be expressed as mRNA for translation to protein. WT-NP-GFP, WTNLS NP-GFP, del20NLS NP-GFP, and del20 NP-GFP were used for confirmation of viral protein localization. pcDNA plasmid

expressing PA, PB1, and PB2 were gifts from Krug lab. All other plasmids were generated in the Newcomb laboratory. PcDNA plasmids: WT-NP-Flag is constructed by Fady Boutros (Newcomb lab) and WT-NP-GFP is constructed by Alan Santana (Newcomb lab); pHH21 plasmids: Flag-M and GFP-M are constructed by Dr. Newcomb (Newcomb lab).

For this study, I constructed WT-NLS-NP-FLAG, del20-NP-FLAG, and del10 NP-FLAG using one-step PCR with WT-NP-Flag as the template. In addition to proper restriction enzymes sites, WTNLS NP was constructed to encode the conventional NLS from SV40 T antigen (PKKKRKV) after the start codon (WT-NLS-NP) while the 3' primer encoded the last amino acids of NP followed by a short glycine linker and the FLAG epitope tag (DYKDDDDK) prior to the stop codon. The del20-NP and del10-NP were constructed in a similar manner using the same 3' primer but in this case the 5' primer will encode the start codon followed by NP codon 21 or 11 (del20-NP, del10-NP, respectively). The PCR products were then digested with restriction enzymes EcoRI and XbaI and ligated into likewise cut pcDNA vector. All plasmids were sequenced at Retrogen (San Diego, USA) to confirm successful plasmid construction.

I also constructed plasmids to express additional NP-GFP fusion proteins, WTNLS NP-GFP, del20NLS NP-GFP, and del20 NP-GFP, which were constructed by one-step PCR using the WT-NP-GFP as template. The 5' primer either encoded the conventional NLS, between NP start codon and NP codon 1 (WT-NLS-NP-GFP) or codon 21 (del20-NLS-NP-GFP) or encoding the start

codon followed by NP codon 21 (del-20-NP-GFP). The 3' primer encoded the last amino acids of GFP and stop codon. PCR protocol is modified from the 'Molecular Cloning-A Laboratory Manual 3rd edition Vol2' text book.

PCR Primers

Primers designed and used to generate the mutant NPs are as follow: (from 5' to 3')

WT-NLS-NP-F/ WT-NLS-NP-GFP-F:

ATAGAATTCATGCCAAAGAAAAAGCGCAAGGTGGCGGCCGCGTCCCAAGG

C;

del20-NP-F/ del20-NP-GFP F:

ATGGAATTCATGCCAAAGAAAAAGCGCAAGGTGGCCGCGGAACAGATGGAA

ACTGATGGG;

del20-NLS-NP-F/ del20NLS-NP-GFP-F:

ATAGAATTCATGCCAAAGAAAAAGCGCAAGGTGGCGGCCGCGTCCCAAGG

C

del10-NP-F:

GCCGAATTCATGCAGATGGAAACTGATGGGGAACGCCAG;

WTNLS-NP-R/ del20-NP-R/ del20-NLS-NP-R/ del10-NP-R:

GACTCTAGATTAACCGTCATGGTCTTTGTAGTCCGCCGCATTGTCGTACTCC

WTNLS NP-GFP-R/ del20 NP-GFP-R:

ATAGAATTCATGGCGTCCCAAGGCACC

DNA Transfection

293T cells or A549 cells were grown to 70% confluency in either 100mm or 6-well dishes. Plasmid DNA was purified using Qiagen QIAprep spin plasmid purification kit as per the manufacturer's protocol. DNA with pcDNA plasmids to express either WT NP, WTNLS NP, del20NLS NP, del20 NP, del10 NP, and no NP control (pcDNA vector) alone or along with the appropriate additional pcDNA and pHH21 plasmids to express reconstituted vRNPs, were mixed with Mirus transfection reagent at a 1:3 ratio of DNA to reagent and cells are transfected as manufacturer's protocol. eGFP was used to monitor transfection efficiency. At ~48 hours post transfection cells are washed with 1X PBS, collected, and pelleted by centrifugation.

