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Influenza A virus: A role for the RNA polymerase in viral particle assembly



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# INFLUENZA A VIRUS:
# A ROLE FOR THE RNA POLYMERASE IN
# VIRAL PARTICLE ASSEMBLY

by

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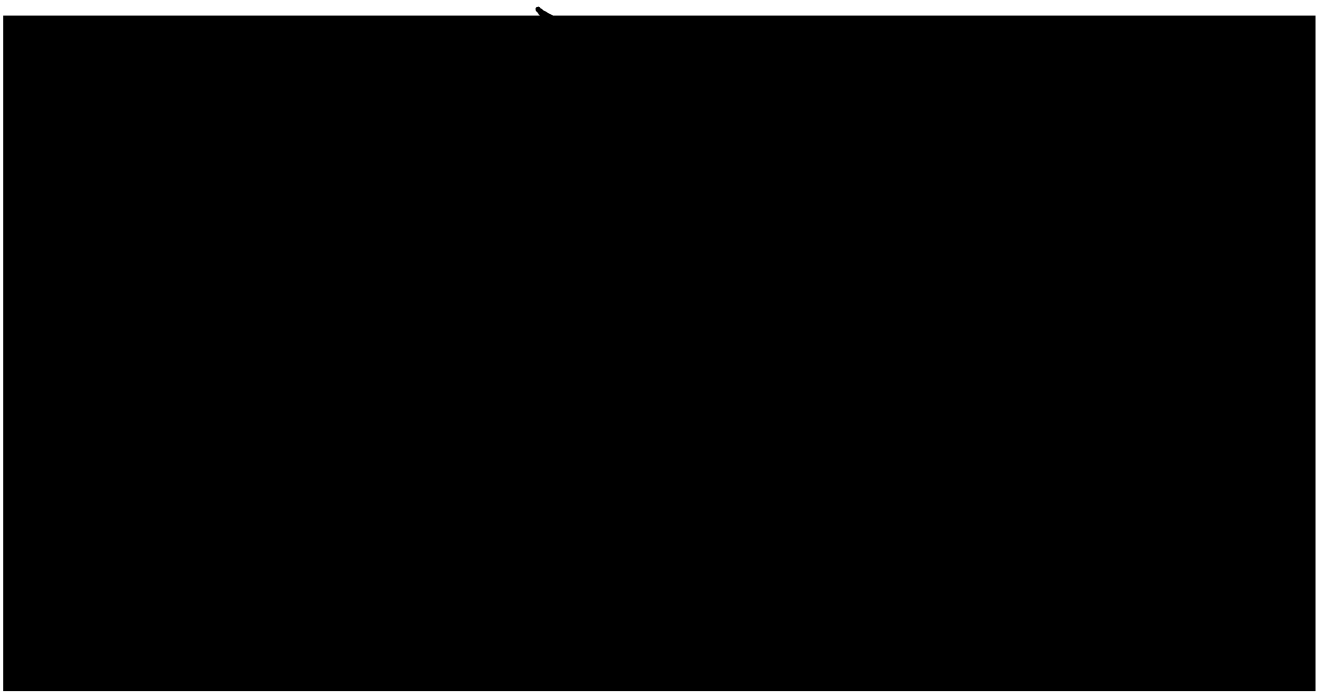

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Influenza A Virus: A Role for the RNA Polymerase in Viral Particle Assembly

John F. Regan

## Abstract

Influenza is an RNA virus whose segmented genome is encapsidated into viral ribonucleoprotein complexes (vRNPs). Upon infection, the vRNPs migrate into the nucleus where transcription and replication take place. The vRNPs contain a RNA-dependent RNA polymerase that is responsible for viral transcription and replication. The polymerase is composed of three subunits, PB1, PB2, and PA. PB1 has polymerase activity and PB2 is involved in viral transcription. The function of PA is unclear. To help elucidate the role of PA in the viral life cycle, 16 conserved regions of PA were targeted for alanine substitution. A plasmid-based transfection system was used to generate recombinant influenza particles bearing each mutation, which were tested for viral viability and the ability of each mutant polymerase to transcribe and replicate a reporter. Mutations in the N-terminus were not well tolerated and resulted in either non-viable or attenuated viruses. One of the mutants, J10, was capable of RNA synthesis, yet did not create viral particles capable of plaque formation in MDCK cells. Specifically, when compared to wild-type, this mutant synthesized 50±7% vRNA, 86±12% mRNA, and 128±18% cRNA. These levels are compatible with viability, as mutants J8 (27%) and J12 (23%), produced significantly less vRNA than J10, yet were viable by plaque assay. The mRNAs generated from J10 polymerase were found to be translationally-active, and both the mutant protein and its RNA products were appropriately localized in the cytoplasm, where influenza assembly occurs. Nevertheless, J10 failed to generate infectious particles from cells in a plasmid-based influenza assembly assay, and hemagglutinating material from the supernatants of such cells contained little or

no nuclease-resistant genomic RNA. These findings suggest that PA has a previously unrecognized role in assembly or release of influenza virions, perhaps influencing core structure or the packaging of vRNAs or other essential components into nascent influenza particles.

Abstract Approved: 

Thesis Advisor

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# Chapter 1. Introduction

Influenza epidemics were originally thought to be due to the "influence" of the stars. Accordingly, the word "influenza" was derived from the Latin word *influentia*, which means, "influence." It is unclear how long influenza has been infecting humans, for its symptoms are similar to other respiratory ailments, but historical records suggest that influenza-like illnesses affected people before the birth of Christ. Over the years, influenza has been called numerous names, but most commonly it is referred to as the flu or the grip (grippe).

Annually, influenza causes approximately 36,000 deaths in the United States. However, because influenza also circulates in other animal populations, on occasion these "foreign" influenza viruses can infect humans and cause a pandemic. Such an event occurred in 1918, when more than 500,000 Americans died, the majority of which were middle aged and healthy. Worldwide, this pandemic caused 20 - 40 million deaths. No other infectious agent or war has caused more deaths in such a short time frame.

Currently, influenza experts are carefully monitoring the recent epidemiological developments in Asia, which have raised the concern that such a catastrophe could occur again. In Asia, a strain of avian influenza (H5N1), which is lethal in chickens, has infected over 100 people since 2003, killing 50% of those infected. Fortunately, this virus is not currently efficient at human-to-human transmission; however, this virus continues to mutate and is becoming more adept at infecting a broad range of animals. To minimize the probability that this virus mutates into a strain capable of causing a pandemic, governments have ordered the slaughter of over 100,000,000 chickens to curb the spread of the disease. Unfortunately, these measures have not eliminated the threat and the virus continues to spread across Southeast Asia.

Presently, our defenses against a H5N1 are weak, since vaccine development is slow and not yet available to the public, and drug studies have suggested that only one of the four available influenza drugs may be effective in prophylaxis. As influenza is

capable of mutating and evolving quickly, one mutation could render this drug ineffective and leave humans extremely vulnerable to this disease. To address these shortcomings, more research is needed to better understand this virus so that additional therapeutics can be developed to combat this potentially deadly pathogen.

## A. Origin of Human Influenza

Birds are the original host for influenza. The probability of avian influenza successfully infecting a mammalian host is small, and a rare event. Nonetheless, avian influenza has successfully been transmitted, often through an intermediate host, into humans and established a stable reservoir that is evolutionarily independent of avian influenza. Cross-species transmission and evolution has generated three different types of influenza (Type A, B, and C) that circulate today in humans [1].

It is not known how long influenza has been circulating in humans. Historical records describe individuals with flu-like illness (Hippocrates, 412 B.C.), but these symptoms are common for many illnesses. Analysis of the evolutionary rate of swine and human NP protein, which is 3.39 substitutions per year, suggests that today's human influenza A virus lineage is slightly more than 100 years old [2]. The evolutionary rate is estimated by creating a regression of the year of isolation against the branch distance from the common ancestor node of the nucleotide phylogenetic tree. Extrapolation of the regression line suggests that today's human influenza A virus lineage began in the late 1800's. This study does not exclude the possibility that human influenza existed centuries ago, and naturally became "extinct" before re-appearing in the 19th century. Similar analysis suggests that the swine influenza began shortly after the human lineage.

Influenza has also created stable reservoirs in pigs, and is capable of infecting other mammals including horses, seals, and whales, but these species to do not maintain stable reservoirs. It is unclear whether original human influenza infection occurred directly from birds or first infected a mammalian intermediary such as pigs for adaptation

prior to infecting humans. The initial jump into a new species must occur "in toto", which means that the parental virus must be able to successfully replicate in both hosts, without the aid of another virus. This does not hold true when cross species infection occurs and the infected cell is already infected by another type of influenza, which enables cooperation between infecting viruses that can result in a hybrid virus. Dual infection of the same cell has allowed for human type A influenza viruses to acquire "parts" of avian influenza, which has generated new subtypes, such as H1N1, H2N2, and H3N2. Such double infections likely occur in pigs that are susceptible to both human and avian influenza.



## B. Discovery of Influenza

The flu virus was first isolated and confirmed as a causative infectious agent of influenza in 1933 by Dr. Wilson Smith. The strain Dr. Smith isolated is commonly used in research today, and bears his name, WSN/33, for Wilson Smith Neurotropic, isolation date: 1933. The strain was found to be neurotropic, after Dr. Smith contracted the virus from an infected laboratory ferret and died from a cerebral infection. To this day, it is unclear what factors make WSN neurotropic. Since its isolation, the strain has been extensively passaged in tissue culture and mice and is no longer considered to be very pathogenic in humans. WSN/33 is the strain studied in this thesis.

## C. Types and Classification

The influenza virus is a member of the Orthomyxoviridae family, which includes enveloped negative-stranded segmented viruses. There are three types of influenza, type A, B, and C. Type A and B are the causative agents of pandemics and as a result are represented in current vaccines. Type A viruses not only infect humans but can also infect a wide variety of avian species and other mammals including pigs, horses, whales

and seals. Type B viruses only naturally infect humans and seals, and type C viruses infects humans and swine, but is not thought to be capable of causing an epidemic. The other genera of the Orthomyxoviridae family include thogotoviruses and the isaviruses, which includes the tickborne viruses and the infectious salmon anemia virus (ISAV) [3], respectively. Due to their structural and genetic similarity, thogotoviruses are sometimes referred to as influenza D viruses.

Influenza types and lineages are determined by antigenic differences in the matrix (M) proteins and nucleocapsid (NP) protein [2].

Influenza type A viruses are classified into subtypes according to the antigenic nature of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Influenza B viruses also have HA and NA proteins, but are not divided into subtypes. In contrast, influenza C viruses have a single multifunctional glycoprotein that is called hemagglutinin-esterase-fusion protein (HEF). HEF organizes into a hexagonal array that is morphologically distinguishable from type A and B viruses by electron microscopy [4]. Due to the presence of HEF, type C viruses have seven genome segments rather than eight as in type A and B viruses.

Influenza strains are named to provide details that describe the strain. The nomenclature of the strains indicates species from which the virus was isolated/the virus type/location of where the strain was first identified/the isolate number/and the year of identification. The subtype of the virus' glycoproteins HA and NA are often included. For example, human A/New Caledonia/20/99 (H1N1).



## D. Mode of Infection, Symptoms

Influenza is a respiratory infection that generally spreads from one infected individual to the next through aerosolized virus-containing droplets that are produced when an infected individual coughs, sneezes, or speaks. The virus enters through the airway and infects the lining of the respiratory tract. This occurs much more efficiently

by inhalation rather than by touching one's eyes, nose or mouth with a contaminated hand. Individuals are contagious one day prior to the onset of symptoms, and remain contagious for three to seven days after symptoms have appeared. Children can pass the virus after the seventh day of symptoms and are often responsible for bringing the virus into close contact with elderly living at home. Symptoms begin one to four days after initial infection. Symptoms can be mild to severe, with most cases being moderate. Some individuals can be infected, yet are asymptomatic and can still pass the virus.

Symptoms can include fever, sore throat, malaise, myalgia, severe prostration (fatigue), chills, headache, nasal congestion, and dry cough. Clinically leukopenia is often observed and C-reactive protein levels rise, which is a marker for systemic inflammation. The dry cough may turn productive upon secondary infection. Such bacterial/viral pneumonia can exacerbate symptoms and greatly increases the mortality rate of influenza. While vomiting, diarrhea, and nausea can sometimes be related to influenza infection, these problems are rarely the main symptoms since influenza is generally a respiratory illness, not an illness of the gastrointestinal tract. However, some strains of influenza are capable of systemic infection. Most people recover from influenza within one to two weeks after the initial onset of symptoms.

Each year, 10-20% of the U.S. population contracts influenza and over the past several years an average of 36,000 people die per year [5]. Approximately 114,000 excess hospitalizations are caused per year by influenza. Young children and the elderly are more likely to develop complications from infection due to underdeveloped or aging immune systems. Infection also exacerbates other health problems such as chronic congestive heart failure and asthma.

Some differences in symptoms exist between the currently circulating type A subtypes (H3N2 and H1N1) and type B viruses [6]. In general, H3N2 infections are more severe than H1N1 or B in terms of fever, leukopenia, and C-reactive protein, but other symptoms are equal among all infections. Gastrointestinal symptoms are more common

in influenza B infections. Type B infections are more frequently associated with Reye's syndrome, which is discussed below. Type C virus is milder than type A and B viruses, and has become uncommon in recent years.

In the Northern Hemisphere, the influenza season generally starts in November and lasts through April, whereas in the Southern Hemisphere it runs from April to September. Influenza can circulate at any time of the year, including in the tropics. Since the flu season overlaps with other respiratory illnesses such as respiratory syncytial virus (RSV), which has similar symptoms, it is often incorrectly diagnosed. Other infections including mycoplasma pneumonia, adenovirus, rhinovirus, parainfluenza viruses, *Legionella spp.*, anthrax, and SARS, all share similar symptoms to influenza and as a result, laboratory tests are required to confirm a diagnosis. Some of the tests that can be used to determine whether influenza is the cause of symptoms include rapid diagnostic tests that take less than 30 minutes to complete. Such tests are available from Becton-Dickinson, Thermo BioStar, Binax, Quidel, and ZymeTx. Other tests include viral culture, DFA immuno-fluorescent antibody staining, RT-PCR, serology, and Enzyme Linked ImmunoSorbent Assays (ELISAs).

The laboratories of the World Health Organization (WHO) that are based in Tokyo, Melbourne, and London and the laboratories of the National Respiratory and Enteric Virus Surveillance System (NREVSS) monitor influenza activity throughout the year. Between September 29th, 2002 and May 29th, 2003, WHO and NREVSS laboratories tested a total of 94,966 specimens that had been isolated from patients around the world. Of these specimens, 11.6% were positive for influenza. Of these 57% were influenza A viruses and 43% were influenza B viruses. Of the type A viruses that were subtyped, 75% were H1 and 25% were H3N2. Influenza A viruses were reported more frequently in the New England, East North Central, Pacific, Mountain, and Mid-Atlantic regions, and influenza B viruses were reported more frequently than influenza A viruses in the West North Central, West South Central, South Atlantic, and East South

Central regions [7].

## E. Treatment and Prevention of Infection

Treatment for influenza usually consists of rest, drinking fluids, and taking medications. The antiviral drugs that are currently available for influenza are more effective for prophylaxis than treatment. The four U.S. available drugs include amantadine hydrochloride (Symmetrel® - Endo Laboratories), rimantadine (Flumadine® - Forest Laboratories), zanamivir (Relenza®), and oseltamivir (Tamiflu® - Gilead Sciences Inc.). Amantadine and rimantadine target the M2 ion channel and zanamivir and oseltamivir are neuraminidase inhibitors. Each drug can be used to treat type A influenza, but only zanamivir and oseltamivir are effective against type B viruses. Unfortunately, studies suggest that drug treatment only shortens the duration of symptoms by approximately one day, and only if treatment is started within the first two days of illness. The narrow window for receiving a prescription, the potential side effects, and the relative lack of relief make these drugs unexceptional. Some of the side effects to amantadine and rimantadine include anxiety, nervousness, and nausea, but more serious side effects are possible. Individuals with a history of seizures should not take these drugs. These side effects are more common with amantadine then rimantadine. Some of the side effects to zanamivir include, but are not limited to diarrhea, nausea, cough, and headache. The most common side effects of oseltamivir are nausea and vomiting. The side effects to these drugs are considered to be transient and will pass within a week of ending treatment.

Zanamivir has not been approved for prophylaxis, but each of the other three drugs has been approved for prevention of type A infection. Relenza (zanamivir) is an inhaled drug that may be more impervious to flu virus resistance than Tamiflu, which is the leading flu drug. However, GlaxoSmithKline cut back its marketing of Relenza in

2000 in response to disappointing sales.

Only oseltamivir can be used to also prevent type B infection. Some age restrictions exist depending on whether the drug is used for treatment or prevention. When used for prophylaxis, amantadine and rimantadine are 70-90% effective in preventing illness from influenza A viruses, whereas oseltamivir is 80-90% effective in preventing illness due to both influenza A and B viruses. The data on the efficacy of oseltamivir as a prophylaxis is mixed since one study showed only a 3.5-4% reduction in serologically confirmed influenza and suggested no sound evidence that oseltamivir is effective against type B influenza [8]. Amantadine, rimantadine, and oseltamivir are taken in tablet or syrup form, whereas zanamivir is inhaled and not recommended for individuals with other lung diseases. Generally these drugs are taken concomitantly with the vaccine, and are used to prevent infection while the vaccine stimulates the immune system.

Other anti-influenza drugs are in development. For example, Peramivir (Bcx-1812, RWJ-270201) (Biocryst Pharmaceuticals) is another orally administered neuraminidase inhibitor. The drug is currently in clinical trials and may have potential for treatment in immune compromised patients [9]. There is evidence to suggest that combinatorial drug treatment may help influenza patients, for treatment with both a neuraminidase inhibitor (zanamivir, oseltamivir carboxylate, or peramivir) and rimantadine has shown to exert an additive and synergistic anti-influenza effect in MDCK cells [10].

Children with influenza like symptoms should not be given aspirin (acetylsalicylate, acetylsalicylic acid, salicylic acid, salicylate), since aspirin may promote the development of a rare and serious illness called Reye's syndrome. Reye's syndrome often follows acute febrile illnesses such as those caused by the influenza virus, especially Type B viruses, or varicella-zoster virus (chickenpox). The syndrome results in fatty accumulation in many tissues, especially the brain and liver. The symptoms

include recurrent vomiting, agitation, and lethargy. If left untreated, the patient can experience edema of the brain, intracranial hypertension, cerebral herniation, coma, and death. The mortality rate of Reye's syndrome is 30-50%, but if it is treated early, recovery is increased to 90%. Survivors may recover fully, but others may sustain permanent neurological damage.

Other classes of anti-viral drugs are in development. Robert Krug's lab at the University of Texas, Austin is developing drugs to target the activity of NS1. A successful anti-NS1 drug would enable the host anti-viral response to effectively curb an infection.

# Chapter 2. Vaccines

Because influenza is highly contagious and the drug treatments are unexceptional, much emphasis has been placed on vaccination to prevent contracting the virus. By vaccinating as much of the public as possible, 'herd immunity' reduces the spread of the virus and often lessens the symptoms of those infected. The first anti-influenza vaccine was developed in 1941 by the U.S. army, and used to vaccinate soldiers departing for WWII.

The traditional vaccine is produced from virus that has been expanded in embryonated chicken eggs. The virus is harvested from chicken eggs and inactivated with formaldehyde. The virus particle is then purified on a linear sucrose gradient, and then chemically disrupted with Triton® X-100 resulting in a split antigen that is further purified by chemical means and suspended in a saline solution. Individuals with allergies to eggs or egg products should not receive the vaccine, nor should those with known sensitivities to the preservative thimerosal that is included at 25 $\mu$g mercury/dose.

As of May 2003, the CDC considers thimerosal containing vaccines safe for both children and pregnant women [11-17]. Since the 1930's, thimerosal has been used as a

preservative in multi-dose vials of vaccines. It is not required for single dose vaccine vials. There is no convincing evidence that the low dose of thimerosal in vaccines causes any harm aside from some minor redness and swelling at the injection site. However, some conflicting data exists. The recent apparent increase in autism and other neurological diseases that has coincided with more widespread thimerosal use in vaccines has resulted in lawsuits being filed against vaccine manufacturers. As a precautionary measure, in July 1999, the U.S. Public Health Service (PHS) agencies, the American Academy of Pediatrics (AAP) and vaccine manufacturers agreed that thimerosal should be reduced or eliminated in vaccines. All recommended licensed pediatric vaccines are currently being manufactured without thimerosal or with only trace amounts. Since 1999, newly formulated pediatric vaccines with reduced thimerosal levels meet the new guidelines established by the FDA, EPA, and Agency for Toxic Substances and Disease Registry (ATSDR). Now, the maximum total exposure due to all recommended childhood vaccines will be less than three micrograms of mercury during the first six months of life, which is small compared to 25 $\mu$g of mercury/dose in the current adult influenza vaccine.

Currently, the majority of adult influenza vaccines still contain thimerosal. For the 2002-03 flu season, a limited number of individually packaged doses of preservative free, reduced thimerosal-content influenza vaccine were available from Evans Vaccines and Aventis Pasteur.

Most influenza vaccines are trivalent, meaning they are comprised of three subtypes; generally two type A strains and one type B strain is included in the vaccine. The widely used trivalent vaccines include Fluzone® (Aventis Pasteur), Fluvirin® (Chiron Corporation, formerly produced by Evans Vaccines, Ltd., a division of PowderJect), and FluShield® (Wyeth/Lederle) and all currently contain thimerosal [18, 19]. Each dose contains 15 $\mu$g of HA from each of the three subtypes contained within the vaccine. Adults are vaccinated intramuscularly in the deltoid muscle, whereas infants

and young children are vaccinated in the thigh. Fever, malaise, myalgia, and other systemic symptoms can occur following vaccination, but rates are no greater than seen with placebo, and resolve within 2 days of the injection [20, 21].

Chiron has a cell culture vaccine in phase II clinical trials and an intranasal vaccine about to enter phase I clinical trials. Chiron also manufactures a number of vaccines that are available in Europe, but have not yet been FDA approved for sale in the U.S. These vaccines include: Agrippal® S1 subunit vaccine, which is the leading product in Europe, Begrivac[], which is a preservative-free split vaccine that is the leading product in Germany for sensitive patients, and Fluad®, which is the leading adjuvant vaccine on the market that enhances protection for elderly and patients with chronic disease.

In March of each year, the strains to be included in the vaccine are selected by the Food and Drug Administration's (FDA) Vaccines and Related Biological Products Advisory Committee (VRBPAC) based on findings of the World Health Organization's (WHO's) Global Influenza Surveillance Network. The committee makes an educated guess as to what strains have the potential to cause the most damage in the coming flu season. Often the committee must choose between prevalent strains and new strains that crop up late in the season and cause a mini-epidemic called a "herald wave." Such waves are thought to precede an epidemic. Included in the decision-making process is the "growability" of the selected strains. Some strains do not grow well chicken eggs and as a result minimize profitability for vaccine manufacturers and can lead to vaccine shortages. However, it is sometimes these more virulent strains that are the ones that can cause the most damage. The committee recommends two type A and one type B virus to comprise the vaccine and the vaccine manufacturers proceed to immediately begin production so that enough vaccine is available for vaccinating the public in the beginning of October of the same year.