Cellular Fractionation

Cell pellets were resuspended in RSB (reticulocyte standard buffer, 10mM Tris HCl pH7.7, 10mM KCl, and 1.5mM $MgCl_2$) and after incubation 0.2% NP-40 is added to burst plasma membranes. Cells are examined under a microscope to confirm burst plasma membranes and intact nuclei. Homogenate is centrifuged 300xg for 5 minutes at 4°C to yield a nuclear pellet and cytoplasmic supernatant.

The cytoplasmic fraction is collected and the nuclear pellet dissolved in Dignam buffer C (20mM HEPES pH7.9, 0.42M Nacl, 1.5mM $Mgcl_2$, 0.2mM EDTA, no glycerol) to puncture the nuclear membrane and allow release of soluble factors. The nuclear fraction is then treated with Buffer E (20mM HEPES: KOH pH7.6, 0.2mM EDTS, no salt) to reduce salt concentration. Both cytoplasmic and nuclear fractions are centrifuged at high speed to pellet cellular debris. The soluble fractions are then collected (protocol modified from Molecular Cloning A Laboratory Manual 3rd edition Vol1).

Isolation of Total Protein

For Western blot, cell pellets were resuspended in 10 volumes of the cell pellet in RIPA Lysis Buffer (25 mM HCl pH 7.6, 150 mM NaCl, 1% deoxycholate, 0.1% SDS) with protease inhibitors. Lysed cells were subject to sonication to shear DNA using Fisher Scientific Sonic Dismembrator for 10 pulses at 30%, output 3-4.

For sucrose gradient sedimentation, cell pellet was resuspended in RSB (reticulocyte standard buffer, 10mM Tris HCl pH7.7, 10mM KCl, and 1.5mM $MgCl_2$) with 0.2% NP-40 and sonicated using Fisher Scientific Sonic Dismembrator for 10 pulses at 30%, output 3-4 to break nuclear membranes and shear genomic DNA. (protocol modified from Molecular Cloning A Laboratory Manual 3rd edition Vol1)

Isolation of Total RNA

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. RNA was quantified and evaluated for purity by taking OD_{260} and OD_{280} readings in duplicate on the NanoDrop ND1000 Nanospectrophotometer (Thermo Fisher Scientific). RNA was resolved on a 1% agarose bleach gel to observe RNA integrity and confirm RNA concentration.

SDS-PAGE and Western Blot

10% SDS-polyacrylamide gel was prepared to separate total protein extracts. Proteins were transferred to nitrocellulose using Fisher semi-dry transfer apparatus. Blots were incubated in primary antibody to recognize target and secondary antibody coupled to HRP. Pierce ECL reagents were used to detect HRP conjugated secondary antibody. Blots were developed using the Chemi-Hi setting on the ChemiDoc XRS (BioRad) System and digital images were obtained using Quantity One software (protocol modified from Molecular Cloning A Laboratory Manual 3rd edition Vol3 - Appendix).

Antibodies

Anti-FLAG antibody was purchased from Agilent, Anti-Hsp90 and anti-Tubulin were purchased from Abcam, all antibodies were used at 1:1000 dilution

per manufacturer's suggestion. Secondary HRP coupled anti-goat were purchased from Pierce and used at a 1:10,000 dilution as per manufacturer's suggestion.

Sucrose Density Gradient Centrifugation

Sucrose gradients were made by layering of sucrose solutions prepared as 5%, 13.75%, 22.5%, 31.25%, and 40% sucrose. The sucrose layers were incubated overnight at 4^{o}C to form a linear gradient. Total protein extract isolated using RSB and sonication was laid atop the gradient and centrifuged at 4^{o}C for 16 hours at 50,000 RPM. Ten fractions were collected and proteins resolved by SDS-PAGE and analyzed by Western blot (Protocol modified from The Laboratory of Andrew Murray at Harvard University).

DAPI and GFP Visualization

To assess NP localization, plasmids expressing NP-GFP or eGFP were transfected into A549 cells grown on poly-L-Lysine cover slips. 48 hours' post transfection the cells were washed with 1XPBS 3 times for 5 minutes each and fixed with 2% formaldehyde for 20 mins at room temperature, and washed again with PBS (protocol modified from Dr. Thompson, CSUSB). Coverslips were mounted onto glass slides using SouthernBiotech™ DAPI-Fluoromount-G™

Clear Mounting Media for 4 minutes, which stains the cell nucleus (protocol provided by SouthernBIotech). DAPI and GFP was observed in cells at 48 hours' post-transfection on a EVOS^{TM} FL fluorescent microscope and images were captured with associated camera software EVOS^{TM} FL Color Imaging System.