The start of the vaccination season is timed with the start of the influenza season,

since antibody levels can begin to decline within a few months after vaccination. Due to the drop in antibody titers, it is recommended that individuals get boosted every year, although the composition of the vaccine may not change from year to year. An weakness in the system is that a new virulent strain may emerge after the FDA has made its recommendations. In addition, there is always the possibility that a new virus could establish itself in a part of the world such as Africa, where surveillance is poor in comparison to Asia, Europe, and the United States. Epidemic flu seasons are more likely if the vaccine being produced does not match with the predominant circulating strains or the vaccine strains grew poorly in eggs resulting in a vaccine shortage.

Antibodies from the vaccine are generally quite specific to the strains included in the vaccine. Cross-reactivity of antibodies to other strains varies from little to none. As a result, since the vaccine can not possibly cover all strains and strain variants circulating in the population, those receiving the vaccine are still susceptible to strains not included in the vaccine, as well as antigenic variants of the strains included in the vaccine. In most years, influenza occurs in less than 15% of healthy adults [22, 23]. Data suggest that this number can be reduced since studies have shown that when the vaccine matches the circulating virus, the vaccine prevents illness in 70-90% of healthy persons younger than 65 years of age. Elderly persons often develop lower post-vaccination antibody titers making them more susceptible to infection. In this population the vaccine is only 30-40% effective at preventing illness, but it does lessen the chance of complications and hospitalization.



## A. Traditional Vaccine Recommendations

Influenza is responsible for more deaths in the United States than any other vaccine-preventable disease. The traditional vaccine is considered to be exceptionally safe, although the vaccine should not be given to those with active infection, illness, a prior history of Guillain-Barré Syndrome (GBS, described later), or those less than 6

months of age. The traditional vaccine is appropriate for anyone greater than six months of age. Among previously unvaccinated children under the age of nine, two doses are administered at least one month apart to achieve a satisfactory antibody response.

The vaccine is also considered safe for pregnant women. The vaccine is recommended to avoid complications that can arise from infection during pregnancy. Retrospective analysis of women in their third trimester indicates that during the average flu season, 25 of 10,000 will be hospitalized for flu related complications [24]. Vaccination is generally offered after the first trimester to avoid the risk of coincidental miscarriages that occur most often in the first trimester. The vaccine is also considered safe for breast feeding mothers. Due to the increased risk of hospitalization between 6-23 months, vaccination is encouraged once the infant reaches six months of age. However, one study has reported that inactivated influenza A vaccine fails to protect healthy children aged 6-24 from influenza infection [25].

Data on immuno-compromised patients, including those with HIV, suggest that HIV infected individuals including pregnant women can safely receive the vaccine [20]. Studies have shown that vaccination in HIV infected individuals with a mean of 400 CD4+ T-lymphocytes cells/mL often induces an antibody titer. Individuals with advanced disease, lower than 200 CD4+ T-lymphocytes cells/mL may not generate antibodies and second doses do not improve the immune response. However, other studies claim that a transient 2-4 week increase in HIV-1 replication is apparent in HIV patients after vaccination. However, deterioration of CD4+ T-lymphocyte cell counts or progression of HIV disease has not been demonstrated among HIV-infected persons following vaccination [20].

## B. Recent Vaccine Strains by Year

The 2001-2002 vaccine was comprised of A/New Caledonia/20/99 (H1N1), A/ Panama/2007/99 (H3N2) (which is a A/Moscow/10/99-like virus), and B/Victoria/504/

2000. The 2002-2003 season vaccine included the same type A strains and changed type B to B/Hong Kong/330/2001. The lack of a significant change in the circulating influenza strains during the 2002-03 season prompted the FDA's VRBPAC to recommend remaking the same vaccine for the 2003-04 influenza season [26].

The 2003-04 season turned out to be relatively severe, killing 120 children. The children who died ranged in age from 4 weeks to 17 years, with a median age of 4 years. Approximately half had no underlying medical condition. Of the children whose flu vaccination status was known, most had not been vaccinated, but at least 6 had had one of two doses suggested for first time vaccine recipients, and at least 1 had been fully vaccinated. Some children who developed severe infection and lived are expected to experience some level of mental retardation.

Despite the apparent severity of the 2003-04 season, the exact toll of influenza cannot be directly compared with previous flu seasons, because reporting of confirmed flu cases and related respiratory deaths in children is not legally required. The CDC is working to require and standardize reporting, however not all flu-like deaths are caused by flu, which can confuse data analysis. Currently, the severity of the flu season is determined by comparing the proportion of deaths attributed to pneumonia and influenza to an epidemic threshold, which changes from month to month as the flu season progresses. During two weeks in December 2003, the proportion of deaths attributed to pneumonia and influenza was approximately 9%, which surpassed the epidemic thresholds of approximately 8% for the month of December, making it an epidemic year.

Epidemic years are caused by a shift in the viral population, which is monitored by collecting and characterizing isolates as the flu season progresses. By February 2004, U.S. laboratories had characterized antigenic nature of 668 strains that included 648 H3N2 viruses, 18 type B viruses similar to strains B/Sichuan/379/99 and B/Hong Kong/ 330/2001, and only 2 H1N1 viruses similar to the New Caledonia strain. Of the 648 H3N2 isolates, 16% were similar to A/Panama/2007/99 that was included in the vaccine

and 84% were similar to A/Fujian/411/2002 (H3N2) [27]. The A/Fujian/411/2002 (H3N2) strain was a new variant and was characterized prior to the VRBPAC meeting in 2003, but due its appearance late in the season and the difficultly of growing it in eggs, the committee voted to stick with the vaccine components of the 2002-03 season for the 2003-04 season. It is uncertain whether vaccine manufacturers would have been able to successfully generate a Fujian-like vaccine in time for the 2003-04 season, but in retrospect, it appears as though the decision not to try was a mistake. In healthy adults, due to the mismatch, the vaccine was found to about 50% of vaccine recipients rather than 90% when the circulating strain is matched to the vaccine strain. The Fujian strain is a good example of how small antigenic changes in the virus can make the vaccine relatively ineffective, for Fujian viruses have only two amino acid changes from the Panama 2002 strain, which was included in the vaccine, yet the vaccine provided only limited protection.

For the 2004-05 season, the VRBPAC recommended a trivalent influenza vaccine that contained A/New Caledonia/20/99-like (H1N1), A/Fujian/411/2002-like (H3N2), and B/Shanghai/361/2002-like viruses. U.S. vaccine manufacturers use "-like strains" that have antigenic similarities to the circulating strains, but grow better in eggs. For the 2004-05 season, the A/Wyoming/3/2003 (H3N2) and B/Jiangsu/10/2003 viruses were used in the vaccine since they are similar to the Fujian and Shanghai strains, respectively. The A/New Caledonia/20/99 virus was retained as the H1N1 component of the vaccine.

WHO has recommended the 2005-06 vaccine to be comprised of A/New Caledonia/ 20/99 (H1N1)-like virus, A/California/7/2004 (H3N2)-like virus which is derived from A/New York/55/2004 and A/PR/8/34 viruses, and a B/Shanghai/361/2002-like virus, which includes the vaccine strains B/Shanghai/361/2002, B/Jiangsu/10/2003, and B/Jilin/

20/2003.

## C. Efficacy of Vaccination to Reduce Mortality

Despite the advent of the influenza vaccine four decades ago, flu-related deaths in the United States have risen since the 1970's. During the 1976-77 season, approximately 16 thousand Americans died from influenza, whereas during the 1998-99 season, 64 thousand Americans died from influenza [28]. This increase has been attributed to a growing elderly population and more virulent circulating strains. The elderly are more susceptible to influenza infection since their immune systems do not mount as strong of an antibody response as seen in younger individuals. In addition, the H3N2 strains have become the predominant circulating strain and H3N2 is generally more virulent than H1N1. During the 1998-99 season, the greatest number of deaths was associated with influenza A (H3N2) viruses, followed by RSV, influenza B, and influenza A (H1N1). Influenza was associated with approximately three fold more deaths than RSV in all age groups except for children younger than one year. Mortality data indicate that 90% of influenza deaths and 78% of RSV-associated deaths occurred in people 65 years or older.

The mortality rate of influenza peaked during the 1998-99 season and the number of Americans dying from flu-related complications has since dropped. In the 2001-02 influenza season, 36 thousand Americans died from flu-related complications. Many of the deaths were caused by type A (H3N2) viruses that predominated early in the season, before influenza B virus became more prevalent at the end of the season [29].

## D. Traditional Trivalent Vaccine Shortcomings

The traditional chemically inactivated vaccine boosts the humoral response, but does little to boost the cytotoxic T lymphocyte (CTL) response. Vaccine manufacturers have been working on addressing this shortcoming. Many new types of vaccines are

being created and in 2003, the FDA approved a new class of influenza vaccine for public use. The new vaccine uses a live attenuated virus to prime the immune system. This type of a vaccine is advantageous since it allows for both humoral and cellular (CTL) immune responses, but due to the "live" nature of the virus, not all people can receive the vaccine and it does pose a threat to the immuno-compromised. This type of vaccine is thought to provide more complete protection and also stronger local immunity in the lung, which is the primary site of infection. In addition, studies have compared the antibody response elicited from inactivated vaccine and compared it to the response to a live attenuated vaccine. The results indicate that live vaccine induces a higher antibody response than the inactivated vaccine [30].

Previously, attenuation was achieved by multiple passages in a heterologous host, yet this process is unrealistic and unpredictable to perform for each strain included in the vaccine. The new approach is to make a "donor strain" that is a cold-adapted virus and attenuated in humans, which can be crossed with the vaccine strain.

The cold adapted vaccine is conditioned to grow effectively at 25 °C, but growth is attenuated at 37 °C, which is the temperature of the lungs. The cold adapted vaccines are produced in a two step process. First, a "donor strain" must be produced that can grow at 25 °C. This virus is made by passaging at progressively colder temperatures until mutants are isolated that grow well at 25 °C. Alternatively, reverse genetics can now be used to recreate strains with PB2 mutations that have been found to favor cold growth conditions [31]. This cold adapted virus is the "donor strain" can be crossed with an "epidemic strain" to acquire its external HA and NA [32]. The "crossing" is achieved by infecting cells with the two viruses and allowing for genetic reassortment to occur and the recombinants are selected for by incubating the co-infection with antiserum against the HA and NA of the donor strain and propagating the virus at 25 °C. Once the vaccine strain has been generated, it is generally grown in chicken eggs at 25 °C to achieve a high titer. The virus is then purified and safety and efficacy tests are preformed, prior to

packaging the virus for delivery to health care providers. A typical adult dose of the live vaccine includes $10^{6.5-7.5}$ $TCID_{50}$ (median tissue culture infectious dose).

The advent of the reverse genetics system removes the "probability" factor for creating a reassortant virus. However, not all viral strains are compatible so it is often necessary to make modifications to some of the strains to retain antigenicity and the cold adaptation feature of the recombinant virus.

The current FDA approved cold-adapted influenza vaccine-trivalent (CAIV-T) is produced by MedImmune Vaccines (formerly Aviron) and is called Flumist[]. Flumist is available to patients 5-49 years old. Contraindications of CAIV-T are more extensive then the traditional vaccine and include those with allergies to egg products, immuno-compromised patients, patients with asthma, pregnant women, and nursing mothers.

Opponents of the vaccine argue that a live vaccine presents the possibility of transmission from a healthy vaccine recipient to an immuno-compromised patient. Proponents argue that the CAIV-T vaccine should be used in children and could have helped prevent some of the 120 deaths in children in the 2003-04 flu season [33]. The vaccine is delivered nasally, 0.25 mL /nostril, by a syringe-like device that creates an aerosol. The vaccine is currently shipped frozen, but phase III clinical trials are underway for a refrigerator-stable vaccine. A drawback of this type of vaccine is that it is more expensive. The increased price is partially due the need to ship the live-attenuated liquid vaccine frozen and then store the vaccine in the refrigerator once it has been thawed to maintain potency.



## E. Guillain-Barré Syndrome

Since the swine flu scare of 1976, the vaccine has been associated with an increased risk of Guillain-Barré Syndrome (GBS). GBS, also known as acute idiopathic polyneuritis, is a neurological syndrome that is probably immune mediated, since its incidence increases after certain viral infections. The median onset of GBS after

vaccination is 12 to 13 days post vaccination. Symptoms and signs include paresthesia of the limbs and muscular weakness or a flaccid paralysis. Acute and severe GBS cases are reported to the Vaccine Adverse Events Reporting System (VAERS). The annual incidence of GBS is 10-20 cases per million adults.

Following the 1976 swine influenza vaccine program, an additional ten cases of GBS per million were observed [20, 34]. This finding scared the public and there have been many studies carried out to examine whether receiving the influenza vaccine puts one at increased risk of acquiring GBS. The published studies on this subject matter have been described below.

Three of four influenza seasons studied from 1977 to 1991 determined the risk of GBS after influenza vaccination to be slightly higher, but not statistically significant. From 1990 through 2003, VAERS received 501 reports of GBS following influenza vaccination in adults. The highest rate of incidence was found during the 1993-94 season, in which 1 in 580,000 vaccine recipients became sick, and the lowest rate of incidence was for the 2002-03 season in which 1 in 2.5 million vaccine recipients became ill. These data indicate that during the worst years, the vaccine may cause slightly more than one *additional* case of GBS per million people vaccinated [20], which possibly represents a causal association between GBS and influenza vaccine [35, 36].

A comparison of the relative risk of acquiring GBS following influenza versus the tetanus-diphtheria (Td) vaccination was performed for data collected between 1991 and 1999. These data suggest that the relative risk for acquiring GBS following vaccination is 1/million and 1/4.7 million for influenza and Td vaccinations, respectively [35]. Similar findings have been found in another study [37]. During these nine years, the risk of acquiring GBS as a side effect of the influenza vaccine was statistically significant over that of the Td vaccine. However, no statistical risk was found between in 1998 or 1999.

The influenza vaccine is manufactured by multiple companies and studies

have shown that the incidence of GBS following vaccination from different influenza manufacturers is statistically significant (manufacturers were not named in the paper) [35]. In addition, the influenza vaccine was found to contain from 125 to 1250 fold higher endotoxin levels compared to the Td vaccine. Increased endotoxin levels may be due to *Salmonella* contaminated embryonated chicken eggs in which the vaccine is grown. Endotoxin has been shown to not only boost the antibody response to unrelated antigens, but also increase the permeability of the blood-brain barrier, which may contribute to GBS. In addition, it has been hypothesized that chicken P2 protein present in the vaccine becomes a target of the immune system. The active immune system then becomes misdirected through molecular mimicry to target the nervous system causing the disease [35]. This concept of vaccine-induced autoimmunity has recently been reviewed by Shoenfeld *et al.* [38].

Despite some conflicting data regarding the risk of GBS following influenza vaccination, the risk of acquiring GBS is small in comparison to the risk of severe influenza. Statistics for the influenza seasons between 1972 and 1981 suggest that in healthy people from 5 to 44 years of age, the risk of hospitalization is 200-300 per million. For those over 65 years of age, the risk of hospitalization increases to 2,000-10,000 per million and 300-1,500 per million die from influenza-associated illness. Such serious illness can be greatly reduced in vaccinated individuals. In short, experts contend that the benefits of influenza vaccination vastly outweigh the small to insignificant risk of GBS.



## F. Chiron Fiasco

During a normal flu season, the CDC recommends that the 185 million Americans who have a high risk of contracting the flu and developing complications get vaccinated. Generally, Chiron Corporation (50 million doses), Aventis Pasteur (55 million doses), and MedImmune (1.5 million doses) supply enough vaccine to cover individuals who choose

to be vaccinated. However, on October 5th 2004, the British Medicines and Healthcare Products Regulatory Agency (MHRA) {equivalent to U.S. F.D.A.} run by the British Department of Health closed Chiron's Liverpool, UK plant that produces influenza vaccine, due to a contamination. Ultimately, all 48 million doses of Chiron's Fluvirin vaccine had potentially been exposed to the bacterium *Serratia marcescens*, which can cause serious illness, and were ultimately deemed unsafe. Due to the contamination, Chiron Corporation's license to manufacture influenza vaccine was suspended until April 2005.

The failure of Chiron to supply the U.S. public with flu vaccine generated a large unanticipated shortage. The severity of the shortage was dampened, when the FDA allowed the distribution of 4 million doses of Fluarix, which is produced by GlaxoSmithKline PLC (GSK), in the United States under an Investigational New Drug (IND) application. In addition, the Department of Health and Human Services (DHHS) purchased 1.2 million doses. The GSK vaccine is produced, licensed, and distributed globally, but it is currently not licensed for use in the United States, but it is expected to win FDA approval. In addition to GSK entering the U.S. flu market, ID Biomedical Corporation from Vancouver, British Columbia has reached an agreement with three wholesalers to supply flu vaccine to the United States, possibly starting in the 2007-08 season.

When vaccine shortages occur, as in the 2004-05 season, the FDA is faced with an ethical dilemma as to how to distribute the vaccine. The issues included: should an elderly individual, who is in danger of dying from the flu, but has already lived much of his or her life, receive the vaccine before a 2 year-old, who is likelier than most people to get sick, but will probably survive? (During the 2003-04 season, approximately 36,000 Americans died from flu, but only 150 were young children). Or, if a nursing home has only enough vaccine for either its staff or its patients, who should receive it [39]? In light of the vaccine shortage, the CDC convened a meeting of their Advisory Committee

on Immunization Practices (ACIP) to discuss these issues and the Committee issued recommendations based on various studies assessing a person's risk of contracting and dying from the flu to direct available vaccine to certain high-risk priority groups. The panel narrowed the recommendations to exclude healthy 50-64 year-old individuals and included very young children.

These recommendations removed 90 million Americans from the high risk groups, leaving an additional 95 million Americans that were suggested to receive the vaccine. In order to help determine which of the 95 million high risk Americans should have access to the available vaccine, five ethicists convened to make further recommendations regarding rationing the vaccine. The new guidelines narrowed the list to include those less than two or older than 65 years of age. Other conditions that placed individuals in the highest risk category included women who are pregnant, individuals with chronic illness, and children that take aspirin daily for medical purposes.

Fortunately, the 2004-05 flu season started slowly and was relatively mild so no significant deleterious effects were felt from the vaccine shortage.

## G. Alternative Vaccines

MedImmune Inc., of Gaithersburg, MD currently holds the patent for reverse genetics vaccine production. Originally, Aviron bought the rights from Mount Sinai School of Medicine (Dr. Peter Palese) in 1993, and MedImmune acquired the rights by purchasing Aviron in 2002. WHO has made an agreement with MedImmune to license the reverse genetics technology to other drug makers to maximize the chances of producing a successful vaccine.

### i. Reverse Genetics Technology

It is generally accepted that reverse-genetics is a faster methodology to reliable create attenuated virus strains that can be used as vaccines. Mount Sinai School of

Medicine, the University of Wisconsin, and St. Jude hold reverse-genetic patents that can be used for vaccine production. St. Jude has licensed their technology to MedImmune, which has licensed the technology for research purposes to Aventis Pasteur and Chiron, which would need to strike an agreement with the patent holders if they ever choose to market vaccines generated by this technology. Of note, if the U.S. government deems it necessary, it can assert its patent rights to produce domestic vaccine, but it could not be sold abroad. Since reverse-genetics vaccines are considered a genetically modified organism, such vaccines are subject to special regulations in both the U.S. and Europe. Country governments need to pass legislation to protect the companies against damages if the vaccine is found to harm its recipients with side effects.

Vaccine production for H5N1 is underway. Dr. Robert Webster's laboratory at St. Jude Children's Hospital and U.K.'s National Institute for Biological Standards and Control (NIBSC) each used reverse genetics to produce an attenuated Vietnam H5N1 virus that can be used as a vaccine strain, and can be grown in eggs [40]. The St. Jude vaccine strain was licensed to Chiron and Aventis Pasteur for production and the U.S. National Institute of Allergy and Infectious Diseases (NIAID) expects to begin trials in 2005. The institute also plans to test a vaccine against H9N2. As part of the pandemic plan, HHS disbursed $50 million in 2004 and plans to spend $100 million in 2005 to ensure companies have enough eggs for vaccine production, and to support development of cell culture fermenters for vaccine production.



ii. Vaccine Strains without NA

CAIV-T vaccines represent just one form of live attenuated vaccines. Viruses can also be attenuated by other means aside from cold adaptation. These means include changing the components of the virus to give the host an advantage to combating the virus. For example, a NA deletion (NA-) virus could be used for vaccine production [41]. NA- viruses are highly attenuated in tissue culture, but can grow in cell lines expressing

reduced levels of sialic acid. Such viruses have been used to immunize mice and these mice are then protected against lethal challenge. These viruses can be engineered to express foreign genes instead of the native neuraminidase gene, and as a result could be used as vaccine vectors against HIV and other pathogens. If necessary, please refer to later sections in the thesis for more complete descriptions of various flu proteins and their functions to clarify why these types of modifications may be effective for vaccine production.

iii. Vaccine Strains with Altered NS1

Similarly, it is possible to create recombinant influenza viruses with altered NS1 genes that are replication deficient and are less able to significantly suppress an immune response. Impairing or deleting the RNA-binding function of NS1 makes replication deficient viruses that are still capable of inducing a Th1-type immune response. In addition, these viruses induce significant serum and mucosal titers of $IgG_{2a}$ and IgA, but $IgG_1$ titers are less prominent. These viruses induced a rapid local release of pro-inflammatory cytokines such as IL-1 beta and IL-6, and higher levels of IFN-alpha/beta in the serum than wild-type viruses. Mice vaccinated with these replication deficient viruses exhibited a faster CD8(+)-T-cell response upon challenge with wild-type virus than when the mice were vaccinated with NS1 altered viruses that were capable of replication [42]. In contrast to replication deficient viruses, the replication capable viruses elicited higher titers of serum and mucosal IgG1, but lower titers of serum IgA.

iv. DNA Vaccines

DNA vaccines encode viral proteins that are highly conserved. These DNA strands are taken up by cells, which express the viral proteins and elicit a cellular and humoral immune response. The response is similar to that obtained from a live virus, but it can be produced more quickly and with less risks. DNA vaccines against M and NP

were shown to protect mice from lethal challenge with avian H5N1 flu [43, 44]. Another study examined a NP DNA vaccine in mice and found the vaccine to elicited both gamma interferon and IL-4 producing CD8 cells [45].

v. RNA Vaccines

The RNA based vaccines aim to exploit the RNA silencing machinery of the cell's innate immune system. For more information, see the section on RNA silencing.

vi. Virosomal Vaccines

U.S. based inactivated vaccines currently only include the hemagglutinin antigen, whereas Invivac[], which is licensed for use in Europe, includes both hemagglutinin and neuraminidase antigens in an inactivated suspension that is injected intra-muscularly. Invivac has been shown to be a safe and effective influenza vaccine in elderly and subjects with low pre-vaccination antibody titers. In subjects with low pre-vaccination antibody titers, Invivac was shown to provide protective hemagglutination inhibiting (HI) antibody titers in 76-99% of recipients depending on their age, health status and the vaccine components [46].

## H. Vaccine Delivery Systems

Vaccine manufacturers are also working on different delivery systems for their vaccines. The traditional needle-based injections are not comfortable to receive and are a deterrent for some potential recipients, making other less-invasive forms of delivery more attractive. One alternative delivery system is intranasal (IN) vaccination, which is currently used by MedImmune to deliver Flumist. Aside from being easier to administer, this vaccine targets the mucosal surfaces and prompts an immune response at the site of infection, theoretically allowing for superior protection. A current drawback to Flumist is that the liquid vaccine requires frozen shipping and short term refrigerated storage, which

increases the price of the vaccine.

To eliminate the necessity of frozen shipping, but still maintain the benefits of IN delivery, some companies are working to create powder formulations of vaccines that will not require refrigeration [47]. Powder-formulated vaccines have been shown to elicit a nasal IgA response and a comparable serum antibody response when compared to the liquid IN or IM injections of similar whole inactivated vaccine.

Similarly, PowderJect has developed a delivery system for epidermal powder immunization (EPI). EPI has been shown in phase I clinical trials to safely and efficiently deliver powdered protein vaccines to the epidermis that elicits a humoral immune response [48, 49].

## I. Lack of Incentive for Vaccine Manufacturers

There are relatively few companies involved in influenza vaccine production. This is largely due to the fact that vaccines are generally not very lucrative and have more pitfalls than designing drugs that are used on a more regular basis. For example, Chiron and Aventis generate approximately $0.3 and $0.6 billion in sales per year, respectively. In comparison, Viagra, Allegra, Zoloft, and Lipitor generate $1.9, $2.2, $3.1, and $9.2 billion in sales per year, respectively. As of early 2004, only three companies were FDA approved to provide influenza vaccine to the U.S. public, including Chiron, Aventis Pasteur, and MedImmune. The reason so few companies make vaccine is that vaccine production generally has a very low profit margin when compared to other drugs that are taken on a daily basis for extended periods of time, such as drugs that target blood pressure, cholesterol, or erectile function. Also, influenza vaccine needs to be re-designed every year and surplus doses can not be saved for the following year. Not only are vaccines administered once per year, but also due to the fact that vaccination is voluntary, demand is variable. During years of low demand when surplus vaccine is not bought, profits can be negligible. These issues have led the number of manufacturers

making influenza vaccine to dwindle significantly [50].

The role of the U.S. government in protecting the population has been debated. Since the public relies on the government for military protection, should the public also expect our tax dollars to provide protection against a pathogen that kills approximately 32,000 Americans a year? Currently, the U.S. government relies heavily on the private sector to provide vaccines to the public. This policy places the public at peril of not receiving vaccine when things go wrong. Recently, the U.S. government has provided some additional minor tax incentives for vaccine manufacturers and these incentives have been increased after the 2001 terrorist attack, when President George W. Bush signed the Project Bioshield Act. However, it is not clear if the Department of Homeland Security will prioritize flu under this Act. In the event of a vaccine shortage, the U.S. government maintains a stockpile of 4 million vaccine doses, which many virologists argue is woefully inadequate if an epidemic were to strike.

Although having more vaccine makers would reduce the severity of future vaccine shortages, should another manufacturer experience a contamination or such that prevents their vaccine from making it to market, there is still little incentive for new manufacturers to enter the market. To provide more incentive, the U.S. government could reduce the financial risk that manufacturers take on by partially stabilizing the demand. This could be achieved if the U.S. government were to guarantee the purchase of unused vaccine at a reduced cost; however the Department of Health and Human Services and Congress have yet to extend this offer to the vaccine manufacturers. In addition, the government could require health insurers to cover the cost of receiving the vaccine and expand the National Vaccine Injury Compensation Program to cover adult vaccines as well as those made for children. Congress has adopted this proposal, but President George W. Bush has not yet signed it into law. A stronger U.S. vaccination system will financially benefit the U.S. economy and healthcare system, since the current healthcare system is strained during non-epidemic years, making epidemic years even

harder to handle, especially considering that small epidemics can easily reach $20 billion in medical payments in the U.S.

The vaccine production system also needs to be modernized to be more efficient in terms of cost per dose and the ability to make vaccine more quickly. Current vaccine production occurs in eggs, which has advantages and disadvantages. On the plus side, influenza grows to a high titer in eggs allowing for the harvest of lots of virus, but on the down side, dealing with 50 million eggs can be cumbersome and difficult to ensure sterility. Cell line-based vaccine production is now technically feasible, however to build plants to make such a vaccine requires approximately $250 million, plus the cost of conducting the clinical trials needed to secure FDA approval. These financial barriers are hard to overcome when current profit margins are slim, since additional investment would require charging the public more per dose than competitors who stick with the old system. Although the U.S. does not currently have any plants for cell-based vaccine production, Europe has already started.

Currently, nine countries in Europe produce 85% of the world's flu vaccine. The world's capacity for making monovalent pandemic flu vaccine is now 900 million doses, enough for only 15% of the world's population [40]. If a pandemic were to break out, there is the potential that these countries would choose to impound the vaccines to protect their own people, as the U.S. government did during the 1976 swine flu episode. This leaves non-producing countries in jeopardy, the U.S. included. Currently, there is only one plant in the U.S. that is making influenza vaccine. The plant is owned by Aventis Pasteur, a French company, and is located in Swiftwater, Pennsylvania.

To stretch the vaccine supply, different adjuvants have been tested that permit less vaccine to be used, while still maintaining efficacy. GlaxoSmithKline found that alum allowed for using only 12.5% of the normal H2N2 vaccine dose. Alum would also be cheaper than MF59, which is produced by Chiron and is the adjuvant that NIAID plans to test. Since no flu vaccine with alum adjuvant has been licensed in the United States,

NIAID is not pursuing this promising strategy.

## Chapter 3. Avian Influenza

Historically, avian influenza has been referred to as fowl plague because it kills many domesticated birds. Avian influenza was first identified in Italy more than 100 years ago. Birds are the primary natural reservoir for all subtypes of influenza A viruses, which includes 15 HA subtypes and 9 NA subtypes. In contrast to humans, infection is normally restricted to the gastrointestinal tract. An infected bird sheds virus in its feces, which can contaminate bodies of water shared by domesticated and wild birds. Contaminated water supplies allow for rapid spread of the virus to other birds drinking the water.

Influenza infection in birds can be asymptomatic to lethal within 24 hours depending on the strain of virus and species of bird infected. Highly virulent strains, including H5 and H7 viruses, can cause systemic infection. Birds with systemic infection, including the respiratory tract, pose an increased risk for the virus being transmitted to humans since the virus can be aerosolized. These virulent strains are capable of recognizing receptors throughout the bird's body, causing capillaries to collapse. A telltale sign of infected chicken is when its comb turns red and appears to melt. Fatally infected birds will literally bleed from all their orifices. These highly virulent strains are often lethal for domestic chickens, but surprisingly can be often asymptomatic in ducks and wild birds.

Ducks have been compared to Trojan horses, since they can carry each of the fifteen strains of avian influenza and can excrete high titers of virus, but not appear ill. The reason for this stark difference in virulence between ducks and chickens is unclear. Asymptomatic ducks not only make the detection of infected birds difficult, but since they are carriers, they undoubtedly play an important role in the transmission of H5N1.

To help prevent the transmission of H5N1 from ducks to chickens, the U.N. Food and Agriculture Organization has suggested raising ducks and poultry separately, however this is difficult to enforce. To date, there have been no confirmed cases of avian influenza being transferred from a duck to human.

## Chapter 4. Cross-species Infection

Cross-species infection between birds and humans occurs rarely due to several barriers. First, avian viruses replicate efficiently at 33°C, which is the temperature of the avian gastrointestinal tract, and have impaired replication at 37°C, which is the temperature of the human lung. This temperature difference provides a thermal barrier. In addition, avian influenza prefers to bind to cells that have cilia, whereas human influenza prefers to infect non-ciliated cells [51]. Furthermore, the receptors for human and avian influenza are different, so in order for an avian strain to infect a human, it must also be able to bind to a human receptor. Lastly, the HA must be able to be processed by the proteases of the new host cell that it infects. These factors and likely other unidentified barriers including incompatibility of type antigens (M and NP) greatly reduce but do not eliminate the amount of cross-species infection. In the rare event that a cross-species infection occurs, these strains generally undergo additional genetic drift to improve infectivity and transmissibility in the new species. As a result, the strain in a new host will genetically drift apart from its parent strain, making it different on an antigenic level.

The probability of cross-species infection increases during an epidemic in birds, since higher levels of virus are being released into the environment. Infected birds shed virus in saliva, nasal secretions and feces, and exposure to these secretions increases the risk of human transmission. The rate of avian to human transmission has recently

increased at an alarming rate. This drastic increase may partially be attributed to increased surveillance, but many experts disagree and feel that it is a foreboding sign of a future pandemic. Since 1997, several bird strains including H7N7, H7N3, H5N1, and H9N2 have jumped into humans (see below for references).

## A. H7N7

Avian influenza has also caused problems in areas outside of Asia. In early 2003, more than 20 million chickens were slaughtered in the Netherlands in an effort to quell an epidemic of avian H7N7 influenza [52]. This strain sickened 80 poultry workers, some with viral conjunctivitis, and killed one veterinarian. The rate of human-to-human transmission appears to be low.

## B. H7N3

In 2004, British Columbia experienced an outbreak of H7N3 in chickens. During this outbreak, two people were confirmed to have been infected with H7N3 and presented with conjunctivitis and mild influenza-like illness [53]. In chickens, some H7N3 isolates were deemed to be low pathogenic avian influenza (LPAI), whereas other isolates were found to be highly pathogenic avian influenza (HPAI). The chicken isolates and the human isolates were analyzed for sequence differences and both the HPAI and human isolates were shown to contain a novel insertion in the $HA_0$ cleavage site. This insertion likely occurred by non-homologous recombination between the hemagglutinin and matrix genes of the LPAI virus, creating the HPAI virus [54]. The 7 amino acid insertion generates an enlarged exposed loop, which contains multiple basic amino acids in the HA0 protein. This sequence increases the rate of cleavage by furin-like proteases and subtilisin-like protease(s) [55]. The ubiquitous nature of these proteases enables HPAI viruses to replicate systemically. In contrast, cleavage of the HA0 precursor in

LPAI viruses is catalyzed only by trypsin and trypsin-like host proteases, which restricts replication to tissues containing these proteases, namely the lungs and respiratory tract.

H7 and H5 can be either LPAI or HPAI, depending on the sequence surrounding the HA0 cleavage site. The avian HPAI and human isolates from British Columbia in 2004 do not conform to the consensus sequence suggested to be the prerequisite for all HPAI viruses [56]. Analysis of the nucleotide sequences of HA genes from HPAI H5 and H7 isolates has shown that in many cases there are direct repeats of a purine-rich sequence (AAGAAA). This sequence may arise due to the polymerase slipping at a site upstream of RNA secondary structure.

## C. H5N1

In 1997, the first cases of pure avian influenza infecting humans were recorded in Hong Kong. During a chicken influenza outbreak 18 people were infected and six died from their infections. The virus was isolated and characterized and found to be phylogenetically related to the H5N1 circulating in chickens in the live poultry markets. Each of the eight segments was of avian descent, suggesting that pure H5N1 chicken influenza can infect humans. This finding has changed the way influenza biologists view cross-species transmission. Previously it was thought that avian flu could not directly infect humans and that pigs serve as mixing vessels for the production of chimeric viruses that are capable of infecting humans (See Swine Flu section).

The HA gene of the 1997 H5N1 virus is similar to the HA gene of a virus isolated the previous year from a goose, A/Goose/Guangdong/1/96 virus [57]. This H5 protein has a motif associated with highly pathogenic avian influenza A viruses, which is a cleavage site with multiple adjacent basic amino acids. Such a characteristic improves the probability that this virus would be able to propagate in humans.

The NA gene of H5N1 is evolutionarily distinct from previously characterized N1 neuraminidases. The NA is avian-like with a shortened stalk due to a 19-amino

acid deletion with two unique amino acids. It is unclear if the loss of these amino acids increases the host range and virulence of the virus [58, 59].

Each of the avian segments tested had features common with human influenza that may have facilitated the transfer to humans. These changes likely occurred over time through antigenic drift. These features are reviewed in Baigent and McCauley, 2003 [60]. For the PA segment, the avian strain adopted Asn 409, which is normally seen in humans [61, 62].

In response to this outbreak, on December 29th, 1997, Hong Kong city officials ordered the killing of every chicken in Hong Kong. Within 3 days, the entire chicken population of Hong Kong was either slaughtered or gassed; and the carcasses were buried and the markets disinfected. These actions removed the H5N1 virus from the poultry markets, brought the outbreak under control, and no further human cases were reported for several years.

Unfortunately, the H5N1 strain was not completely destroyed by the slaughter of poultry in 1997. The H5N1 strain is thought to have survived by circulating in wild birds, non-chicken hosts, and/or waterfowl. In 2002, H5N1 re-emerged and returned to the markets and also began killing some wild birds.

In 2003, a father and son who lived in Hong Kong were infected with this avian virus (A/HK/212/03 and A/HK/213/03). Both were hospitalized and the 33 year old father died [63].

Laboratory experiments demonstrated that the 1997 and 2003 human H5N1 viruses induce macrophages to overproduce pro-inflammatory cytokines, when compared to earlier isolates of the H5N1 virus. Sequence analysis of the hemagglutinin gene demonstrated that it had undergone significant antigenic drift [64].

During the 2003-04 influenza season, H5N1 became more prevalent in Southeast Asia. Outbreaks were confirmed in Cambodia, China, Hong Kong (in a single peregrine falcon), Indonesia, Japan, Laos, South Korea, Thailand, and Vietnam. Genetic

sequencing of virus samples from South Korea and Vietnam demonstrated that the viruses isolated in these two countries were slightly different. The increase in the outbreaks in birds has been followed by an increase in the number of humans infected with H5N1.

In the spring of 2004, more than 100 million chickens were slaughtered to try to eliminate the H5N1 outbreak in Thailand and Vietnam that had begun to infect and kill people. The slaughter seemed to bring the outbreak under control, but the disease re-appeared during the summer, and human infections and deaths resumed. By the end of 2004, H5N1 had killed 32 humans (12 in Thailand and 20 in Vietnam) and greater than 120 million poultry have been killed or culled in Eastern Asia [65].

Until recently, Japan has been free of highly pathogenic avian influenza since 1925. However, between the end of December 2003 and March 2004, four geographically separated outbreaks of acute, highly transmissible and lethal H5N1 disease occurred in chickens. These outbreaks were caused by similar viruses [66]. These viruses were found to be genetically different from the viruses prevalent in China in 2003 (genotype Z), but closely related to A/chicken/Shantou/4231/2003 (genotype V) isolated from Guandong Province in China.

Viral isolates from Japan were tested in the laboratory and found to kill chickens within 1 to 3 days of inoculation, when infected intravenously or intranasally, respectively. In mice, these viruses replicated well in the lungs and spread to the brain without prior adaptation, and were easily transmitted to crows. These viruses were found to be more pathogenic than the viruses isolated from humans in Hong Kong in 1997.

In December 2004, Japan had its first cases of humans infected with H5N1. Four employees of an infected farm and one governmental health official tested positive for antibodies to H5N1. Interestingly, only one of these individuals became mildly ill and the others were essentially asymptomatic. These were the first documented cases of mild or asymptomatic infections in humans to emerge from the H5N1 outbreak, whereas in Vietnam and Thailand, more than 70% of the cases were fatal. Some scientists have

argued that the asymptomatic individuals were exposed but never actually infected [67], or that the H5N1 strain is sufficiently different from the strain that infected people in Thailand and Vietnam, to cause little to no infection.

### i. H5N1 Summary

From 2003 to June, 2005, there have been 108 reported H5N1 related causalities in Vietnam, Thailand, and Cambodia. The mortality rate in humans infected with H5N1 is 50%. The reason for the high mortality rate is that H5 viruses have not circulated previously in humans and the entire population is naïve; and as a result, the severity of disease is not be tempered by any pre-existing immunity [68]. These H5N1 viruses trigger inappropriate innate immune responses in humans, leading to severe respiratory disease and multi-system failure [69, 70]. It has been estimated that the crisis has cost the region $8-12 billion, and due to the uncontrolled epidemic, the European Union has extended its ban on poultry meat and live bird imports from eight Asian nations until September, 2005.

### ii. H5N1 Drugs

The H5N1 virus presents a significant risk to humans. This virus has been examined to determine whether current antiviral drugs would be effective. Genetic sequencing of virus samples from human cases in Vietnam and Thailand suggest that the M2 protein contains a mutation that makes the virus resistant to the ion channel pore blockers, amantadine and rimantadine [71, 72]. In addition, H5N1 viruses are resistant to the anti-viral effects of interferons and tumor necrosis factor alpha. This resistance has made these viruses extremely virulent and lethal in humans. The increased resistance is likely due to the action of the NS1 protein, which has been found to be responsible for circumventing the host anti-viral cytokine responses [73]. The increased virulence of the H5N1 NS gene has been attributed to either the glutamic acid residue at position 92

[73] or to a 5-codon deletion [59]. Although these viruses appear to be resistant to many of the current antivirals available, they do appear to be sensitive to the neuraminidase inhibitors, oseltamavir and zanamavir [71].

Currently, oseltamivir (Tamiflu), is the only antiviral drug that has been shown to be effective against the H5N1 virus [40]. Tamiflu was originally created and patented by Gilead, who later licensed the manufacturing rights to Roche. Roche has a Tamiflu manufacturing plant in Switzerland. If H5N1 were to successfully jump into humans, prior to the production of an effective vaccine, Tamiflu would be the only defense. Modelers have estimated that a course of antivirals given prophylactically to 80% of the exposed U.S. population for 8 weeks could be as effective as a vaccine in preventing death and disease [74]. However, this would require 2 billion doses, which is currently unrealistic to achieve. Japan, Australia, and the U.S. have begun to stockpile Tamiflu, but at $8 - $10 per five day course, it is expensive. Despite the price, the demand is relatively high. Roche is currently capable of making 7 million treatments a year, which Gilead contends is not sufficient to meet the demand and as a result, Gilead has recently filed suit claiming that Roche has not adequately manufactured Tamiflu to meet the demand.

### iii. H5N1 Vaccine

Developing vaccines in eggs against new H subtypes has proven difficult since the virus often kills the eggs prior to generating a significant titer. In the past, the FDA has been resistant to approving non-egg based vaccine production, including tissue culture based development, but in light of current events in Asia, approvals have been granted. However, tissue culture based vaccine production is not yet refined and has been hampered by lower titer production.

Dr. Klaus Stöhr, the director of WHO, has pushed for a reverse genetics vaccine program to address the bird flu threat and laboratories now working to address this issue are located at the CDC, the UN Health Agency, and in London and Memphis. Some

of these labs are attempting to make slight changes to H5N1 in a WSN/33 background. Mass production is likely to occur by culturing eggs at get a high titer virus and processing HA as done normally for the inactivated vaccine.

Aventis-Pasteur and Chiron Corp. will begin the first ever human testing of a vaccine against bird flu starting in early 2005 at seven U.S. university sites.

### iv. H5N1 Human-to-Human Spread

In order for a pandemic to occur, avian influenza must first infect humans and then be able to transmit from person to person. To date, most humans infected with avian influenza have been dead-end infections. However, in the fall of 2004, a probable human-to-human transmission of H5N1 was documented in Thailand [75]. An 11 year old girl became ill with a fever, severe cough and a sore throat, three to four days after her last exposure to dying household chickens. Her mother came from a distant city to care for her in the hospital and had no recognized exposure to poultry. The mother provided 16 to 18 hours of unprotected nursing care before the girl died, and per Thai custom, she was cremated immediately. The mother, who had not encountered chickens, soon afterwards became ill and twelve days after the death of her daughter, she too died from pneumonia. The aunt also provided unprotected nursing care and also became ill but recovered. Disease in the mother and aunt probably resulted from person-to-person transmission from the infected girl. However, because no blood or sputum samples were collected from the girl prior to her cremation, it was not possible to genetically confirm that both women died of the same strain of influenza.

Vietnamese officials are investigating another suspected case of person-to-person bird flu transmission involving two brothers in Hanoi.

### v. H5N1 Evolution

The most recent H5N1 isolates have shown that the H5 gene is the only gene of

the eight that resembles the original H5N1 virus that first began killing people in 1997 (Richard Webby at St. Jude Children's Research Hospital in Memphis). A recent report harvested 21 H5N1 samples from apparently healthy ducks between 1999 and 2002 and used these viruses to infect chickens, mice, and uninfected ducks in a controlled environment to monitor whether the virus has become more virulent. The researchers found that the severity of the illness was linked to the year of the viral isolation; the later viruses were found to be more virulent than the early isolates. In addition, data from mice suggest that in the past few years the virus has become more adept at infecting mammals and is becoming more virulent [76]. These data have been supported by genetic analysis, which has demonstrated that the virus mutated in 2001.

Furthermore, field data suggest that the H5N1 virus has become more lethal for wild birds. At the start of the outbreak in 1997, relatively few wild birds were found dead. However, it has recently been reported that hundreds of thousands of wild migratory birds have been lethally infected with H5N1 [77]. This development may spur the spread of H5N1 beyond Asia.

Evidently, the genetic changes that have made H5N1 more virulent have also made the virus hardier. Viruses isolated in 2004, were shown to survive in the environment for 6 days at a temperature of 37 °C, compared to 2 days for older strains. In addition, the 2004 isolates were found to be shed from infected ducks for a longer period of time [78].



vi. H5N1 Host Range

Since 2003, H5N1 has infected a variety of different birds and other species of animals. During the 2003-04 outbreak of avian influenza, there were anecdotal reports of fatal infection in domestic cats, a species previously considered resistant to infection. To test whether the influenza host range had expanded, naïve cats were inoculated intrathecally with H5N1 or given virus-infected chickens for their feed. Both groups of

cats excreted virus, developed severe diffuse alveolar damage, and transmitted the virus to sentinel cats [79]. Furthermore, in 2004, forty-five black-striped Bengal tigers in a zoo near Bangkok died from avian influenza and 100 other sick tigers were destroyed [80, 81].

In 2004, in China, there was a report that the H5N1 virus had been isolated in pigs. However, Chinese officials have not been as forthcoming with information as WHO officials would like. A senior Chinese health official at the World Health Organization in Beijing issued the report that pigs were found to be infected with avian H5N1 in the southeastern Fujian province at several farms. In addition, the chief of the China National Avian Influenza Reference Laboratory has commented on pigs infected with H5N1, which has been denied by officials at China's Agriculture Ministry. It has not been confirmed whether the virus was living and replicating in pigs or if it was just isolated from their nasal passage from inhaling contaminated poultry feces. It was also not clear if the pigs had become sick, or if the virus had the ability to pass from pig to pig. If the reports are true, the ability of the avian H5N1 virus to infect pigs is troubling for pigs are considered to be a mixing vessel for influenza, which facilitates the transmission into humans.

In all, H5N1 has been reported to have caused death in many animals that were previously thought to be resistant to avian influenza, including: blue pheasants, black swans, turtledoves, open-billed storks, eagles, clouded leopards, mice, pigs, domestic cats, tigers, and humans.

### vii. H5N1 Geographic Spread

The H5N1 virus has spread since its original outbreak in Hong Kong in 1997. In 2003, H5N1 spread to Thailand, Vietnam, and China; and by 2005, H5N1 was reported in Indonesia and North Korea. H5N1 has been able to spread due to the trafficking of infected birds (chickens and ducks) and likely due to infected migratory birds that can

spread the disease over distant regions.

### viii. H5N1 Mortality Rate

The reliability of the mortality rate is in question since only the most seriously ill get tested. Additionally, the extent of mild or asymptomatic H5N1 infection is unclear. Traditional testing for exposure to avian influenza has relied on hemagglutination inhibition tests to detect antibodies in serum samples; however this assay is not often sensitive enough. Micro-neutralization assays are more sensitive, but also more time consuming, expensive, and require live H5N1 virus, which should be confined to BSL 3(+) equipped laboratories. The extent of sub-clinical H5N1 infection is being examined in collaboration between the Thai Ministry of Health and the CDC and the preliminary analysis suggests that there does not appear to be a large number of sub-clinical cases [82].