For reconstituted vRNP assays, the visualization of GFP-M fusions was done with a Nikon Eclipse TS100 (Nikon Intensilight C-HGFI for fluorescence) inverted microscope and images captured with the Nikon DS-Qi1Mc camera with NS Elements software.

Reverse Transcription

Prior to reverse transcription RNA was first treated with RNase-free DNase (Promega) to degrade any contaminating plasmid DNA. 1 µg total RNA was subjected to reverse transcription using oligo dT and AMV reverse transcriptase. Promega reverse transcription system was used as per manufacturer's protocol with the modification of increasing the reverse transcription time to 59 minutes to facilitate complete cDNA synthesis. Reverse transcription reactions were aliquoted from a master mix to ensure all samples received equivalent AMV-RT enzyme.

Quantitative PCR: Protocol and Primers

Quantitative PCR (qPCR) reactions were run in the Applied Biosystems StepOnePlus Real Time PCR system using SYBR Green Master mix (Applied Biosystems), with ROX as the reference dye.

Gene specific primers are as follow:

M detection:

(F) CGAGTATCATTGGGATCTTGCA, (R) TTCCTTTCGATATTCTTCCCTCATA

PA detection:

(F) GGACAAATGGAACATCAAAGATTAAA,

(R) CAGAAGACTCGGCTTCAATCATG

Statistical Analysis of qPCR Data

Melt curve was confirmed to contain just one prominent peak above 72 ^{o}C before analyzing the qPCR data, to confirm specific PCR product amplification. Raw CT values were analyzed in Microsoft Excel using the ΔCt/ΔCt formula of 2^CTaverage control sample/2^CTaverage tested sample. Therefore, PA expression is used to normalization expression because PA is the limiting factor for vRNP expression during transfection. The $\Delta\Delta$Ct formula is: 2^ ((CT average control sample) – (CT average housekeeping control)) – ((CT average tested sample) – (CT average housekeeping tested sample)). Error bars (standard error) were obtained by calculating the standard deviation of the

sample set divided by the square root of the sample set size. WT-NP samples were set at 100% and mutants analyzed by comparison to this reference.

CHAPTER SIX

DISCUSSION AND FUTURE RESEARCH

Discussion and Conclusion

The Nucleoprotein of Influenza A plays multiple critical roles within various stages of the Influenza viral life cycle. Pertinent to this project is the role of NP in viral RNA expression. Specifically, DEAD-Box RNA helicase UAP56 was identified in yeast-two hybrid assay to identify the interaction of host factors and NP. Yeast-two hybrid techniques were further employed to demonstrate sufficient binding between the first 20 amino acids of NP and DEAD-Box RNA helicase UAP56 (Momose et al., 2001). A cell-free system was then used to study the role of NP in viral replication and it was found that addition of UAP56 to the in vitro assay resulted in enhanced viral RNA synthesis (Kawaguchi et al., 2011). Consistent with these findings, an N-terminal deletion mutant, termed del20NLSNP validated the significance of the N-terminus of NP aside from vRNP and NP nuclear import by finding this N-terminal deletion with additional nuclear localization signal maintained nucleotide binding and nuclear localization but displayed diminished influenza RNA expression and vRNP formation, exacerbated by vRNA template length (Sanchez et al., 2014). These studies support further characterization of the importance of the N-terminus of NP and its

role in viral RNA expression to reveal new antiviral targets within NP and interacting factors.

In NP nuclear localization study, our results demonstrated the additional NLS of NP is not essential for newly expressed NP nuclear localization in reconstituted vRNP assays (Figure 11). Further, we show addition of an NLS to the N-terminus of NP results in a more severe defect to vRNP activity as assessed by gene expression from the vRNA template. This result is consistent with an unpublished observation that encoding a FLAG tag at the N-terminus of NP results in non-functional vRNPs (unpublished data from Fady Boutros, Newcomb lab). To obtain a stable NP crystal for crystal structure analysis, the N-terminus of NP had to be deleted (Ye Q et al., 2006), which suggests the N-terminus of NP is a flexible region and might play an important role for NP function. Our results demonstrate adding amino acids to the N-terminus of NP results in inhibit of viral gene expression.