It is possible that more deaths have been caused by H5N1 that were either not reported or incorrectly diagnosed as acute encephalitis, since victims of H5N1 have presented with diarrhea, followed by seizures, coma and death, without significant respiratory distress [83]. In such cases of acute encephalitis, the source or cause of inflammation is not always determined. In the confirmed H5N1 cases, the virus was isolated from the cerebrospinal fluid, fecal matter, throat samples, and the blood.

### ix. Other H5 Activity

H5N1 activity has not been solely confined to Asia. In 1983, the H5N1 strain killed 17 million chickens in Pennsylvania. These chickens experienced systemic infection and died from massive hemorrhaging throughout their bodies, but no human infections were recorded. From 1983 to 2002, H5 outbreaks have been sporadic and uncommon in the U.S. However, in 2002, in Texas, there was an outbreak in chickens (H5N3), and in California, there was an outbreak in turkeys (H5N2). These H5 strains

seem to be the result of separate introductions from the wild bird reservoir. The Texas H5 has a unique hemagglutinin cleavage site (REKR/G), which differs from other recent isolates that have the non-virulent motif (RETR/G). In addition, the Texas isolate had a 28 amino acid deletion in the stalk of NA [84]. The Texas and California isolates share high sequence identity, and phylogenic analysis has placed them into a separate clade from the Pennsylvania/83 lineage.

## D. H9N2

The culling of all the chickens in the Hong Kong markets in 1997 not only removed H5N1 from circulation, but also removed a H9N2 virus (A/Chicken/Hong Kong/G9/97) that had been prevalent in the markets. The H9N2 strain was not detected in the markets in 1998, but in 1999, it returned as two separate lineages, represented by A/Quail/Hong Kong/G1/97 -like and A/Duck/Hong Kong/Y280/97 -like viruses.

The re-emergence of H9N2 resulted in another episode of cross-species transmission. In Hong Kong, two children were infected and hospitalized with a virus similar to the A/Quail/Hong Kong/G1/97 strain. Characterization of the strains demonstrated that the virus contained the six internal genes from the H5N1 A/Hong Kong/156/97 strain [85-87]. This finding suggested that the internal components of H5N1 appear to be better adapted to growth in humans than the internal components of the original A/Quail/Hong Kong/G1/97 virus. Fortunately, the H9N2-like virus was not found to spread from human to human [88]. As of 2003, no additional cases of illness caused by H9N2 have been reported, although evidence exists that humans have been exposed to the virus [89].

Although this virus does not currently appear be to a threat to humans, a cold-adapted influenza A H9N2 reassortant vaccine has been generated, which is specific for A/Chicken/Hong Kong/G9/97 viruses [90-92]. This vaccine virus utilized the six internal genes of A/Ann Arbor/6/60 (H2N2) and may be useful if there is a re-emergence

of this strain. Furthermore, the NIAID Pandemic Influenza Preparedness Program has awarded Chiron Corporation a contract to produce a vaccine against avian H9N2, which it will produce at its manufacturing facility in Siena, Italy. The vaccine strain is based on an inactivated strain of the H9N2 virus developed by the CDC. Clinical trials will be performed using different doses of H9N2, with and without MF59, which is an adjuvant that Chiron has developed that boosts the immune response to the vaccine. Trials are expected to begin in early 2005.

# Chapter 5. Influenza and Global Climate

Little is known about the association of influenza epidemics with global climate variability. However, a recent study investigated the co-variations between Él Nino Southern Oscillation (ENSO) and morbidity and excess mortality from different viral subtypes collected in France from 1971 to 2002 [93]. These data suggest that the circulating subtype and the magnitude of ENSO, which is a major component of climate, are associated with the impact of influenza epidemics.

# Chapter 6. Why is Asia the Starting Point of Pandemics?

If a pandemic were to occur, the site of origin would likely be Southeast Asia, which is considered a breeding ground for influenza. Southeast Asia is home for 60% of the world's population and roughly 6 billion chickens and millions of pigs. These animals and their farmers spend a considerable amount of time in close proximity, which enhances the likelihood of influenza transmission.

In particular, both Hong Kong and Guangdong province have been hotbeds for emerging viruses. Guangdong province alone has 86 million residents and large amounts

of poultry are transported to Hong Kong, which is the most densely packed city on earth. Hong Kong is believed to be the point of origin for both the 1968 flu pandemic and the 2003 SARS outbreak.

Although the CDC has funded ten laboratories in China to help monitor flu activity in this region, the surveillance in other countries in Southeast Asia is relatively weak. Many of the countries lack the capital and the support facilities that are required for surveillance. In addition, many regions in these countries lack credible public-health systems, and the network for influenza communication and education is weak.

## A. Economics and Politics in Asia

In order to limit the spread of H5N1, the European Union and the United States have suspended chicken imports from several countries in Southeast Asia. Furthermore, three international agencies including the World Health Organization (WHO), the UN Food and Agriculture Organization, and the World Organization for Animal Health have asked for increased funding to improve surveillance for avian influenza. Overall, the foreign aid to monitor and help Southeast Asia against avian influenza has been minimally acceptable. To this cause, the United States, under the direction of the CDC and HHS is planning to spend $5.5 million to help countries in Asia improve surveillance.

These organizations have also recommended regulations and restrictions on the farming and marketing of domesticated birds, some of which have been adopted by local governments. The imposed regulations are designed to confine outbreaks and protect the people. Some of these regulations include requiring coops to be made on dry ground and enclosed, and to eliminate the use of coops on stilts over the ponds. Also, larger facilities are required to have daily inspections and the workers are required to change clothes entering and leaving the chicken houses. There are also restrictions within Asia on transporting poultry. In the event of an infection, governments urge that infected birds be slaughtered. In addition, governments are urged to crack down on widespread cockfights,

which are immensely popular, but promote the transmission of viruses.

Due to the farming culture in Southeast Asia, change is difficult. For example, Thailand has an estimated 200,000,000 small family farming businesses. These family farms keep an average of fifteen birds, including ducks, chickens, geese, turkeys, and quail. Most of these birds are free-range and prone to infection from migrating fowl that contaminate the local water systems. The birds raised on these family farms are often sold to larger conglomerates, which do business on a larger scale. Imposing regulations on this style of farming is nearly impossible.

The H5N1 outbreaks have either outright killed chickens or prompted their slaughter. To prevent future outbreaks, governments have implemented restrictions that have made farming an extremely difficult livelihood to pursue due to slim profit margins. Many chicken farmers are out of work and their coops remain empty. However, compliance of new recommendations is far from complete. In some countries, such as Indonesia, the government has resisted international pressure to slaughter infected poultry. The same holds true for many small farmers in rural areas of other countries were enforcement is difficult to achieve. These deviant practices will make the H5N1 virus difficult to control.

The countries in Southeast Asia are generally economically strapped or under communist control where liberties are restricted. In such areas, there is concern that failure to be forthcoming regarding cases of avian influenza could have devastating consequences, by permitting a pandemic-causing strain to take route and spread before containment can be achieved. This culture was particularly evident when Chinese leaders covered up the SARS outbreak for months in fear of reduced trade and tourism. In the case of SARS, the outbreak ultimately caused billions of dollars in lost revenue, unemployment reached record levels, and Hong Kong, which neighbors Guangdong province and its seven million people, was pushed to the edge of its third recession in a

decade.

## B. Steps to Reduce Avian Influenza in Asia

World Organization for Animal Health (OIE) and the United Nations Food and Agriculture Organization (FAO) recommended vaccinating poultry to contain H5N1 spread and to reduce the risk of transmission of H5N1 to humans. Different countries have responded differently to these recommendations: Hong Kong requires vaccination of all poultry, China and Indonesia are selectively vaccinating in regions where the virus has appeared, and in August 2005, Vietnam will do the same. In contrast, Thailand, which is the fourth largest exporter of poultry, does not endorse bird vaccination.

The Thai government does not endorse vaccination because it makes it more difficult to track infected birds, and in order to export poultry, the birds must be shown to be free of disease using a laboratory test. The current test includes examining the blood for the presence of antibodies, which indicates the bird has been or is infected. However, vaccination also elicits an antibody response, which clouds the diagnostic test, making it more difficult to determine whether a bird is actually infected. However, this problem can be avoided by creating a vaccine that carries the same HA of the circulating strain, but a different NA subtype. The diagnostic test then focuses on the NA antigen, rather than the HA antigen, which allows for successful discrimination between infected and non-infected vaccinated birds. Alternatively, this diagnostic problem could be addressed using an RT-PCR based assay.

Although Thai officials have not endorsed vaccination, they have established strict guidelines designed to reduce avian influenza. These guidelines include using netting to keep wild birds out of chicken ponds and copious amounts of cage and clothes washing. In addition, transport of birds is kept to a minimum and contact with humans is also minimized. At the live animal markets, there is a movement to stop the entrenched custom of slaughtering chickens, in favor of a central slaughtering facility. Lastly, as an

alternative to vaccination, some Thai villages have chosen to use herbs to protect their flocks. However, these guidelines are more easily enforced in commercial chicken farms than in small family farms with backyard chickens.

The aim of vaccination is to reduce the amount of virus in the environment, which reduces the chances of further spread and possible infection of humans. It is important to note that vaccination does not always prevent disease, but it reduces mortality and reduces the amount of virus shed from an infected bird or human. If a bird is found to be infected, regardless of the health of the bird, culling is recommended to preserve the health of the entire flock.

Recent work with avian vaccination has suggested that a H5 vaccine strain offers cross protection to other strains [94]. In addition, in the laboratory, a vaccine based on an H5N2 strain was found to protect against clinical H5N1 disease and reduce viral excretions by 1000 fold [95]. Further field evidence supports the efficacy of avian vaccination. On Hong Kong chicken farms, H5N2 vaccine was shown to protect against H5N1 infection and there was no evidence of asymptomatic shedding if the vaccine was given 18 days prior to exposure to the virus. In early 2003, Hong Kong implemented universal vaccination and began placing sentinel chickens into each flock to monitor for circulating avian influenza. In early 2004, H5N1 swept through China, but Hong Kong remained virus free, suggesting the vaccine was effective. However, Hong Kong has only 150 chicken farms and is small in comparison to China, making enforcement of vaccination more practical.

The cost of vaccination is also an issue. Not including labor, the vaccine costs 2.5 - 7 cents per dose, which becomes prohibitively expensive for large scale farms. In addition, vaccination presents a logistical challenge considering hundreds of millions of birds require vaccination in Southeast Asia, so rather than attempt to vaccinate all the birds, China and Indonesia resort to spot vaccination. Unfortunately, these countries are

using H5N1 as a vaccine strain, which makes it difficult to track the disease.

Although vaccination has been shown to be effective, it also has its risks. Previous work in Mexico has highlighted the dangers of bird vaccination. For 7 years, chickens were vaccinated with inactivated H5N2 virus, which was circulating. Over time, the circulating virus mutated and although the vaccine continued to prevent clinical disease, it failed to reduce the amount of virus shed by infected animals, which endangers unvaccinated chickens [96]. This vaccination practice may have contributed to the virus becoming endemic in Mexico, Guatemala, and El Salvador. To avoid this, the virus must be monitored and the vaccine updated regularly to make sure that vaccinated birds either resist infection or shed fewer viruses if infected.

### C. Vaccination versus Culling of Birds

Prior to 1997, chicken flu was relatively rare. Since 1997, the H5 and N7 subtypes have killed, directly or by culling, over 120 million birds in just six years. Most of these deaths have occurred in Southeast Asia. Prior to 2003, agriculture authorities have favored culling over vaccination. However, on February 5, 2004, the United Nations Food and Agriculture Organization (FAO) advised governments in affected areas to vaccinate their poultry flocks , since mass culling of infected birds has failing to halt the disease, which since the 2003 re-emergence has become endemic. Currently, both culling and vaccination are encouraged to curb the spread of H5N1.



## Chapter 7. U.S. Wild Bird Influenza

Surveillance of North America's wild ducks and shorebirds has demonstrated that wild ducks have a high frequency of influenza A inflection virus during their southern migration, whereas shorebirds have a high frequency of infection during their northern

migration [97]. Shorebirds may be the leading source of some viruses since they were found to be infected with a broader range of subtypes.

# Chapter 8. Influenza versus SARS

In 2003, an unidentified virus began sickening people in Hong Kong. The virus was termed Severe Acute Respiratory Syndrome (SARS) and was eventually classified as a new corona-like virus [98, 99]. Ultimately, SARS infected nearly 8,000 people, 774 of whom died. SARS was found to infect the upper respiratory tract and gastrointestinal system, and the virus can be spread by inhalation of aerosolized virus or via a fecal-oral route.

Retrospective analysis of the outbreak suggested that when the virus first emerged, only 3% of the individuals who came in contact with an infected patient became ill. Seven months later, the virus had mutated to allow better transmission, and 70% of susceptible hosts that came in contact with an infected individual became ill. In one case, an infected individual living in an apartment complex with faulty plumbing that aerosolized the virus caused 321 new cases.

Despite the contagious nature of SARS, the virus has a relatively long incubation period, 6 days, during which time; the infected individual is asymptomatic and can not spread the virus. This time interval allows for notification and quarantine precautions to be put in place. Once a SARS infected individual becomes sick, they are generally ill enough where they are bed-ridden. From a population standpoint, the advantage of a long non-contagious incubation period and having an abrupt onset of serious symptoms is that it reduces the number of people the may inadvertently come in contact with an infected patient. This greatly reduced the ability of SARS to spread.

In contrast, influenza has a very short incubation period of two days, after which the infected individual can pass the virus. In addition, it is possible for an infected

individual to be asymptomatic and still pass the virus to a susceptible individual. This feature of influenza makes it a more difficult virus to contain than SARS.

Interestingly, although SARS was a newly emerged clinical entity, analysis of the market workers in Guangdong suggest that the virus has been circulating for years since 40% of the workers have antibodies. Presumably, these infections were largely asymptomatic.

# Chapter 9. Human Influenza

The current belief is that all human influenza originated from avian influenza. Classification of current human influenza strains suggests that avian influenza (or segments of avian viral genomes that have undergone reassortment in another species) have successfully crossed into humans and established residency only five times in recent history. These events have lead to the generation of type B and type C viruses, as well as type A viruses, which includes subtypes H1N1, H2N2, and H3N2. These now-human viruses have evolved enough so that it is equally hard to them to cross back into birds. As a result, the relationship between human HA and NA antigens and their avian counterparts is distinct, in other words, sera against human H1 will not completely neutralize avian H1, although there may be some cross-reactivity.

Previously it was thought that pigs were a necessary mixing vessel for avian flu to infect humans. This belief grew out of analysis of 1957 and 1968 strains, each of which are thought to be the result of reassortment within pigs, in which the currently circulating human strain acquired some avian segments. However, it now appears as though

reassortment in pigs is not essential, for H5N1 has been shown to directly infect humans.

## A. Pandemic Predictions

Many experts feel that the trend we are observing in Southeast Asia with avian influenza infecting humans also happened in the years leading up to the 1918 pandemic. The enhanced virulence of H5N1 and the potential for a wider distribution by infecting migrating birds are causes for renewed pandemic concerns. H5N1 meets the two of the three criteria that are required in a pandemic strain. The first criterion is that humans must have little to no immunity against the emerging virus, generally because it originates from another animal reservoir. The second criterion is that the virus must make humans sick. And the last criterion is that the virus must be contagious from human to human. So far, the H5N1 virus meets the first to criteria, and question remains as to whether the third criterion will be met.

Scientists at the CDC and WHO have been deliberately cautious in predicting the severity of the next global pandemic, since dire predictions that never materialize are damaging to their agency's credibility, as was the 1976 Swine flu scare. It is important to emphasize the danger and the need for continued surveillance and work on vaccines and research to help combat future pandemics, but firm estimates on worldwide mortality range from as low as 2 million in a mild pandemic to as high as 100 million deaths in a worst case scenario [100]. Some scientists believe that if a H5N1 pandemic were to start, it will be impossible to stop, but there is hope that proper antiviral medications, vaccines, quarantine procedures, and medical preparedness can reduce its impact.

## B. Pandemic Modeling

Pandemic modelers incorporate a number of parameters in their calculations. Some of these include: 1) the basic reproductive number (R0), which is the number of

secondary infections resulting from one patient, 2) the attack rate, which is the percentage of people who get sick after being exposed to the virus, 3) the chance of becoming infected when in close contact with a patient, 4) the incubation period, 5) the mortality rate, and 6) the rate of spread, which is dependent on the travel patterns of infected individuals. Another uncertain parameter is whether the virus is free to travel between two animal hosts.

It is estimated that if the 1968 pandemic surfaced in 2000, due to increased world travel, the pandemic would spread across the world twice as fast, in 6 months rather than a year [101, 102]. A relatively mild pandemic like the 1968 virus would cost the United States between $71 and $166 billion if it were to strike today [103]. When faced with limited vaccine during a mild pandemic, ethicists must consider whether the primary goal is to reduce mortality or reduce the economic fallout. If the goal is to reduce mortality, the elderly should be vaccinated first; and if the goal is to reduce the economic fallout, the school children should be vaccinated first [74].

# Chapter 10. Genetic Shift, Drift, and Recombination

Pigs, like humans, contract influenza through the respiratory tract and symptoms are similar to what is seen in humans. The epithelial cells of the pig's lungs possess the receptors for both avian and human influenza, and as a result, it is possible to have one pig infected with swine, human, and avian influenza. Consequently, pigs are considered a mixing pot for avian and human influenza, whose segmented genomes can undergo reassortment when the same cell is infected with both viruses [104]. Such reassortment of two viruses can create a chimeric virus. By definition, chimeric viruses have acquired genetic diversity, and this process is called antigenic shift. Antigenic shift causes a major change in the virus, which often results in a new influenza subtype. Some chimeric viruses have the potential to cause a pandemic since they are capable of replicating in

humans, but are not recognized by the human immune system due to the presence of foreign glycoproteins acquired from either bird or pig virus. Recombination occurring in pigs is thought to have spawned the pandemics of 1957 and 1968. Pure swine influenza rarely crosses species into humans. In fact, only 18 cases of pure swine influenza infecting humans have been documented. Generally reassortment viruses contain some avian genes.

In addition to undergoing change through genetic shift, viruses can also undergo a more gradual change through mutation. Acquiring genetic diversity through mutation is called genetic drift. Viruses that undergo antigenic drift can acquire the ability to re-infect a person previously infected by the ancestor virus, due to changes in their antigenic makeup.

Type A viruses can change by both shift and drift, whereas type B viruses only change by antigenic drift. This is because type B viruses only infect humans and do not have other reservoirs in other species that can evolve independent of human influenza. The constantly changing nature of influenza necessitates yearly vaccines.

Genetic diversity in influenza is thought to predominantly occur through genetic drift and shift. However, there have been reports that the influenza polymerase can undergo recombination between viral genes and even with cellular genes. For example, there have been reports that recombination has occurred between the HA and matrix segments [54], between HA and NP segments [105], between NP and HA segments [106], and between the HA gene and host cell 28S ribosomal RNA [107]. There have also been reports of recombination occurring within the HA gene between swine and human segments [108], but this finding is controversial.

# Chapter 11. Swine Influenza

Between 1931 and 1997, the predominant subtype of influenza in North American

pigs was H1N1. Due to its dominance, it is referred to as "classical swine flu", and it is thought to be related to the strain that caused the 1918 Spanish flu. However, since 1997, three different novel subtypes (H3N2, H1N2, H4N6) and five different genotypes have been isolated in infected pigs in North America [109].

During the 1997-98 season, four severe outbreaks occurred that sickened and killed many pigs. Hemagglutination inhibition (HI) assays of serum samples indicated that new reassortant viruses were to blame. The causative viruses were identified and shown to be chimeras that had acquired human coat proteins, H3N2. One virus was a double reassortment that contained human and swine segments, but the other was a never before identified triple reassortant. The triple reassortant viruses possessed a combination of the following: PB2 (avian), PB1 (avian or human), PA (avian), HA (human or swine), NP (swine), NA (human or swine), M (swine) and NS (swine) [61, 110].

Since 1997, H3N2 has continued to circulate in North American pigs and has become a major cause of swine influenza. The H3N2 swine virus has been particularly active since it has undergone reassortment with the H1N1 classical swine flu to make a H1N2 strain. Furthermore, in 2003, the H3N2 virus jumped from pigs into turkeys in two geographically distant farms [111]. These isolates, A/turkey/NC/16108/03 and A/turkey/MN/764/03, are better adapted to avian hosts then their closest swine counterparts, suggesting the virus already has begun to evolve in the new hosts [111].

In 1999, an avian H4N6 strain was isolated in swine in Ontario, but has not spread beyond the farm of origin.

The spread of new subtypes into pigs is not restricted to North America. Northern Europe was free of swine flu until 1979, when severe swine flu broke out and subsequently formed a new stable lineage. The swine flu was found to be of avian origin [112] and has the highest evolutionary rate measured [113]. Specific monoclonal antibodies against A/Swine/Germany/81 (H1N1) have been generated that neutralize the swine strain and may be therapeutic for humans if they were to become infected with

swine flu. Furthermore, in 2003 in the Netherlands, there was an outbreak of avian H7N7 that infected both humans and pigs [63].

The apparent increased incidence of reassortant viruses is a serious concern. The increase may be attributed to better surveillance, but many feel it is an indication of a rapidly changing virus. The virus may be changing more rapidly because changes in animal husbandry may permit a faster rate of evolution. Today's pig farms are larger than they were three decades ago and pigs are often kept in outside pens that are exposed to the droppings of migratory waterfowl. In addition, prior to 1995, swine vaccination was in its infancy, but now greater than 50% of pig farms vaccinate their animals. Some epidemiologists argue that vaccination may be putting evolutionary pressure on the virus to evolve more quickly, but most health officials and scientists feel that vaccination is necessary to protect the animals. Not only are vaccinated pigs more resistant to the circulating viral strains, but also vaccinated sows pass protective maternal antibodies on to their young. Although swine influenza poses a major threat to the human population, no official surveillance system currently exists. In acknowledgement of this fault, WHO has agreed to make a priority list of all viral subtypes for vaccine production, although it is unclear if progress has been made in generating any vaccines.

# Chapter 12. Seasonality

In temperate regions, the incidence of influenza infection oscillates throughout the year, with the highest incidence of infection occurring in middle of winter. In tropical regions with rainy and dry seasons, the incidence of infection is greatest during the rainy months, although outbreaks can occur during the dry season.

Many hypotheses have been posited to explain this oscillation phenomenon, some of which include: that cold weather favors influenza survival; that cold weather increases mixing patterns by forcing people inside (schools) and into close contact while

runny noses facilitate the spread of the virus, and that the virus replicates better in a host that lives in colder weather. Laboratory and epidemiological studies have failed to establish whether seasonal transmission changes are due to the effects of temperature and humidity [114], changes in mixing patterns [115], or increased viral production under winter conditions [116]. Although no one parameter has been defined that affects the transmission rate, the combination of all parameters is called seasonal forcing.