Our data comparing two templates of varied length provide evidence that the N-terminus of NP is important for optimal gene expression especially as the vRNA template is lengthened. This data fit with the proposed role of UAP56-NP interaction to facilitate NP encapsidation of vRNA and cRNA templates, resulting in more templates for increased gene expression (Kawaguchi et al., 2011). While other groups have shown NP-UAP56 interaction, this was done either with yeast two-hybrid assay (Momose et al., 2001), *in vitro* purified proteins (Kawaguchi et al., 2011), or in cells using expressed FLAG-UAP56 (Wisskerchin et al., 2011).

All these approaches can result in false positives and are not representative of endogenous NP-UAP56 interaction. Unfortunately, our laboratory was unable to co-immunoprecipitate endogenous UAP56 with expressed NP-FLAG in 293T cells, even with cross-linking (Sanchez, Newcomb lab, data not shown) or demonstrate interaction using sedimentation on sucrose gradient (Figure 17). While a negative result on the sucrose gradient does not disprove interaction, the lack of interaction in cross-linking and co-IP together with the sucrose gradient are strong evidence these factors do not interact in the cell. However, given complexes can separate during sucrose gradient analysis, a control showing two proteins maintaining interaction through the sucrose gradient will help confirm the negative result. Indeed, we are able to demonstrate NP-PB2 interaction with this approach (data not shown). We conclude that in our hands, NP expressed alone in 293T cells do not show demonstrable interaction with UAP56 (Figure 17). We speculate another host factor, perhaps the UAP56 paralog URH49, interacts with the N-terminus of NP in the cell.

In conclusion, the N-terminal NLS1 of NP is not essential for NP nuclear localization in our reconstituted vRNP assay. Addition of SV-40 NLS was shown not only to be unnecessary for proper NP localization, but also resulted in further defect to viral gene expression. Our results indicate modification of N-terminus of NP perturbs NP and inhibits gene expression, especially as template is lengthened. We speculate adding peptide sequence to the N-terminus disrupts an important N-terminal NP interaction. Interestingly, we narrowed the region

responsible for the phenotype observed with del20NP as possibly located within NP amino acids 11-20. This is based on an indirect approach using protein gene expression with del10NP vRNPs, which quantitatively appear to function as well as WTNP vRNPs. However, we have not elucidated the exact mechanism of the defect. Furthermore, we were unable to demonstrate interaction between WTNP and host factor UAP56, and therefore cannot assess interaction with our NP mutants. However, our data disputes existence of NP-UAP56 interaction and we hypothesize a different interaction is of importance, and that this interaction is disrupted with our N-terminus NP mutants.

Future Direction

Future studies will build off this work to narrow down and locate the specific amino acids responsible for the defect of viral gene expression observed with reconstituted vRNPs. The del10 reconstituted vRNP GFP expression suggest the region is located within NP amino acids 11-20 (Figure 15). However, direct RNA analysis is required for confirmation.

Given multiple attempts to confirm the reports of NP-UAP56 interaction, future research will first determine if the paralog of UAP56, URH49, is the *in vivo* host factor interacting with the N-terminus of NP and relevant in our experiments. If not, the plasmids constructed for the tri-partite split GFP assay could be used with a cDNA library fused to GFPd11 to identify additional potential interacting

factors. Further, an N-terminal NP-GFPd10 could be constructed and used with a cDNA library fused to GFPd11 in the tri-partite split GFP assay to specifically select for proteins which interact with the N-terminus of NP.

Lastly, I contributed to attempts to make a recombinant virus expressing NP-FLAG. I constructed a pHH21 plasmid to express the NP-FLAG coding sequence as a vRNA genome segment. For this construct a section of the coding sequence needed to be repeated to ensure NP-FLAG vRNA genome packaging in the new virions. Although our initial attempts to generate recombinant NP-FLAG expressing virus was not successful, future research aims to generate this NP-FLAG virus to serve as a tool for studying NP host factor interaction during actual virus infection

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