Seasonal forcing may be too small to measure, but overall it is thought to resonate with the oscillatory nature of viral epidemics, causing the seasonality of influenza infection [117]. Influenza has a natural oscillation in the transmission rate of the virus. This natural oscillation is due to the fact that immunity to the virus is not long lasting due to the fact that the virus is constantly evolving. Although, immunity to a particular strain of influenza can be long lasting, the virus continues to evolve and previously resistant hosts eventually become susceptible. As a result, an infected person is generally susceptible to a variant (progeny of original infecting virus) within a few years [118]. The average duration of immunity is thought to be between 4 - 8 years. Other factors that contribute to the oscillatory nature include the mean infectious period, which is approximately 6 - 10 days, and the basic reproductive number as defined by the expected number of secondary infections that a single infectious individual will cause in a wholly susceptible population, estimated at 4-16 infections [119-121]. These parameters can be modeled mathematically to give an endogenous oscillatory period of 0.4 to 1.5 years for influenza, which is close to the annual period of seasonal forcing. As a result, the natural oscillatory period of influenza and seasonal forcing resonate with one another, and this is manifested as a prominent influenza season.

# Chapter 13. 1889 and 1900 Epidemics

Historical records describe flu outbreaks in 1889 and 1900. Epidemiologic data

obtained from analyzing sera suggest that the 1889 strain was subtype H2N2 and the 1900 strain was H3N8.

# Chapter 14. 1918 Spanish Flu

The 1918-19 "Spanish flu" (H1N1), was the most catastrophic of the three major pandemics in the past century. The pandemic coincided with the end of World War I and it is thought that the social disruptions during war time likely exacerbated the pandemic and made it more deadly [122]. The 1918 virus was so virulent that it was capable of causing massive damage to the lungs of infected individuals, which would fill with blood and fluid. Victim's faces would turn ashen blue from lack of oxygen and they would cough violently with projectile nosebleeds. Most 1918 victims died of pneumonia caused by the virus and a secondary bacterial infection, for which there was no treatment. Some victims died solely from influenza and within 24 hours of symptoms onset. Essentially, the victims suffocated from excessive fluid in their lungs.

The pandemic lasted 18 months and in the end approximately 30% of the world's population had been infected [123]. For the infected, the mortality rate varied tremendously from region to region, from 1% to 80%, but overall it was thought to be approximately 2.5% [124]. Once the pandemic began, the incidence of infection and the ensuing deaths double about every three days [125]. By the end of the pandemic 20-50 million people had died worldwide and half of the victims are thought to have died within India's borders. In the United States approximately 500,000-675,000 died and in Philadelphia alone, 7,500 died within two weeks. Forty percent of the 43,000 U.S. servicemen and women who died in WWI, died from influenza. Most deaths occurred in adults from ages 15-35. In contrast to typical death curves for influenza, 99% all "excess" influenza deaths (those above annual averages) were in people younger than 65 years old. Since the young had a higher mortality then the old, it suggests that the older

generation had previously been exposed to a similar strain and had some prior immunity. In cities that were hit hard, hospitals became overwhelmed, coffins ran out, morgues filled up, and mass graves were dug to help bury the dead.

At the time, the causative agent was unknown, but many doctors suspected it was caused by the bacterium *Haemophilus influenzae* (Pfeiffer's bacillus). Fifteen years after the pandemic, the true causative agent of influenza isolated.

Detailed accounts of the pandemic can be found in Alfred Crosby's "America's Forgotten Pandemic" [126] and Gina Kolata's "Flu" [127] and John Barry's "The Great Influenza: The Epic Story of the Deadliest Plaque in History" [128].

## A. Origin of 1918 strain

Most pandemics receive their name due to their place or species of origin. The 1918 pandemic was mistakenly called the "Spanish flu", because Spain was stricken early in the outbreak and the publicity stuck with the virus. The 1918 pandemic came in two waves. The first wave or "herald" wave arrived in late 1917 and lasted through the spring of 1918 [129, 130]. Anecdotal evidence suggests that those who fell ill during this herald wave, were resistant to infection when the brunt of the pandemic hit in the fall and winter of 1918-19. However, historians disagree as to whether the 1918 strain that is responsible for the pandemic first emerged in France in 1917 or not until spring of 1918 in the United States.

There is some evidence to suggest that the virus may have first emerged in the winter of 1917 at a British base camp in Etaples, France. The Etaples camp was overcrowded with troops that were living in close proximity to pigs, geese, ducks, chickens, and horses, which made ideal conditions for interspecies viral transmission. Furthermore, the soldiers in the camp were under significant respiratory distress due to gas warfare that occurred in the months immediately prior to the winter of 1916/1917. Some historians claim the viral outbreak that occurred here initiated the 1918 pandemic

when the soldiers returned to their homelands in the autumn of 1918 [131].

Alternatively, other historians believe that the first recorded outbreaks occurred in Kansas. U.S. soldiers fell ill on March 4, 1918 at Camp Funston and seven days later at nearby Fort Riley. The virus spread quickly, and by April, most American cities had reports of infection. However, the virus subsided in midsummer, only to come back with a vengeance as a more deadly virus in the fall. At the time, hundreds of thousands of American soldiers were landing in Europe for WWI, bringing with them the virus.

The first case of the second wave was recorded on August 22nd, in Brest, France, which was a major port for incoming American troops. Within a matter of weeks it spread through Europe and it lingered long enough in Spain to be erroneously dubbed the Spanish flu [132]. It quickly re-appeared in the States and spread worldwide. Every corner of the world was hit, including Eskimo villages near the Artic, Africa, India, New Zealand, and beyond.

## B. 1918 Virulence and Transmissibility

The 1918 virus spread very quickly, leaving some to speculate as to whether the 1918 strain was transmitted only through the usual aerosol route, or also via the drinking water [124], which would be unconventional for human influenza. In addition, the 1918 pandemic was very widespread, reaching even Eskimo villages, and considering the more limited travel in comparison to modern times, one might wonder if migrating birds may also have played a role. However, these hypotheses have largely been dismissed and the current thinking is that the 1918 strain was transmitted by conventional human to human "contact".

The mortality caused by influenza pandemics is largely dependent on the transmissibility and virulence of the virus. Virologists measure the transmissibility of a virus by its reproductive number, which is the number of secondary cases produced by each primary case [115]. The larger the reproductive number, the harder it is to stop a

potentially epidemic outbreak. Reproductive number estimates for pandemic influenza ranges from 1.68 to 20 [74, 133, 134]. The reproductive number for the 1918 pandemic was calculated by using data collected by 45 US cities in a deterministic SEIR model (susceptible-exposed-infectious-recovered) [125]. When immunity to H1N1 prior to 1918 was incorporated, the reproductive number was found to be less than 4. This value is comparable to an estimate of the R value for the SARS outbreak [135-137] and it is slightly higher than R values for 1957 A/H2N2 and 1968 A/H3N2 pandemic R estimates [74, 138], but well below many other infectious diseases [115].

The level of transmissibility affects the doubling time for an epidemic, which is dependent on both the reproductive number and the serial interval. The serial interval is the average time between a primary case and its secondary cases. The mean latent and infectious periods for 1918 influenza is 1.9 days and 4.1 days, respectively [74, 139].

The lethality of the 1918 pandemic was not due to its average level of transmissibility, but instead to its high virulence, which resulted in a mortality rate ten times higher than that of all other influenza pandemics [139, 140].

## C. Finding the Spanish Flu

The Spanish flu was unique in that almost half its victims were young healthy adults. The reason for the increased virulence and pathogenicity of the 1918 flu is still unclear. In order to unravel this mystery, it is necessary to obtain the genetic sequence of the 1918 virus. Two different approaches have been taken to find and isolate the sequence of the 1918 virus. One approach has been to extract the genetic material from paraffin embedded blocks of lung tissue taken during autopsies of soldiers who died from the virus; and the second has been to look for victims of the 1918 flu who were buried in permafrost and have remained frozen.

Dr. Jeffrey K. Taubenberger is a pathologist at the Armed Forces Institute of Pathology, in Washington, D.C. that examined paraffin embedded samples of victims

from 1918. He and his technician, Ann Reid, searched through the National Tissue Repository at the Walter Reed Army Medical Center in Maryland. The repository holds more than 2.5 million samples, many of which are preserved in formaldehyde and encased in a block of paraffin wax. Samples from autopsies date back to before WWI. They found seven samples from victims who had died rapidly from influenza in 1918. In 1997, a sample from a 21 year old Army private who died six days after coming down with pneumonia at Fort Jackson in South Carolina turned up positive for influenza RNA.

The first attempts to find the 1918 flu virus from frozen victims were carried out in 1951. The Army carried out a secret mission code-named "Project George" to Nome, Alaska, but was unsuccessful since the bodies had decomposed. During the same year, a graduate student by the name of Johan Hultin went to Brevig Mission, Alaska and recovered lung tissue from four frozen bodies but failed to remove live virus from the tissue.

More recently, in 1997, there were two more expeditions to find the 1918 virus. One group was lead by 30 year old Canadian geographer Kirsty Duncan who had compiled a team of world-class scientists from England, Canada, and the U.S., including Dr. Taubenberger who agreed to help analyze any frozen samples. The project took four years to plan with much publicity and cost. To avoid the potential of releasing a live virus to start another pandemic, they took all precautions including biohazard suits and tents. They planned to use ground radar to locate the bodies and once the tissue sample had been extracted, to send it to BSL-4 facilities located in either England or at the U.S. Army's infectious-disease research facility at Fort Detrick, Maryland. Duncan followed all the rules and took all precautions any individual could hope for in her efforts to unearth a deadly virus.

The group went to a cemetery in the town of Longyearbyen on a Norwegian island called Svalbard, which is north of the Artic Circle, less than 800 miles from the North Pole. In the cemetery lay seven coal miners, ages 19 to 28, who died of the flu and

had been buried during the first week of October in 1918. Unfortunately, exhumation determined that the bodies had not been buried deep enough into the permafrost and had thawed over the years so that no tissue could be recovered that contain viral sequence.

Also in 1997, 46 years after his first trip to Alaska, at the age of 72, Dr. Jonah Hultin, returned by himself to Brevig Mission, Alaska. In 1918, 72 of the 80 residents at Brevig Mission had died from influenza within five days of one another [141]. Again, he found the frozen bodies that were buried seven feet deep and removed lung tissue using his wife's pruning shears from a corpse he named Lucy. Lucy had been an obese woman who died from flu in her mid-20s and after 89 years, her lungs were frozen and filled with blood. He placed the tissue in preservative and built two crosses, 7 and 11 feet tall, to mark the grave site. Dr. Hultin's mission generated no publicity and cost only $3,200, which he paid himself. He returned to the States and mailed the specimens to Dr. Taubenberger for analysis. One of the samples was positive for influenza sequence.

## D. Characterization of 1918 segments

Dr. Jeffrey Taubenberger has led an effort to sequence the 1918 virus from RNA isolated from several sources including two positive paraffin samples of soldiers who had died, lung tissue from a woman frozen in the Alaskan permafrost (Johan Hultin), and autopsy tissue samples from victims who were stored in the Royal London Hospital. All isolates were found to be genetically very similar, suggesting one strain was responsible for the outbreak. As of March 2004, five of the eight segments have been sequenced, including HA, NA, M, NP, and NS segments. The genetic codes for these segments are available on the internet. The sequences of PB2, PB1, and PA have not been made public, presumably due to bioterrorism concerns. None of the publicly available segments have shown obvious anomalies that might explain the virus lethality.

### i. HA Segment

Early work on the HA segment concluded that despite the fact that the HA

segment shares many characteristics with subsequent human and swine strains, it appeared more closely related to avian HA than to other mammalian strains [142]. The 1918 HA has four glycosylation sites that are conserved in all avian H1 HAs and none of the additional sites that have accumulated by antigenic drift in human strains. Furthermore, the antigenic areas of the 1918 HA are nearly identical to avian strains, whereas human strains from 1933 and beyond show extensive drift at these sites. The analysis suggested that the 1918 HA either entered the human population directly from birds or through a mammalian host just prior to the pandemic.

Two groups have solved the crystal structure of the 1918 HA. Crystal structures provide insight into the dimensions of the receptor binding pocket of HA. In human strains of influenza, the HA binding pocket is slightly wider than in avian strains. This difference likely presents a barrier for cross-species infection. The HA structure of the 1918 strain has an avian-like sequence. The binding pocket of the 1918 HA contains either one or two amino acid changes, depending on the strain examined, that makes the avian-like pocket slightly wider. Such subtle differences may be sufficient to allow avian-type HA proteins to bind to human receptors [143, 144]. These structures explain how the 1918 HA, is able to bind human receptors while retaining many amino acids and features that are characteristic of avian HA.

Another study that examined the primary sequence, claimed that a recombination event occurred that transferred the globular domain of a swine HA onto a human HA stalk [108]. This finding is controversial among experts in the field.

More recent data encompass the HA sequence from influenza infected birds prior to 1918 and concludes that the HA segment did not come directly from birds since it is different from sequence obtained from 1917 avian influenza, and the avian HA sequence has not drifted much from 1917 to the present [145]. The current thinking is that HA had

acquired many changes that distinguished it from avian strains, prior to entering humans.

ii. NA Segment

Similarly, phylogenetic analysis of the 1918 NA gene segment suggests that the 1918 NA gene possesses many avian features, yet appears to have acquired a few mammalian features. The avian sequence and structural characteristics include the conserved active site, wild-type stalk length, glycosylation sites, and antigenic sites [146]. Overall, these data suggest that the NA segment was introduced into mammals just before the 1918 pandemic.

iii. NP Segment

The origin of the 1918 NP gene still is unclear, despite its sequencing. At the amino acid level, the 1918 NP sequence differs at only six residues from the current avian consensus sequence, which implies that possibly reassortment occurred with an avian source shortly before 1918 [147]. However, at the nucleotide level, the sequence of the 1918 NP gene has more than 170 differences from avian strain consensus sequences, suggesting substantial evolutionary distance from known avian sequences. As a result, it appears as though the 1918 strain did not reassort with an avian strain to acquire its NP gene. Comparison to other available sequences suggests that the 1918 strain did not retain the previously circulating human NP. These results are consistent with the existence of a currently unknown host for influenza and phylogenetic analysis would predict that the host would have belonged to the mammalian clade.

iv. NS Segment

The NS segment from A/Brevig Mission/1/18 (H1N1) virus was sequenced and phylogenetically it appears to be close to the common ancestor of subsequent human and classical swine strain NS genes [148]. To test the hypothesis that the 1918 virus NS1 gene played a role in virulence, recombinant viruses were generated with the 1918 NS gene in a background of influenza A/WSN/33. These recombinants were tested for virulence in tissue culture and in mice in a BSL3+ laboratory. The 1918 NS-WSN virus

replicated well in tissue culture but was attenuated in mice as compared with the isogenic control virus. These data suggest the 1918 NS gene does not cause increased virulence, however it must be taken into consideration that the experiment was done in the context of a WSN background and in mice.

v. M Segment

Phylogenetic analysis of the matrix gene suggests it is a mammalian gene similar to the common ancestor of all subsequent human and classical swine matrix segments. The sequence of the M1 RNA-binding domains, NLS, and M2 transmembrane domain match current consensus sequences. However, the amino acid changes that are thought to correlate with high yield and high pathogenicity in animal models were not found [149].

## E. Phylogeny of 1918

Phylogenetic analysis of the 1918 virus has placed the virus within the human family, but on the side that is most closely related to the swine family. In contrast, the avian family of influenzas is more distantly related to human family than to the swine family. In other words, pig influenza is evolutionarily in between human and avian influenza. Despite this genetic separation, the 1918 viral genes do have some avian features, making it probable that the virus originally emerged from an avian reservoir prior to 1918. However, due to the significant number of differences from bird sequences, it is likely that the virus spent some length of time in an intermediate host, where it accumulated many changes in HA from the original avian sequence prior to infecting humans.

Because the 1918 strain has significant differences from avian viruses, it is assumed that it spread to an intermediate source, where it acquired genetic variation prior to infecting humans. It is not known when this first jump occurred or the animal in which it occurred. It is reasonable to assume that a jump did occur, since when a virus enters a foreign host, it generally acquires mutations that enable it to replicate more effectively.

Because avian viruses are well adapted to growth in birds, they undergo a relatively slow rate of genetic drift. This has been shown by analyzing avian sequences gathered from 1917 (infected Brant goose from Alaska) to the present, which suggests that avian strains have changed relatively little over the past 80 years. Of course, it is possible that the 1918 strain came directly from a bird that was evolutionarily isolated from typical waterfowl.

For a long time it was speculated that pigs were the intermediate animal since they are susceptible to both human and avian influenza and there were simultaneous outbreaks of influenza in both humans and pigs during the 1918 pandemic. However, Taubenberger's group, which has carried out most of the analysis on the 1918 strain, believes that the mammalian intermediate was not pigs.

Unfortunately, there are very few pre-1918 influenza samples, which makes it difficult to determine what changes occurred that made the virus so virulent. To this end, it would be helpful to find pre-1918 influenza samples from both humans and other species. Of note, there have been expeditions to find 1889 flu victims that are buried in the permafrost, but the lack of any published work suggests these searches were unsuccessful. Furthermore, it would be helpful to find post-1918 samples to fill in the gap to 1933 when influenza began to be more regularly preserved. Post-1918 samples may shed light on what changes were made to the 1918 strain that made it less virulent. A comparison of the 1918 and WSN/33 strains for the HA gene indicates that they similar, but do have significant differences; they are 90% and 86% identical at the nucleotide and protein level, respectively.

## F. Reverse Genetics and 1918

The reverse-genetics system allows scientists to create any virus for which the sequence is known. This ability does pose a risk to the human population, for it would not be unprecedented for a scientist to allow a virus to escape from the laboratory. The

consequences of releasing a 1918-like virus could be devastating. However, in order to better develop therapeutics to viruses, it is important to clearly understand what makes one virus more virulent than another. To minimize these risks, "live" virus experiments with virus that have pandemic-potential are carried out in BSL 3 or 4 laboratories, depending on the perceived risk.

This is particularly true for animal experiments. Both mice and ferrets have been used as animal models when studying influenza. Mice are not a natural host for influenza, and as such, most human viruses need to be adapted for growth in mice before they become virulent. Because mice are not a natural host of influenza it is extremely rare to have a non-adapted virus be virulent in mice. Recent human isolates have been tested in mice and found to not be lethal, regardless of dose [150, 151].

In regards to the 1918 strain, several recombinant viruses have been created that contain two to five 1918 segments, but none have been generated that contain the 1918 polymerase. These recombinant viruses have been generated in a WSN/33 background. Examination of the internal proteins, excluding the polymerase, has shown that the addition of NS seems to decrease virulence of the virus, suggesting that the NS gene is not very well adapted to growth in mice [148]. However, the addition of the matrix gene offsets the decrease virulence, and the addition of NP does not seem to affect the virulence.

The 1918 external glycoproteins, HA and NA, were examined in mice and compared to native WSN/33, which been adapted for growth in mice and is virulent, and non-lethal New Caledonia HA/NA in a WSN background. Both the 1918 and original WSN were highly lethal for mice and displayed severe lung pathology in comparison to the New Caledonia recombinant. Microarray analysis on infected lung tissue showed activation of many genes involved in the inflammatory response for each of the three viruses. The virulent strains distinguished themselves from the non-virulent strain by showing increased up-regulation of genes associated with activated T cells and

macrophages, as well as genes involved in apoptosis, tissue injury, and oxidative damage [152].

The finding that 1918 HA and NA themselves are sufficient to confer virulence is mice is significant [150, 151]. However, from pure sequence analysis, it is still unclear what features make the 1918 HA and NA proteins so virulent. One hypothesis is that the 1918 HA is easily activated by ubiquitous proteases like furin, which would expand its tissue tropism. This is true for the avian H5 and H7 , as well as the WSN strain, however, the 1918 HA does not possess a furin cleavage site. Another hypothesis is that the 1918 NA recruits a protease to cleave HA. This strategy is seen in the WSN strain, which is a descendent of the 1918 strain. The WSN neuraminidase protein has a carboxyl-terminal lysine and the absence of an oligosaccharide side chain at position 146 (N2 numbering). These features enable NA to bind plasminogen, a protease precursor, and the increased local concentration of plasminogen facilitates the cleavage of HA [153]. However, the 1918 neuraminidase protein does not have these features. Ultimately, it is still unclear how the 1918 HA is activated. These data suggest that an unidentified ubiquitous protease may be responsible for cleaving 1918 HA.

Although WSN/33 is thought to be a descendent of the 1918 virus, there are sequence differences that make these viruses very distinct. Despite the sequence differences of 1918 HA and NA, both viruses can be passaged in MDCK cells without exogenous trypsin. WSN is unique in this regard for viral strains used in the laboratory.

## G. 1918 Vaccines

To determine whether current vaccines would provide protection against the 1918 strain, mice were vaccinated with different strains and challenged with the 1918 recombinant. The H3N2 vaccination provides no protection, whereas the H1N1 and H5N1 strains provided partial protection [151]. The partial protection of current H5N1 vaccines was hypothesized to be attributed anti-HA immunity, since recombinant H5 HA

also provided some protection. Of interest, the strain that provided the best protection was isolated in 1930 and is the oldest available swine flu, (A/Swine/Iowa/30 (H1N1)). The A/Swine/Iowa/30 (H1N1) protected all mice from lethal challenge. This finding lends credibility to the theory that the swine flu outbreak in 1918 may have been caused by the same virus, or one closely related.

Hemagglutination inhibition assays demonstrated that antisera raised in ferrets and chickens against A/Swine/Iowa/30 (H1N1) provided a high level of cross-reactivity with 1918 HA. The high level of cross reactivity exists since of 22 amino acid differences, only 4 fall in antigenic sites. In contrast, WSN/33, PR/8/34, and Tx/91 exhibit low levels of reactivity, and USSR/77, Chili/83, and New Caledonia/99 exhibit no cross-reactivity with the 1918 HA. PR/8/34 has 15 antigenic amino acid changes from the 1918 HA, and the acquisition of glycosylation sites may attribute the lack of cross-reactivity. Antigenic cross-reactivity between 1918 virus and A/Swine/Iowa/30 was also demonstrated in humans. Humans born in the first three decades of the 20th century still contained antisera specific for 1918 influenza that would also neutralize A/Swine/Iowa/30. However, no individuals born after 1944 contained antisera that reacted with either 1918 virus or A/Swine/Iowa/30.

These data suggest that the currently circulating H1N1 viruses have drifted significantly from the 1918 strain [151]. In addition, these data suggest that A/Swine/Iowa/30 (H1N1) would serve as the best vaccine against a re-emerging 1918 virus.

If the virus were to re-emerge in the absence of effective vaccination, current antiviral strategies may be effective; as both neuraminidase and ion-channel inhibitors have been shown effective *in vivo* and *in vitro* against 1918 recombinants [150].

# Chapter 15. 1957 Asian Flu

Variants or the 1918 H1N1 strain circulated in humans until 1957, when a

reassortant virus, H2N2, displaced it. The reassortant virus contained avian HA (H2), NA (N2), and PB1 gene segments while the remaining five gene segments were of human H1N1 virus origin [154-156]. This H2N2 virus spread quickly and caused the "Asian flu" pandemic of 1957, which killed 70,000 Americans and a million people worldwide. This virus likely was created from a reassortment event in pigs [155].

## Chapter 16. 1968 Hong Kong Flu

Variants of the H2N2 strain persisted for ten years until another reassortment event occurred in 1968, and displaced the Asian strain. In 1968, H2N2 and avian H3 viruses recombined in pigs to create a H3N2 strain, which caused the "Hong Kong" pandemic. The H3N2 viruses that spread in humans had PB1 (newly acquired) and HA gene segments of avian (duck) origin while all other genes were inherited from human H2N2 viruses [154-159]. The 1968 Hong Kong flu killed approximately 34,000 Americans.

## Chapter 17. 1976 Swine Influenza Scare

On February 5th, 1976, 19-year-old Private David Lewis of Ashley Falls, Massachusetts died at Fort Dix in New Jersey, within 24 hours of symptoms onset. The cause of death was determined to be swine influenza A (H1N1), which sparked fear of a return of the 1918 epidemic. On August 12, by advice from federal public health officials, President Ford approved a $135-million vaccination program. By October 1st, a nationwide vaccination program began. Within days of the first inoculations, several people fell seriously ill, and on October 12th, three elderly suffered heart attacks within hours of receiving the vaccine. On December 16th, the vaccination program was halted

on the report that associated the vaccine with an increased risk of a neurological disease called Guillain-Barré syndrome. In all, 40 million of 220 million Americans had been vaccinated and 32 people are thought to have died from side effects, which is less than one in a million. The threat of another swine outbreak never materialized and the disease disappeared after infecting 500 people, many of whom never got sick. Some viewed the vaccination program as the finest hour of America's public health programs, but most viewed it as a fiasco. The industrial vaccine makers were accused of creating the hysteria for financial profit and in the end lost the public's confidence in the safety of the influenza vaccines. In addition, the administration was accused of using the swine flu scare as a spring board for a re-election campaign, which did not work since Ford did not get re-elected and CDC Director, Dr. David J. Sencer was dismissed.

## Chapter 18. 1977 Russian Flu Scare

In 1977, there was a resurgence of the 1950's H1N1 that is thought to have escaped from a research laboratory [160-162]. H1N1 spread worldwide, infecting mostly children and young adults under 23 years of age that had not yet been exposed to H1N1 viruses and developed immunity. Since the virus predominantly just infected children, it is not considered a true pandemic. In contrast to the pandemics of 1918, 1957, and 1968, the 1977 Russian strain (H1N1) did not displace the previously circulating strain, but instead began to co-circulate with variants of the Hong Kong 1968 virus (H3N2). Over the last 28 years, variants of H1N1 and H3N2 have continued to co-circulate.

## Chapter 19. Influenza in the Past Two Decades

Type B viruses are generally milder then type A, yet type B viruses have become

increasingly common within the past two decades, during which two strains have predominated, B/Victoria/1987 and B/Yamagata/1988. Victoria was dominant from 1987-1990 but restricted to Asia. From 1990-2000 Yamagata dominated, only to have Victoria regain dominance in 2000 and spread globally. Since 2000, there have been two clades of antigenically distinct HA and NA. There is not thought to be cross-reactivity between Victoria and Yamagata. In addition, reassortants have been isolated.

### A. Avian Flu Scare

In four of the past seven years (1997-2004) pure avian flu has infected humans. Similarly the 1997 H5N1 and 1999 H9N2 avian flu outbreaks never caused pandemics. Subsequent studies found the H5N1 virus to be very contagious among chickens, and although it had a death rate of 30% in humans (6/18 died) it did not spread from person-to-person. HK/H5N1 was able to replicate in the human intestine, causing severe gastrointestinal symptoms [60, 163]. The H5N1 influenza viruses, unlike other human, avian and swine influenza viruses, are resistant to the antiviral effects of interferons and tumor necrosis factor alpha, and this resistance has been mapped to the NS gene [69]. The failure of these avian viruses to cause human pandemics may be due to the natural barriers involved in cross-species infection as well as prompt government action in ordering the slaughter of all infected birds. Such action eliminates or greatly reduces the viral reservoir and diminishes the chances of cross-species infection and the probability that the virus will mutate into a more contagious form among humans.

## Chapter 20. Immunology

### A. Innate Immune System

A/WSN/33 has been shown to invade brain of WT mice through the olfactory

neurons [164]. Mice lacking the recombinant activation gene 1 (RAG-1 -/-) develop lethal infection, whereas mice without genes for IFN-α/β receptor, IFN-γ receptor, inducible nitric oxide synthase (iNOS), IgH, the transporter associated with antigen processing 1 (TAP 1), and natural killer cell depleted mice all survived infection. Viral RNA was found in 80% of mice lacking iNOS, IFN-γ receptor, and TAP 1 suggesting persistent infection. Taken together, these data suggest the cellular immune response prevents establishment of persistent infection in the olfactory bulbs, and the innate response contributes to keeping the infection under control.

A recent report has identified residues 224-233 of PA (HKx31 and PR8 strains) as being presented by major histocompatibility complex class I H-2D$^b$ [165]. PA specific CD8(+) T-cells that recognize this epitope were found to may a prominent role in the primary response to infection, but its role in a secondary challenge was found to be greatly diminished.

i. RNA Silencing

Mammalian cells have several innate mechanisms by which to protect them from viral infection, one of which is RNA silencing, also known as RNA interference (RNAi). When a mammalian cell recognizes dsRNA, either a nonspecific host shutdown occurs that leads to apoptosis, or the dsRNAs are processed into small interfering RNAs (siRNA), which specifically degrade the cognate RNA. However, influenza attempts to disguise its dsRNA by coating it with the NS1 protein, which has a dsRNA binding domain. In *Drosophila* cells, the double stranded RNA-binding domain of NS1 can inhibit RNA silencing, allowing the virus to successfully replicate [166].

RNA silencing is through the actions of Dicer-RDE-1, which is a dsRNA-specific endonuclease that processes long dsRNA into double-stranded fragments 21-25 nucleotides in length (siRNA). These siRNAs are incorporated into a protein complex, and siRISC, which is the proposed multi-protein complex that orients the antisense strand of the siRNA and the cellular mRNA together to allow for endonucleolytic activity,

which causes silencing. Similarly, microRNAs (miRNAs) are small, RNA molecules encoded in the genomes of plants, animals, and viruses (EBV) [167]. MicroRNAs are transcribed as roughly 70 nucleotide hairpins called pre-miRNAs, which are exported from the nucleus, where Dicer cleaves them into highly conserved, ~21-mer RNAs. These mature miRNAs are assembled into a ribonucleoprotein complex (miRNP), which binds to the 3'-untranslated regions (3'-UTR) of specific host mRNAs and prevents translation. In a few cases, the miRNA is exactly or nearly exactly complementary to the site in the mRNA, and this results in cleavage and degradation similar to that observed with siRNAs.

siRNA complexes silence mRNAs in a sequence specific manner. Due to the short length of siRNAs, the IFN response pathway is not activated. The RNA silencing machinery of the mammalian cell can be utilized to specifically down regulate a particular target gene. The target gene can be down-regulated by transfecting a synthetic 21-nt siRNA into the cell (or a plasmid that encodes a hairpin miRNA) that is sequence specific for target gene, thereby bypassing the role of Dicer.

The discovery of RNA silencing has introduced another branch of the innate immune system that may be taken advantage of to help fight influenza infection. In order to successfully utilize RNA silencing to fight influenza infection, it is important to identify the specific dsRNA sequences of influenza that are the most effective at reducing the ability of the virus to replicate. To this end, a panel of influenza specific siRNAs were examined in both MDCK cells and embryonated chicken eggs to determine if they could specifically target viral transcripts and reduce the efficacy of the virus to replicate [168]. Of the panel of siRNAs tested, NP-1496 and PA-2087 not only down regulated the level of mRNA, but also cRNA and vRNA of all the segments tested. This drop in RNA species correlated with a drop in the production of virus from 200 to 30,000 fold depending on the MOI used to infect MDCK cells. The authors conclude that siRNAs directly affect the level of mRNA, and as a downstream effect, the level of cRNA and

vRNA drop. Possibly mRNAs are targeted since they are exported out of the nucleus, whereas cRNA and vRNA remain in the nucleus and are encapsidated with NP. This effect was found to be independent of INF, since VERO cells deleted of the α, β, and ω genes exhibited the same effect. In addition, siRNA was shown not to affect the level of phosphorylation of PKR.

Additional work examining siRNA for influenza has utilized a lentivirus vector that expresses a 21 nt duplex of the M gene. This siRNA was shown to be effective at inhibiting M1 protein expression in transfected 293T cells and in virally infected MDCK cells [169]. Furthermore, other experiments utilized short interfering RNAs that are specific for conserved regions of the influenza virus genome are potent inhibitors of influenza virus replication in both cell lines and embryonated chicken eggs [170].

RNA silencing may also be used as a potential vaccine. Experiments using siRNAs that are specific for conserved regions of influenza virus genes, NP or PA, were tested in mice and found to inhibit influenza virus production [170, 171]. Further experimentation demonstrated that siRNA can not only prevent, but also help treat influenza virus infection in mice, when given either before or after initiating virus infection, In these experiments, the siRNAs were mixed with a polycation carrier and delivered intravenously. This mode of treatment appears promising, as siRNA treatment was found to protect against lethal challenge and the protection was specific and not mediated by an antiviral IFN response. In addition, protection was found to be broadly effective and protected animals against lethal challenge with highly pathogenic avian influenza A viruses of the H5 and H7 subtypes. Similar effects are also observed when mice are given DNA vectors intravenously or intranasally, from which siRNA precursors can be transcribed. Although a delivery system for siRNA in humans has not yet been developed, these data suggest that siRNA treatment may be an effective way to treat

human influenza.

ii. NF-kB

NF-kappa B controls the expression of many proinflammatory and antiviral cytokines. Contrary to expectation, influenza efficiently infects Epstein-Barr virus-immortalized B cells, which are high in NF-kappa B activity, yet has a difficult time infecting Burkitt's lymphoma cells, which are low in NF-kappa B activity. A study using these two cells lines demonstrated that the Burkitt lymphoma cells become susceptible to infection upon activation of the NF-kappa B signaling pathway, whereas blocking activation of NF-kappa B in EBV-immortalized B cells severely impairs infection. These data suggest that an active NF-kappa B signaling pathway is a prerequisite for influenza virus infection [172].

Additional experimentation has shown that influenza activates signaling through NF-kappa B, which induces the expression of the pro-apoptotic factors TRAIL and FasL [173]. These pro-apoptotic factors were shown to enhance virus propagation in an auto- and paracrine fashion.

iii. MxA

Mx proteins belong to the dynamin superfamily of high molecular weight GTPases and interfere with multiplication of a wide variety of viruses. Earlier studies show that nuclear mouse Mx1 and human MxA designed to be localized to the nucleus inhibit the transcription step of the influenza virus genome. One study has shown that the nuclear MxA suppresses the influenza virus transcription by interacting with not only PB2, but also NP[174]. MxA was not shown to interact with PB1 or PA.

iv. Toll-like Receptors

The pathways linking virus recognition to IFN induction remain poorly understood. Toll-like receptors have been shown to mediate the recognition of many types of pathogens, including viruses. The genomes of viruses possess unique characteristics that are not found in mammalian genomes, such as high CpG content and double-

stranded RNA. These genomic nucleic acids serve as molecular signatures associated with viral infections, and can be ligands for TLRs. TLR7 has been shown to recognize ssRNA viruses, including VSV and influenza [175]. In plasmacytoid dendritic cells and B cells, the Toll-like receptor (TLR) 7 and MyD88 respond to ssRNA in the endosome and signal to help induce IFN alpha in response to wild-type influenza virus [175, 176]. In addition to RNA, lipids were viral associated can also serve as TLR ligands.

Toll-like receptors (TLR) are expressed on many cell types including B and T cells, natural killer cells, dendritic cells, and epithelial cells. It is thought that binding to these receptors initiates a signaling pathway that mediates the release of anti-viral cytokines. The release of pro-inflammatory cytokines, including IFN-alpha, beta, and gamma, is thought to play an essential role in the innate immunity. In regards to influenza infection, TLR 3 and 7 have been examined in a number of cell types, and in response to both natural infection and different types of RNA. The response in each of these cells seems to be largely cell-specific and dependent on the type of activating factor used [175-179].

# Chapter 21. Design of Genome/Virus

The genome of influenza A viruses is approximately 13,600 nucleotides with some fluctuation between different strains. The genome is broken up into eight segments for type A and B viruses [180], and seven segments for type C viruses, which has the multifunctional HEF protein, rather than HA and NA (Intro. Fig. 1). The genome of the virus is termed viral RNA (vRNA) to distinguish it from complementary RNA (cRNA), which is an exact copy used in replication. The vRNA coding region is in the negative-sense orientation and can only be transcribed or replicated by the influenza polymerase. The influenza polymerase is made up of three subunits, PB2, PB1, and PA. The polymerase is thought to bind to each segment at the promoter. The promoter is made

up of the terminal 3' and 5' ends of the genome that interact with one another through partial complementarity. The 3' end contains 12 nucleotides that interact with the 13 nucleotides of the 5' end to make a structure that is considered to be the promoter. The ends of the genome are conserved between each segment, allowing for the polymerase to bind and recognize each segment. Interior to the terminal ends is additional noncoding sequence that is unique to each segment and conserved among different strains of influenza. The entire noncoding terminal ends make up the untranslated regions (UTRs) of each segment. The coding sequence of each segment encodes one protein except for the polymerase basic 1 (PB1), matrix (M), and nonstructural (NS) genes. There are 9 known structural proteins and two non-structural proteins including NS1 and the recently described PB1-F2 protein [181].

## Chapter 22. Viral Lifecycle

Influenza binds to the surface of cells via a sialic acid linked receptor (Intro. Fig. 2). The virus is endocytosed and while in the late endosome, the HA protein facilitates the fusion of the viral and endosome membrane. Membrane fusion permits the release of the viral genome into the cytoplasm, where each segment is individually transported into the nucleus. Once inside the nucleus, the viral polymerase transcribes and replicates the genome. Newly synthesized viral RNA and protein are assembled in the nucleus and exported as a viral ribonucleoprotein complex (vRNP). vRNPs are assembled into virions at the plasma membrane, where viruses bud out of the infected cell. This process

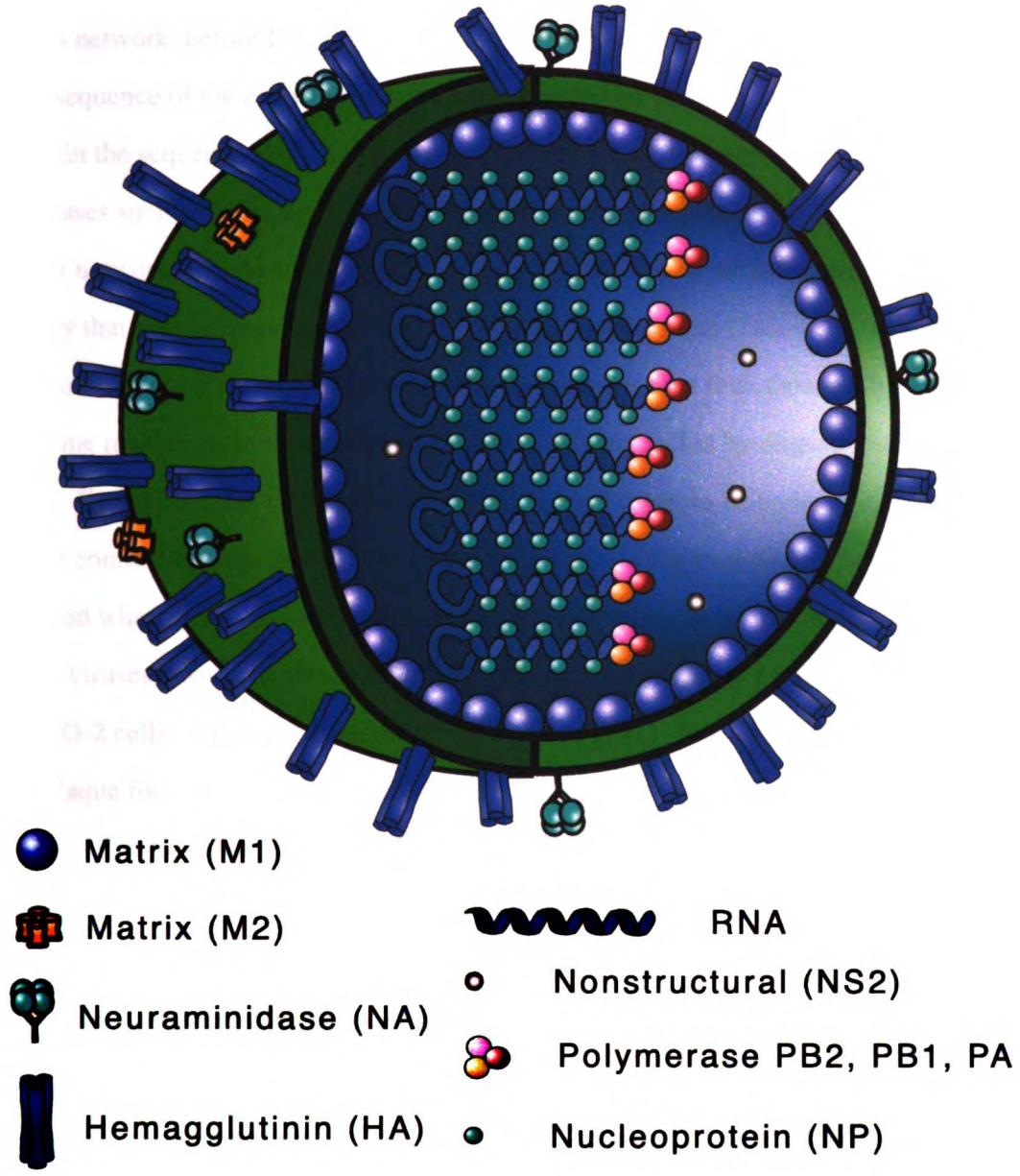
can occur in less than 16 hours.

# Chapter 23. Hemagglutinin - Entry

Hemagglutinin (HA), named for its ability to agglutinate red blood cells, is the most abundant surface glycoprotein of the influenza envelope. HA is a homotrimer of noncovalently linked monomers that appears as a rod shaped spike by electron microscopy. The homotrimer is anchored to the lipid bilayer by palmitoylated cysteines near the C-terminus of the protein [182-184]. To date, 15 antigenic subtypes, H1-H15, have been characterized and many variants exist within each subtype. Historically, the influenza subtypes that infect humans have been restricted to H1, H2, and H3. However, since 1997 subtypes H5, H7, and H9 from the avian reservoir have been added to the list, yet these avian viruses do not appear to be very communicable amongst humans. The intent of yearly vaccines is to stimulate the production of neutralizing antibodies against the most prevalent subtypes of HA in circulating strains of influenza. A properly matched antibody can bind to the virus and prevent viral attachment and penetration into host cells.

## A. Cleavage

Prior to binding a target cell, HA must be activated by proteolytic cleavage. The uncleaved HA is called $HA_0$, and the cleavage products are disulfide linked $HA_1$ and $HA_2$. The structures of $HA_0$, $HA_1$, and $HA_2$ have been solved by X-ray crystallography. Analysis of the structural requirements for cleavage activation of HA has shown that the cleaving proteases recognizes the connecting peptide sequence and at least one other structural feature [185]. The proteolytic cleavage can occur in the extra-cellular environment such as the airspaces of the lung, or it can occur prior to budding out of a



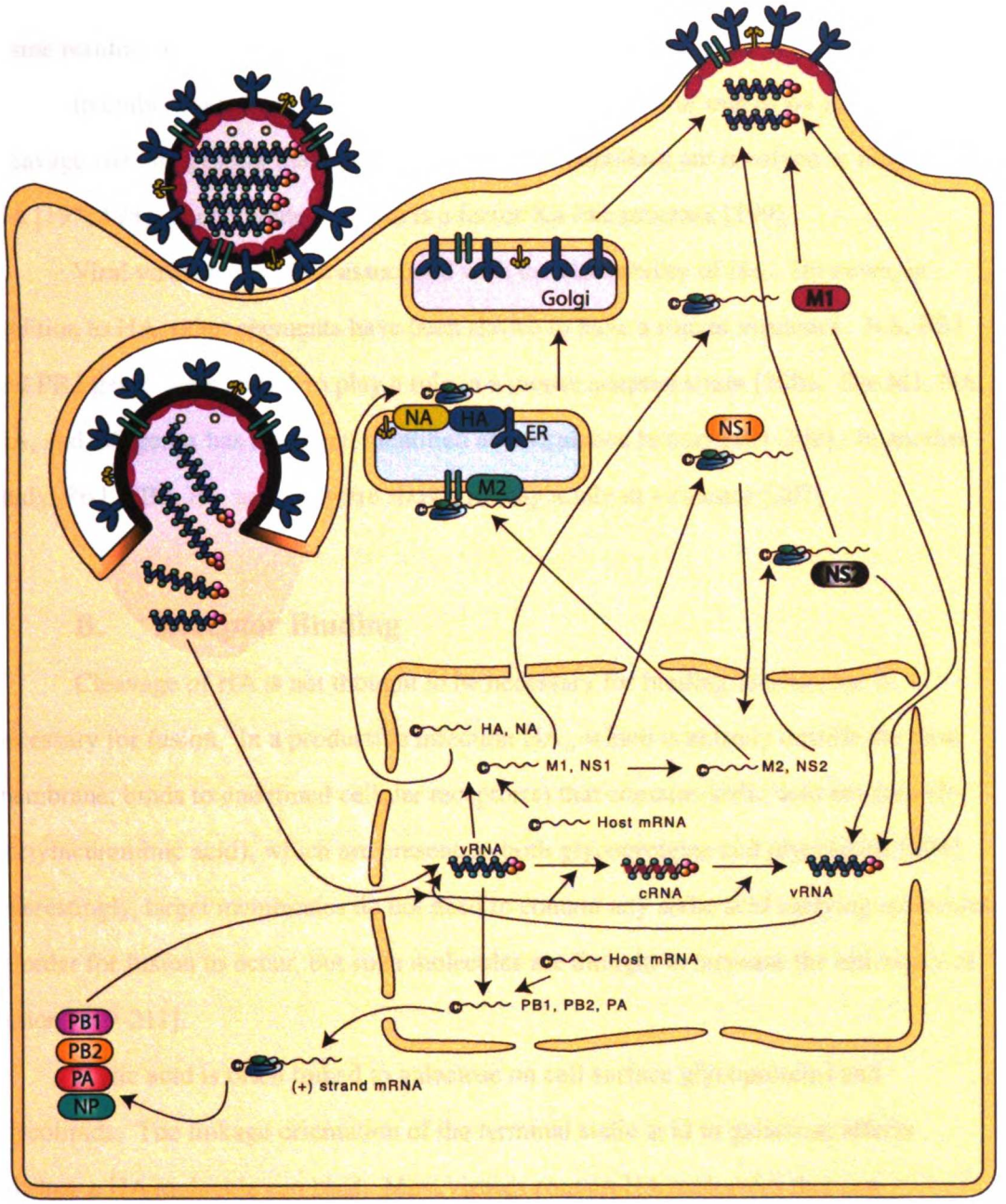

**Intro. Fig.1 Influenza Virus Schematic**

Schematic representation of an influenza A virion.

cell.

When processing of HA occurs prior to budding, it generally occurs in the trans-Golgi network, before HA is transported to the plasma membrane for virus assembly. The sequence of the cleavage site dictates the proteases that can activate HA. HAs that contain the sequence R-X-K/R-R, such as H5 and H7, are cleaved intracellularly by proteases such as furin, plasmin, or PC5/PC6, which are proteases resident to the trans-Golgi network and endosome [55, 56, 186, 187]. Work in avian influenza supports the theory that the cleavage site dictates the virulence of the virus [186, 188]. In general, strains containing furin recognition motifs are more virulent than those with a single arginine residue, as long as the cleavage site is not obstructed by oligosaccharides [189, 190]. Each of the three human subtypes H1, H2, H3 contains a single arginine residue in the connecting peptide and is not cleaved by furin in the trans-Golgi, nor are they cleaved when grown in tissue culture cells, unless exogenous trypsin is added. However, these viruses have been shown to undergo effective cleavage by a trypsin-like protease in CACO-2 cells, which are intestinal epithelial cells [191]. The addition of trypsin allows for plaque formation in otherwise non-permissive host cell monolayers [192, 193]. It is thought that most human strains of influenza possess HAs that are activated by proteases in the extracellular environment. Most proteases that activate HA are found only in the lungs, tracheobronchial tree, and throat, which restricts infection to the respiratory pathway. Some scientists believe that the most virulent strains of influenza have HA molecules that can be activated by proteases such as plasmin found throughout the body, which can result in a systemic infection [185]. The highly pathogenic 1918 strain does not appear to have been activated by plasminogen.

An exception to the rule that human strains cannot replicate without the aid of an extracellular protease is the strain A/WSN/33. WSN/33 is the only human virus (H1N1) that can replicate in a variety of cultured cells without adding trypsin [194]. Genetic studies indicate that WSN NA is essential for WSN HA cleavage [195]. WSN NA binds




**Intro. Fig.2 Viral Lifecycle**

Lifecycle of the influenza A virus.

to and sequesters the serum protease precursor plasminogen through a unique C-terminal lysine residue, absent in other N1 subtype NA molecules [153, 196].

In embryonated eggs, HA cleavage results in the loss of one to six residues at the cleavage site, suggesting that a protease and an exopeptidase are involved in activation of HA [197, 198]. A candidate protease is a factor Xa-like protease [199].

Viral virulence is often associated with the cleavability of HA. However, in addition to HA, other segments have been shown to have a role in virulence. NA, PB1 and PB2 genes were shown to play a role in a mouse adapted strain [200]. The M1, NA, HA, and NS genes has also been identified as a virulence factors [201-206]. In another study, PB1, PB2, PA, and NP were shown to play a role in virulence [207].

## B. Receptor Binding

Cleavage of HA is not thought to be necessary for binding to cells, but is necessary for fusion. In a productive infection $HA_1$, which is entirely outside the viral membrane, binds to undefined cellular receptor(s) that contains sialic acid residues (*N*-acetylneuraminic acid), which are present on both glycoproteins and glycolipids [208]. Interestingly, target membranes do not need to contain any sialic acid carrying molecules in order for fusion to occur, but such molecules are thought to increase the efficiency of fusion [209-211].

Sialic acid is often linked to galactose on cell surface glycoproteins and glycolipids. The linkage orientation of the terminal sialic acid to galactose affects whether a HA molecule can bind. Most viruses contain HA molecules that can only bind to the linkage orientation that is found most predominantly in their native host. The human trachea has predominantly *N*-acetylneuraminic acid-α2,6-galactose (NeuAcα2,6Gal), with lesser amounts of *N*-glycolylneuraminic acid-α2,6-galactose (NeuGc-α2,6Gal) [212], and very sparse amounts of α2,3 linkage [213]. In contrast, duck intestine has predominantly α2,3 linkage, with more NeuAcα2,3Gal than NeuGcα-

2,3Gal. Similarly, horse trachea has predominantly α2,3 linkage, but in the reverse abundance as that seen in bird intestines. This may explain why horses are susceptible to direct transmission of avian viruses, although this has been reported only once, A/Equine/ Jilin/1/89, H3N8. Interestingly, the swine trachea contains NeuAc and NeuGc in both SAα-2,3Gal and SAα-2,6Gal linkages [104]. These linkages make pigs susceptible to both avian and human viruses [60], making them a mixing pot for influenza. A pig that is simultaneously infected with both a human and an avian virus may generate a reassortant (chimeric) virus that has both human and avian segments [104]. Such chimeric viruses are more likely to cause a pandemic than a virus that changes slowly through genetic drift, since humans generally have relatively little pre-existing immunity to them.

Cross species infection can occur when mutations arise in the binding pocket of HA that change the linkage specificity. The heart of the HA receptor-binding site has been identified as Tyr-98, Trp-153, His-183, Glu-190, Leu-194, and Gln/Leu-226, and these residues are essentially invariant features of type A viruses [212]. The specificity of the HA binding pocket is largely dependent on HA residue 226, which in human H3 viruses is a leucine and in avian and equine H3 viruses is a glutamine [214]. In addition to the specificity of the binding pocket, the adjacent residues also help determine host range.

The attachment and entry of influenza has been thought to be dependent on a viral receptor that consists of a surface carbohydrate sialic acid, which can be present as glycoprotein or glycolipid. However, Lec1 cells cannot be infected by influenza. Lec1 is a mutant CHO cell line that is deficient in terminal N-linked glycosylation, due to a mutation in the N-acetylglucosaminyltransferase 1 (GnT1) gene. Expressing wild-type GnT1 in this cell line restores susceptibility to infection. In Lec1 cells, the viral particles bind to the cells, but are not endocytosed. These data suggest that influenza virus entry is dependent on host cell N-linked glycoprotein [215], and that sialic acid may act as an

attachment factor, but is not sufficient as a influenza virus receptor *in vivo*.

## C. Viral Entry

Upon binding to the cell surface, the virus is endocytosed. Endocytosis is not thought to be a result of signaling through the viral-bound receptor(s), but due to normal constitutive cycling of the plasma membrane [216, 217]. The half time of bound viruses on the cell surface is 10-15 minutes [218, 219]. Influenza can enter the cell through numerous pathways which all lead to efficient entry. Initially it was discovered that influenza can enter via clathrin coated vesicles. It was later discovered that such clathrin-coated pits were formed *de novo* upon virus binding, and such formation occurred faster than elsewhere on the cell surface [220]. However, it has been shown that the virus can also enter through clathrin- and caveolin-independent endocytic pathways [220, 221, Lakadamyali, 2003 #1663, 222]. Viral entry has been shown to be dependant on dynamin [223].

Virus entry has been examined using real-time fluorescence microscopy, and entry has been shown to occur in a three-stage active transport process [222]. First, the endocytosed virus containing vesicle undergoes actin-dependent movement in the cell periphery, followed by a rapid, dynein-directed translocation to the perinuclear region, and finally an intermittent movement involving both plus- and minus-end-directed microtubule-based motilities in the perinuclear region [222]. Interestingly, it was shown that the majority of viruses experienced their initial acidification, changing from an early to a late endosome in the perinuclear region immediately following the dynein-directed rapid translocation step [222]. Viral fusion occurs in the late endosome, prior to fusion with the lysosome [224].

The ubiquitin-vacuolar protein sorting system of the endocytic pathway has been shown to be selectively required during entry of influenza into host cells [225]. Treatment of host cells with proteosome inhibitors MG132 and lactacystin directly

affects the early stages of influenza replication, but do not affect the budding process. In contrast, Semliki Forest virus and vesicular stomatitis virus, which are also pH-dependent viruses, are not affected by MG132, and as such, the authors conclude that influenza communicates with a specific cellular machinery for intracellular sorting during the initial phase of virus infection.

The mechanism by which influenza enters cells via a pathway that is independent of clathrin and caveolin mediated endocytosis is still under investigation. The clathrin-mediated endocytosis pathway can be disrupted by chlorpromazine treatment or potassium depletion, whereas the caveolae pathway can be disrupted by nystatin, methyl-beta-cyclodextrin, or genistein treatment, as well as by transfection with dominant-negative caveolin-1. In a study that combined inhibitory methods to block both clathrin-mediated endocytosis and uptake by caveolae, it was demonstrated that influenza virus may infect cells by an additional non-clathrin-dependent, non-caveolae-dependent endocytic pathway [226-228].

## D. Viral Fusion

The low pH nature of the late endosome activates the M2 ion channel. Once the channel has been activated, protons flow into the core of the virus and disrupt the association of M1 to the internal vRNP complexes [229-233]. This dissociation is important since M1 bound to the vRNP prevents their nuclear localization.

In addition to disruption the core of the virus, the low pH nature of the late endosome allows for HA to undergo an irreversible conformational change. During this change, the top domains of the $HA_1$ chains dissociate from each other, exposing hidden hydrophobic moieties of the N-terminus of $HA_2$ [234]. These hydrophobic moieties are the fusion peptides that are essentially inserted or injected into the lipid bilayer of the late endosome. This injection process brings the two membranes closer together. The penetration of influenza is dependent on the transition to a low pH that occurs in the

late endosome. The conformational change in HA occurs between pH 5.1 and pH 5.8, which is reached at approximately 20-35 minutes after viral entry [235]. HA is the only protein component required for fusion activity [236]. If HA has not been proteolytically processed, $HA_0$ will undergo a similar conformation change under low pH conditions, but a fusion peptide will not be exposed [237].

Pretreatment of virus with mature HA in low pH media causes premature exposure of the fusion peptide, which enables the virus to fuse with the plasma membrane, but does not cause infection [221, 238]. The failure to infect is thought to be due to the inability of the incoming nucleocapsids to penetrate the underlying membrane cytoskeleton. These data suggest that endocytosis is a requirement for infection.

Inhibitors of vacuolar-proton ATPases, but not of plasma-proton ATPases, have been shown to selectively prevent the acidification of the endosomes, which stops the exposure of the fusion peptide and ultimately the entry of the virus into the cytoplasm [239]. In addition, acidotropic weak bases (ammonium chloride, chloroquine, amantadine, and methylamine) and carboxylic ionophores (monesin and nigericin) raise the pH of the endosome and block viral uncoating, but this effect is not specific to influenza infection and will block most viruses entering through to endocytic pathway [219, 240]. Weak bases and carboxylic ionophores do not affect binding or endocytosis, nor do they affect the fusion reaction directly [217].

The insertion of the fusion peptide into the lipid bilayer of the late endosome allows for close apposition of the two membranes, which lowers the hydration force between the two polar surfaces. The proximity, reduced repulsion forces, and connectivity provided by the inserted fusion peptides allows for local lipid reconfiguration to occur and fusion of the membranes [241]. The lipid compositions of the HA-containing membrane and the target membrane are not critical to fusion [209, 242, 243]. HA can be reconstituted into artificial membranes without loss of activity [244, 245]. The fusion rate for influenza with the endosomal membrane has been

determined to be 0.02 s(-1) at 37 °C and 0.0035 s(-1) at 20 °C [246].

Once fusion has occurred, the components of the viral core are released into the cytoplasm. Upon release into the cytosol, the vRNPs can be transported into the nucleus, since they are free of M1 that initially facilitated the export of the vRNPs out of the nucleus in the prior infected cell. Nuclear transport of the vRNPs is facilitated by NLSs on each of the proteins constituting the vRNP [247]. Microinjection and immuno-electron microscopy experiments indicate that the vRNP enters the nuclear pore intact.

## E. Determinants of Host Cell Range

HA is thought to be the major determinant in host cell range for its role in binding and fusion as mentioned earlier. The complete molecular mechanism that allows HA subtypes 1-15 to replicate in the avian pool, but only HA subtypes 1-3 to replicate well in the human pool, is unknown. Similarly, the swine pool is restricted to HA subtypes 1 & 3 and the equine pool is restricted to HA subtypes 3 & 7. In addition to HA other viral genes play a role in determining host cell range. In fact, host range discrimination is thought to be a polygenic trait. Data generated prior the development of a complete reverse genetics system suggested that each of the eight segments may play a role in host cell determination [248].

The reverse genetics system will allow more extensive study on the segments that play the most significant role in host cell determination. The system enables the creation of reassortant viruses that carry one 'exogenous' segment. These recombinants can be tested to determine if they are competent to replicate in different hosts. The ability of the recombinant to replicate is dependent on successful interactions with the other viral proteins and the host proteins.

Aside from HA, NP has been shown to be a determinant in host cell range. Reassortants of fowl plague virus that have a human NP gene do not replicate in chicken cells, but replicate effectively in MDCK cells [1, 249]. The phosphorylation pattern of

NP may affect the ability to this protein to facilitate replication in different hosts [250]. NA has also been implicated in playing a role in host-range discrimination. Changes in NA can sometimes alter the virulence of a virus [251] and the ability of a virus to plaque in tissue culture [195]. Generally, human and swine viruses are N1 and N2 restricted, whereas equine viruses are N7 and N8 restricted. The features of NA responsible for this restriction are unknown.

For a review of host range and interspecies transmission see the review by Baigent and McCauley [60].

## F. Determinants of Influenza Evolution

Influenza is an RNA virus with a very high mutation rate that requires us to develop vaccines yearly to target specific strains that are circulating in the environment. Each of these strains is subject to different levels of selective pressure and mutate at different rates. The dominant antigen involved in immunity is the HA antigen. Phylogenetic analysis of the strains of influenza that have circulated since 1977 has provided insight into influenza evolution [252]. The evolutionary dynamics of the HA protein revealed that the subtypes act differently. H3 subtypes essentially undergo serial replacement since roughly 2+ strains co-circulate, and the average survival time of side lineages is only 1.6 years [253]. H1 side lineages are also short lived, but the H1 trunk has bifurcated allowing for co-circulation of several antigenically distinct lineages. Type B viruses diverged earlier and have more extant lineages. Sequence analysis suggests the fixation rates for H3 is 0.0037, H1 is 0.0018, and B is 0.0013. This equates to one nucleotide substitution per 270/555/769 nucleotides per year for H3, H1, and the HA of type B viruses, respectively. Immunological pressure differences may contribute to the higher rate of mutation of H3 over H1 and type B HA. Many of the mutations occur in just 18 of the 300 codons.

Despite influenza's high mutation rate, the overall diversity of extant strains

is surprisingly limited. In fact, less that 1% of all influenza strains make up 90% of all disease incidence [252]. The diversity of influenza is influenced by the immune system of its host. Since antibody recognition of the HA protein decreases with genetic divergence, the more two strains are different, the less cross-protection antibodies will provide. If cross-immunity were the only form of interaction between strains, the expected result would be an exponential growth in diversity, which is not observed. This paradox has been explained with a mathematically model, that allows for the re-creation of the phylogenetic tree of HA diversity that has occurred over the past three decades. The model argues that, in order to have such limited diversity in HA, there must, in addition to long-lived strain specific immunity, be short-lived nonspecific immunity that plays an essential role in limiting diversity. In other words, infection from a H3 strain will protect against infection from other strains for six months, regardless of antigenic diversity.

There is scientific evidence for short-lived nonspecific (heterosubtypic) immunity [254-260]. Such a nonspecific immunity limits infection and promotes the dominance of a few strains. The predominant strains generally only survive for 2-3 years [252], whereas non-predominant strains (approximately 95% of all influenza strains) survive for less than a year.

For influenza, competitive exclusion among subtypes is more common than coexistence between subtypes. The pandemics of 1957 and 1968 were due to the introduction of a new subtype into the population, and this subtype became competitively dominant and excluded its predecessor. However, coexistence is possible in situations where subtype introduction does not cause a pandemic. This occurred in 1977, when H1 was re-introduced and began to circulate with H3. The re-introduction of H1 did not cause a pandemic, since the older population retained immunity against this subtype,

which had circulated prior to 1957.

## Chapter 24. Nuclear Import and Export of vRNPs

Nuclear import of vRNPs has been examined using sensitive fluorescence microscopy to monitor, in real-time, the localization of microinjected vRNPs. vRNPs were found to interact with the nuclear pore complex and to have dissociation rate constants spanning two orders of magnitude. From these observations, it is thought that vRNPs are transported to the nuclear envelope by diffusion [261]. Additional experiments that included M1 displayed a reduced rate of diffusion, suggesting that M1 inhibits the vRNPs from entering the nucleus.

Nuclear export of the vRNPs is thought to be mediated by the action of the M1 and NS2 proteins. For additional information see the sections describing the matrix and nonstructural proteins.

## Chapter 25. Budding

Influenza buds from the apical surface of polarized epithelial cells and all three envelope proteins, HA, NA, and M2 are targeted independently to the apical surface. Accordingly, envelope glycoproteins are believed to be a major determinant in selecting the viral budding site since the majority of the viral glycoproteins are directed to the budding site independent of other viral components. To determine if the most abundant glycoprotein, HA, is responsible for defining the site of budding, HA was re-directing to the basolateral surface; yet >99% of the budding continued to occur from the apical surface, suggesting that HA alone does not determine the site of budding [262]. The other major surface glycoprotein, NA, has conserved N-terminal cytoplasmic tails among

type A viruses and is considered a candidate for nucleating the budding site. Further work is needed to determine whether the cytoplasmic tails of the envelope proteins play a role in determining the site of budding.

Influenza has been shown to bud from ordered, raft-like plasma membrane domains while VSV buds from domains without lipid rafts [263]. Sequences within the HA transmembrane domain are important for raft association [264]. By electrospray ionization mass spectroscopy (ESI-MS), the influenza envelope was found to be enriched in glycosphingolipids more so than VSV [265]. Budding generally occurs in a area that contains little to no cellular membrane protein. Examination of particles has found very few cellular contaminants, but some particle preparations have been shown to contain actin and actin-binding proteins of the ezrin-radixin-moesin family, which supports to the requirement of the actin network for budding [266, 267]. In addition, DMSO treatment, which reduces membrane viscosity, decreases budding and suggests the physical state of the membrane is important in the budding process.

Budding has also been shown to be dependent on ATP [268]. Infected MDCK cells treated with metabolic inhibitors and non-hydrolyzable ATP analogues have demonstrated that both ATP binding and hydrolysis are required for viral budding [268]. Inhibitors of ion channels and protein ubiquitinylation did not affect viral budding, suggesting that these processes are not required for budding. In addition, budding has been shown to be stimulated by signaling through G proteins and protein kinases [269].

The influenza matrix protein has been determined to be a major driving force in virus budding [270]. These data were generated by examining VLP formation in COS-1 cells that had been infected with vTF7-3, which provides the T7 polymerase, and later transfected with plasmids that drive the expression of flu proteins under the control of the T7 promoter. M1 alone was found to be sufficient for VLP formation, in the context of a vaccinia infection. This observation suggests that M1 has all the necessary qualities for self-assembly and budding, and that NP, M2, HA, NEP, PB2, PB1, PA, and viral RNA

are not required. Of note, VLPs are simply structures that resemble viruses via EM and do not necessarily possess all the component of a virus. Additionally, if HA is included to generate VLPs, NA is also required to mediate the VLP release from the surface of the cell.

Additional work has confirmed that a recombinant baculovirus that drives the expression of M1 is sufficient for VLP formation in Sf9 cells, and that a quadruple baculovirus recombinant that expresses NA, HA, M1, and M2 will generate VLPs that closely resemble wild-type influenza by electron microscopy [271].

During the later stages of infection, NP and the polymerase have been shown to localize to the apical, but not the lateral or basolateral membranes, even in cell types where HA was found on all external membranes [272]. NP localized specifically to the apical surface even when expressed alone, indicating intrinsic targeting, and detergent-resistant floatation experiments suggest that NP may localize to lipid raft microdomains. Such localization may help determine the polarity of influenza virus budding. Other viral proteins were examined and NEP was found to be distributed throughout the cytosol and M1 did not localize solely to the apical plasma membrane in all cell types, suggesting that these proteins play a less pivotal role in determining the budding sites.

# Chapter 26. Neuraminidase

Once influenza infects its target cell, it replicates inside the cell and eventually progeny virions bud out of the cell. The time between infection and maximal budding varies among strains and host cell types, but generally maximal budding occurs between 12-24 hours, and earliest budding may begin as soon as 9 hours after infection. There is a natural propensity of the progeny virions to stick to or re-bind the producer cell. To prevent this interaction, the virus possesses a second major glycoprotein that carries out the function of severing the tie with the parent cell. The protein that is thought to

be responsible for this function is neuraminidase (NA). NA is a type II transmembrane glycoprotein. It forms a homotetramer that looks like a mushroom spike by electron microscopy. NA is less abundant than HA by about one homotetramer to five homotrimers. The neuraminidase (also called sialidase or acylneuraminyl hydrolase) activity resides in the head domain of NA and catalyzes cleavage of α-ketosidic linkage between a terminal sialic acid residue and an adjacent D-galactose or D-galactosamine [273]. The current suggested model is that NA removes sialic acid residues from HA, NA, and the cell surface, enabling the virus to bud free of the infected cell. NA may also permit transport of the virus through the mucin layer of the lung to reach infectable epithelial cells. How neuraminidase distinguishes between the host cell from which it has just budded and a target cell is unclear.

Early work on neuraminidase demonstrated that viruses lacking sialidase activity can undergo multiple cycles of replication if sialidase activity is provided exogenously [274]. Interestingly, such neuraminidase deficient viruses can adapt to grow in the absence of exogenous sialidase [275]. These viruses do not grow as efficiently as wild-type viruses, but they will grow in cell culture, eggs, and mice. This finding suggests that neuraminidase activity is not absolutely required in the influenza A virus life cycle, but it does appear to be necessary for efficient virus replication. These NA-deficient viruses were found to adapt to the absence of sialidase activity by changes in the HA receptor-binding pocket that reduces the virus's affinity for cellular receptors [275].

In tissue culture, early work has isolated temperature sensitive mutants in the NA segment [276]. Later work has utilized bacterial neuraminidase (CPNA) and zanamivir to study the role of HA in replication. The ability of NA to facilitate replication has been linked to the glycosylation state of hemagglutinin and stalk length of neuraminidase [277]. The study found that glycosylation at residue 158 on HA makes a virus less dependent on NA for release from its receptor. Generally, NAs with longer stalks are more efficient than those with shorter stalks. For optimal growth, a balance must be

struck between the glycosylation status of HA and the stalk length, since too much neuraminidase activity is disadvantageous.

The role of NA in viral morphogenesis and budding was examined by mutating the highly conserved transmembrane domain and cytoplasmic tail[278]. Mutations toward the ectodomain-proximal sequence of TMD progressively caused reduction in NA enzyme activity, affected lipid raft association, and attenuated virus growth. These viruses remained aggregated and bound to the infected cell surface, unless provided exogenous sialidase. Mutations to the extreme N terminus of the CT as well as chimeric NA containing the TMD of the transferrin receptor caused reduced viral growth and elongated particles, supporting the role of NA in morphology.

In type B viruses, the NA segment encodes both NA and NB, which have overlapping reading frames. NB is a integral membrane protein that has been speculated to have ion channel activity [279]. This theory is appealing since NB is a type III integral membrane protein, similar to M2. Electro-physiologic recordings from bacterially expressed NB suggest ion channel activity, however amantadine does not effectively block the channel [280].

## A. HA and NA Compatibility

A virulent virus must have compatible HA and NA molecules, since the release of a virus from an infected cell is dependent on the ability of NA to the sialic acid residue to which HA in bound. Since human virus HAs have less affinity for their respective receptors than avian virus HAs [281], it is likely that human virus NAs have reduced specific activity in comparison to avian virus NAs, in order to maintain an optimal balance between glycoproteins. The specific activity of NA can be influenced by mutations in the active site [163] as well as by the length of the NA stalk, which holds the active site above the viral envelope [282]. A short-stalked NA is inefficient in disaggregating progeny virus because the active site cannot access its substrate efficiently.

Most NAs can cleave sialic acid in both α-2,3 and α-2,6 linkages, but often at different efficiencies. For example, during the evolution of N2 avian influenza viruses in man, the N2 has increased its activity on α-2,6 linkages, while still maintaining the ability to cleave α-2,3 linkages [283]. Overall, the mechanism of substrate specificity is not well understood [60].

# Chapter 27. Packaging the Genome

Inside the virion, each segment is thought be associated with at least one of each of the three polymerases subunits and nucleoprotein. Nucleoprotein wraps the genome, but does not coat the RNA, leaving it sensitive to chemical and enzymatic digestion. The RNA, polymerase, and NP structure is referred to as the ribonucleoprotein complex (RNP). RNP complexes from partially disrupted virions have been visualized with electron microscopy and have been shown to often exhibit a loop on one end and a periodicity of alternating major and minor grooves [284, 285]. Others have described the RNP in virion to be a continuous strand of 6 nM in diameter, arranged in the form of a double coil or helix [286]. Such visual data suggest the vRNP structure is formed by the RNA strand folding back on itself to form a twin-stranded helix, adding credibility to the notion that the promoter is formed by the joining of the two ends of the genome segments. These twin-stranded helices have been observed to interact with one another in an ordered fashion, and may represent ordered complexes of the eight separate RNPs inside the virion [284-287].

It is generally accepted that the segments are present in roughly equimolar concentrations within the virion. However, some studies have suggested that vRNAs 4-8 are present in equimolar ratios in virions, whereas segments 1-3 are underrepresented, possibly due to the presence of defective genomes [288]. The exact number of segments that can be packaged by influenza virions is still unknown, although at least 9 can be

definitively packaged [289]. If virions incorporate 10-12 segments [289, 290], by chance alone, 2.8-9.3% of the particles would contain a full complement of segments, and hence be infectious. This mathematical prediction is supported by biological data that suggests only 5-10% of particles are infectious [4, 291]. The ratio of infectious to noninfectious particles was determined by mixing a known concentration of microbeads with an influenza preparation and counting both the number of particles and beads by electron microscopy. Once the particle concentration had been determined, a plaque assay was used to determine the number of particles that are capable of forming plaques within the preparation.

Packaging studies have been traditionally performed by mutating the putative packaging signal and looking for the absence of the genome in budded virions. However, studying packaging in influenza has been complicated by the construction of the packaging signal, which is believed to overlap with the promoter for transcription and replication. Since the same region is involved in three aspects of the viral lifecycle, mutating the region invariably affects packaging, transcription, and replication, making any observed phenotypes difficult to interpret.

Not only must the virus incorporate a full complement of segments, but it must also distinguish between packaging cRNA and vRNA. The polymerase can differentiate between the vRNA and cRNA, and much of this is attributed to the finding that PB1 has distinct regions that recognize the vRNA and cRNA templates [292, 293]. In addition, the polymerase recognizes an adenosine bulge at A10 in the 5' end of the vRNA, which is not present in the 5' cRNA and thought to be a sense specific packaging signal [294]. By mutagenesis, this bulge structure can be transferred to the 5' cRNA UTR, which results in the exclusive packaging of cRNA over vRNA. If the bulge is removed, both vRNA and cRNA are packaged indiscriminately at 1:1 ratios, but at a lower level. Only the species of RNA that is packaged makes it out of the nucleus, suggesting that nuclear export is a barrier for RNA species lacking the correct packaging signal. The uridine stretch in the 5'

vRNA UTR that is responsible for the addition of the poly (A) tail has been shown not to be essential for packaging. These data support a theory that the "sense" of the packaged RNA is determined by the sequence and structure in the conserved portion of the UTR. It also lends some support to the random packaging model, since this packaging signal resides in the common section of the UTR, although these data do not exclude the possibility that regions outside the UTR contribute to specific packaging of the segments.

Initially, the packaging signal of the segments was thought to occur in the terminal untranslated regions (UTR) of each segment, since the polymerase has been shown to bind to the ends of the vRNPs [295] and foreign genes containing influenza UTRs are be packaged and expressed by the viral polymerase [296]. The UTRs of each segment are composed of a conserved and non-conserved part. The conserved part is at the extreme 5' and 3' ends of the genome. For WSN/33, the extreme ends include the thirteen nucleotides at the 5' end and twelve nucleotides at the 3' end of the genome. These two ends are partially complementary and are nearly identical for each of the eight segments. Interior to these extreme ends are the non-conserved portions of the UTRs. The non-conserved portions are different for each segment, yet are conserved across subtypes of influenza.

Due to the nature of the UTRs, which contain both a "common" and "unique" region, two models have been proposed to explain the mechanism by which influenza packages its genome. The two models are aptly called the "random" and "specific" models for packaging the genome. The random packaging model predicts that influenza does not differentiate among the segments, and acquires a full complement of vRNAs by chance. In contrast to the random packaging model, the specific packaging model states that each segment is selectively incorporated into the virion.

In addition to the ratio of infectious to noninfectious particles that supports the random packaging model, Bancroft and Parslow recently published data supporting the random model for packaging. In their work, they constructed artificial virus-like

reporters in which the UTRs of NS, NA, and NP were maintained, but the coding regions replaced with those for GFP, YFP, and RFP. In a reverse genetics system to reconstitute virus-like particles, these reporters were included under conditions that promote competition. These recombinant particles were then used to infect MDCK cells that were analyzed for color by FACS. The generated data suggests that influenza packages segments with different UTRs in nonspecific manner [297]. Because the native influenza coding sequence was replaced by that of GFP, YFP, or RFP, no information was gathered on whether or not the coding region participates in packaging.

However, the specific model is also supported by data suggesting the concentration of various vRNAs may differ within an infected cell, yet vRNAs are found in equimolar concentrations in the particles budding from these cells [288]. Most of the data that support the specific model for packaging come from work on defective interfering RNAs, which are internally truncated influenza genes. Data from several reports suggest that defective interfering vRNAs specifically compete for packaging with their normal counterparts, but not with other vRNAs [298-302]. In addition, other experiments have shown the exclusion of certain defective RNA segments from packaging, which further supports the selective packaging theory [300].

Recent data has provided more support for selective incorporation of vRNAs into virions [303]. Using the 17 plasmid reverse genetics system, neuraminidase deletion constructs were created that contained either a FLAG or GFP tag. The deletion constructs were found to be propagated as long as bacterial sialidase was provided exogenously [274, 275, 304, 305]. The work with these deletion constructs resulted in several conclusions. First, virions are more efficiently made in the presence of all eight segments, and although infectious virions can be made with 6 segments (-NA, -HA), the titer is reduced and only one round of infection can occur. Second, using constructs containing a mutated start codon, protein expression was found to not be necessary for packaging of a truncated gene. And third, a neuraminidase deficient virus will selectively

package a truncated neuraminidase gene from which no neuraminidase protein is expressed, and the minimum sequence required for selective packaging includes part of the coding region. The packaging signal of the NA segment was determined to reside predominantly in the 3' vRNA UTR plus the first 21 nucleotides of the coding region, although the coding region at the 5' end was found to slightly enhance the packaging efficiency. The constructs in which the entire coding region was omitted, as done by Bancroft and Parslow [297], were packaged at <2% of the packaging efficiency observed when the coding region was included. These data suggest the UTR may contain a nonspecific packaging signal in the promoter and a specific packaging signal in the coding region. Subsequent packaging studies on the vRNA HA segment has demonstrated that 9 nucleotides at the 3' end of the coding region and 80 nucleotides at the 5' end are sufficient for efficient packaging and maintenance [306]. In this study vesicular stomatitis virus glycoprotein VSVG and green fluorescent protein GFP replaced the internal coding region of HA and NA respectively and these two foreign genes were stably maintained in virions.

The role of the non-conserved UTRs in replication and packaging is still unclear. In a report that introduced mutations into the non-conserved noncoding region of the neuraminidase segment found that such segments exhibited diminished levels of replication, yet these segments were packaged equally well [307]. These data suggest that the non-conserved coding region plays a role in replication but not in packaging. Other data suggests that this region may play a role regulating the translation of the viral transcripts, which will be discussed later.

At the budding site, HA and NA may play a role in packaging the genome, since their cytoplasmic tails have been shown to be required for normal packaging [308]. Influenza A virus preparations lacking the cytoplasmic tails of HA and NA have reduced vRNA to protein content and contain an increase in cellular RNA contaminants. HAt-/ NAt- virion populations contain a broader range of packaged RNA segments than wild-

type virus.

Recent advances in studying influenza through a plasmid based viral reconstitution system, has allowed for vRNA production in a manner that is independent of the viral polymerase. This system will improve the progress made in this field of influenza biology to bring it up to par with other viruses which have had their packaging signals elucidated, including: coronavirus [309], sindbis virus [310], retrovirus [311, 312], hepatitis B virus [313].

## Chapter 28. Influenza Associated Protein Kinase Activity

Purified influenza has protein kinase activity, which has been attributed to a 47 kD polypeptide that radiolabels with ATP [314]. The polypeptide phosphorylates a synthetic peptide that is a substrate for casein kinase II. Antiserum directed against casein kinase II reveals a positive signal in immunoblots of purified virus, suggesting that host cell casein kinase II associates with purified virus.

## Chapter 29. Defective Interfering Genomes

Upon high multiplicities of infection, the influenza polymerase will create defective interfering (DI) genomes, also called subgenomic RNAs (sgRNA). The DI genomes have internal deletions in the coding region of the genes, which makes the gene product non-functional. However, these genomes can compete with full-length genomes for transcription and replication and also for packaging. Particles that incorporate DI genomes are often non-infectious; but in a process called multiplicity reactivation, different DI particles can infect one cell and complement each other to re-create an

infectious virion.

DI genomes are thought to be created when the polymerase jumps at transitions between adjacent regions that are brought together by the vRNP tertiary structure. Generally, when the polymerase jumps, it is thought to stay on the same strand of genomic RNA, yet a chimeric DI has been found in which 30 nucleotides from segment 1 were found in a DI for segment 3 [315].

Examination of DI segments has found that most are approximately 400 nucleotides long, containing roughly 200 nucleotides from each end. Overall, the genomes of the polymerase gene are found more often to be truncated in DI particles than the other segments. Reports differ in whether the PB2 segment [316] or the PB1 segment [317] is most frequently truncated. Interestingly, DI genomes do not appear to be equal in their ability to be passaged. Although DI genomes for each segment can be created, when DI particles are propagated in mice, only the polymerases and matrix DI RNA are maintained [318].

A study on the initial central deletion in DI genomes has shown that they occur at specific regions in the 3' vRNA end, but in a nonbiased manner at the 5' vRNA end. Portions of both the 3' and 5' vRNA are required for a DI to be passaged. The minimum amount of 3' vRNA genome has not been carefully examined, but a study did examine the minimum amount of 5' sequence required for passage of the DI genomes [319]. The study found that 150 nucleotides of the 5' portion of vRNA of PB2 was sufficient to allow stable passage through ten rounds of infection using a helper virus, but most isolated DI genomes contained at least 178 nucleotides of the 5' end of the vRNA. If the 5' end was shortened to 80 nucleotides, the segment was lost during passaging, suggesting more 5'

vRNA sequence is necessary for stable propagation.

# Chapter 30. Influenza Morphology

Influenza viruses can vary in size and shape, from long filamentous particles to spherical particles [320, 321]. Spheres have a mean diameter of 100-150 nM in length, and filamentous particles can be from 500 nM to 15 $\mu$M in length and often visualized by immuno-fluorescence and light microscopy. Ultrahigh-resolution scanning EM of influenza infected MDCK cells have shown filamentous and bacillary-like particles budding out, which sometimes appear as a twisted rope-like structure [321]. The filamentous particles have been shown to sediment faster than spheres through sucrose gradients. Filamentous particles have also been reported to exhibit a higher specific infectivity and higher RNA content than spherical virions [322].

Most laboratory strains grown in eggs or tissue culture, including WSN, produce spherical particles [320], with the exception of A/Udorn/72, which produces a large number of filamentous particles [320]. Early egg passages of primary human or animal isolates exhibit a greater degree of heterogeneity and pleomorphism and are predominantly filamentous. The molecular mechanisms responsible for filamentous particle formation and the role of such particles in viral transmission and pathogenicity are still not well defined.

Viral morphology has been shown to be largely dependent on host cell type [320]. Non-polarized cells produce almost exclusively spherical virions that assemble at lipid rafts in a process that is independent of microfilaments. In contrast, filamentous virions are only produced from polarized cells, and their production is dependent on an intact actin microfilament network. Cytochalasin D treatment of MDCK cells disrupts the actin microfilament network and results in a 15-fold reduction in the number of filamentous particles released, but interestingly does not change the titer in tissue culture

[323, 324]. Similarly, disrupting the cortical actin by jasplakinolide causes HA, NP, and M1 to redistribute around beta-actin clusters to form a novel annular membrane structure [325]. Analysis of these structures has led to the hypothesis that the actin cytoskeleton is required to maintain the correct organization of lipid rafts for incorporation into budding viral filaments.

In contrast, nocodazole treatment of MDCK cells, which disrupts the microtubule network, was found not to alter the ratio of filamentous to spherical particles. It has been reported that nocodazole treatment re-directs a fraction of the influenza viral proteins from the apical to the basolateral plasma membrane domains in polarized epithelial cells [326].

Early work claimed that the genes that specify spherical morphology segregate independently of HA and NA [327, 328]. The cytoplasmic tails of HA and NA are highly conserved in sequence for all virus subtypes and it is believed that assembly depends on interactions of these domains with cytoplasmic viral components. Virion particles lacking HA and NA cytoplasmic tails have a ten fold reduction in formation, yet have a fairly normal protein composition, and are greatly elongated and irregular in shape [329]. HAt-/NAt- virion populations contain a broader range of numbers of RNA segments packaged than wild-type virus. Sucrose gradient centrifugation confirms the presence of a subpopulation of virions with pronounced deformation in virion morphology and reduced infectivity. Upon expression of wild-type HA and NA, with infection of HAt-/NAt- virions, both morphology and proper genome packaging is rescued [308].

Viral morphology has been shown to be largely dependent on the amino acid sequence of the matrix proteins M1 and M2. Reverse genetics has demonstrated that switching the amino acid at position 41 from A to V removes the filamentous phenotype of A/Victoria/3/75. In addition, three mutations can be made in the amino and carboxyl regions of M1 to convert a virus containing the WSN M gene to a filamentous phenotype [330]. The A/Udorn strain is normally filamentous, but antibodies against M2 protein

inhibit this viruses from producing filamentous particles in infected cells. Similarly, a single amino acid substitutions in the cytoplasmic domain of M2 will allow Udorn strains to remain filamentous in the presence of the M2 antibodies. In addition, Udorn strains have been isolated that contain a single amino acid substitution in M1 that confer spherical morphology. Also, high growth viruses were found to have a stronger M1-RNP interaction than low growth viruses, and were also found to be predominantly spherical in nature, while low-growth strains more commonly were of filamentous nature. These data indicate that the binding property of M1 to RNP may affect viral growth speed and morphology [320, 331].

Analysis of the genetic content of reassortants between parent viruses differing in their ability to form filaments suggests that primarily HA, M, and NP genes are involved in the control of the filament forming ability. A difference in the UV-inactivation kinetics between filaments and spherical virions suggests that the infectious filamentous forms are probably represented by multigenomic particles or partial heterozygotes [322].

# Chapter 31. Matrix

The matrix protein (M) is a type specific antigen of influenza viruses. The matrix gene encodes two proteins, M1 and M2. M1 is the most abundant protein of the virus and is thought underlie the lipid bilayer and provides rigidity to the membrane [332]. M2 is a Type III transmembrane protein that forms a tetramer that has ion channel activity. The channel allows ions to enter the core of the virus to facilitate uncoating, once the virus has been endocytosed. The M2 protein is named according to its type: M2, BM2, and CM2 for influenza A, B, and C, respectively. Interestingly, each of these gene products is produced by a slightly different mechanism. The M2 protein is produced by alternative splicing of the matrix transcripts, so that the N-terminal 14 residues of M1 are common with M2, then M2 proceeds in a +1 reading frame. The BM2 protein is translated

from a tandem bicistronic mRNA in an open reading frame that is +2 nucleotides with respect to the matrix (M1) protein. And the matrix transcript of type C viruses can be translated into an internal membrane protein, p42 protein. p42 can be N-glycosylated, in which case it is designated p44 and it is capable of forming disulfided linked dimers and tetramers. p42 can be processed by a signal peptidase to generate M1 (p31) and the CM2 protein, which is the C-terminal 115 amino acids of the ORF. Alternatively, the C matrix gene can be transcribed and spliced to encode the matrix protein. In addition, the type A matrix gene contains an open reading frame that potentially encodes a nine amino acid peptide that would be identical to the C-terminus of the M1 protein, but to date, no transcripts or gene products have been identified in virus infected cells.

## A. M1 Protein

The M1 protein is a major structural protein of the virus and may play a pivotal role in viral assembly. The M1 protein associates with the lipid bilayer and also interacts with M2 [333], NS2 [334], and the vRNPs [335]. To date, there is little data to support the interaction of the cytoplasmic tails of HA and NA with M1, but without such interaction, it is thought that it would be difficult to have efficient viral assembly. The expression of M1 with the aid of a recombinant vaccinia virus has shown that M1 alone is sufficient for VLP formation [270].

Aside from M1's apparent role in the assembly of the virus, M1 also regulates transport of vRNPs into and out of the nucleus. During acidification of the virus in the endosome, M1 dissociates from the vRNP to permit localization of the vRNP into the nucleus [336].

M1 is translated late in infection and much of the new protein intrinsically associates with the plasma membrane [232]. A fraction of M1 migrates into the nucleus via its NLS [337] and is found in association with vRNPs [336, 338, 339]. The migration of M1 toward the plasma membrane or toward the nucleus may be

determined by the phosphorylation state of the protein, since a variant of M1 that gets hyperphosphorylated is retained in the nucleus [340]. While in the nucleus it is thought to inhibit transcription, which has been shown *in vitro* [341, 342].

Aside from M1 role in inhibiting transcription, there is conflicting data on whether M1 facilitates the export of vRNPs out of the nucleus. One study has shown that M1 does not affect nuclear export of vRNPs[343]. However, other data suggests that M1 does facilitate the export of vRNPs out of the nucleus [336]. Additional work has shown that M1 participates in the regulation of bi-directional nuclear transport of the vRNPs in Chinese hamster ovary cells [344]. For this action, M1 is thought to interact with the RNA [341, 345]. Nuclear export is then mediated by M1 interaction with the NS2 protein, also called the nuclear export protein (NEP) [346, 347]. M1 contains a zinc-binding motif, but the x-ray crystallographic structure indicates this sequence does not form a functional zinc finger [348].

The N-terminal 164 residues of M1 are involved in three functions: membrane binding, self-polymerization [349], and nuclear export of RNPs. This region also contains the NLS motif, which is positively charged. The charge of the motif has been implicated in being important for each of the three functions of the N-terminus. A mutant M1 protein was created that lacked the positively charged NLS and was functionally defective. X-ray crystallography indicated the structure was not significantly different from WT, suggesting that the charge of this region is important for its function [350].

The RNA-binding domains of M1 map to the following two independent regions: a zinc finger motif at residues 148 to 162 and a series of basic amino acids (RKLKR) at positions 101 to 105. A mutation of the zinc finger motif has created temperature-sensitive phenotype and greatly reduced the ratio of M1 to NP in viral particles, and a weaker binding of M1 to vRNPs [351].

Initial temperature sensitive M1 mutants were used to study vRNP export [337, 340]. Most of these mutants appeared to be restricted to the nucleus. These studies

suggested that M1 facilitates vRNP export from the nucleus by allowing their release from a nuclear retention mechanism, which contradicts other studies that have claimed that M1 and NS2 bind to the vRNP to mediate nuclear export.

## B. M2 Protein

The M2 protein has two functions, to modulate the pH of the Golgi and to initiate viral membrane dissociation during entry. These two functions have been found to be separately genetically encoded [352]. It has been suggested that there must be functional complementarity between the activation characteristics of the M2 channel and the pH of membrane fusion by hemagglutinin during virus entry.

The M2 protein is a disulfide-linked non-glycosylated homotetramer that forms a channel that has ion activity [353, 354], which is highly selective for H+ ions [355, 356]. The M2 cytoplasmic domain is post-translationally phosphorylated on a serine residue [357] and palmitoylated on a cysteine residue [358, 359], but neither modification is necessary for ion channel activity [357, 360, 361]. The ion channel activity is regulated by changes in pH and is active in both the endosome and the trans-Golgi network [229-231, 355, 362, 363], where it may also modulate the pH [354]. M2 may maintain a neutral pH in the trans-Golgi network, which is thought to be important to prevent an early conformational change in HA that would prematurely expose the fusion peptide. The M2 ion channel is the target of amantadine (1-aminoadamantane hydrochloride) and has been used as prophylaxis and treatment of type A virus infections [364]. For type A viruses, amantadine binds to the transmembrane domain and prevents acidification of the core of the virus, which prevents M1 from dissociating from the vRNPs [336]. As a result, amantadine blocks viral entry between penetration and uncoating [365, 366]. Mutants resistant to amantadine contain amino acid changes in the transmembrane domain [367].

Similar to M2, the BM2 protein is a Type III integral membrane protein since

it contains a single hydrophobic domain and lacks a cleavable signal sequence [368, 369]. BM2 is most likely a tetramer and shares structural and sequence similarity to M2 and has been shown to possess ion activity. The BM2 protein exhibits a N (out) C (in) orientation in the membrane. The open reading frame of BM2 has been knocked out using reverse genetics and these viruses are not viable, unless BM2 is provided in *trans*, suggesting that the BM2 protein is required for replication [370].

Interestingly, type B viruses also possess the NB protein that has ion channel activity. Whether the roles of NB and BM2 are redundant or distinctly separate has not been determined.

The matrix protein of influenza C virus that is encoded by segment 6, CM2, has been shown to possess voltage-activated ion channel activity in *Xenopus laevis* oocytes, which could not be blocked by amantadine hydrochloride. The channel was found to be permeable to Cl (-) ions and the amplitude of the inward current could be decreased by an anion channel blocker [371, 372].

When M1 is expressed alone in COS-1 cells in the context of a T7 recombinant vaccinia virus, M1 has been found to not only bud from cell surface, but also form internal long electron-dense filamentous structures that are observed in both the nucleus and cytoplasm [270]. In addition, when HA was co-expressed with M1, the formation of the filamentous structures decreased and more M1 was found associated with membranes. This suggests that M1 and HA interact to promote viral assembly at the plasma membrane, which has been supported by other groups [373]. Association of influenza glycoproteins may start in the secretory pathway, as HA and NA are transported to the cell surface via the Golgi network.

# Chapter 32. Nonstructural Proteins

The nonstructural segment (NS) is processed into two different gene products,

NS1 and a splice variant NS2 [374, 375]. Briefly, NS1 is a multifunctional protein involved in inhibiting the anti-viral response and regulating host and viral protein synthesis, and NS2 is involved in vRNP export. In type A viruses, both products share the same nine N-terminal amino acids, before NS2 reaches its intron and begins translation in a +1 reading frame [376].

## A. NS1 Protein

The NS1 protein is thought to be absent in virions, despite its high level of expression in infected cells [377-379]. NS1 has been shown to possess a two part NLS [380] and a nuclear export signal (NES) [381]. In transfected cells the NES is inhibited by adjacent sequence and NS1 localizes to the nucleus. However, in virally infected cells, another viral protein is likely responsible for unmasking the NES of some of the NS1 proteins, causing them to localize to the cytoplasm.

NS1 possesses a unique-RNA-binding domain that has been examined by X-ray crystallography [382]. NS1A binds nonspecifically to double-stranded RNA, and this activity has been localized to the N-terminal 73 amino acids [383]. Interestingly, the same region of the protein is also thought to be involved in dimerizing the protein. The NS1 protein binds as a dimer in the absence of an RNA target and also in the presence of a specific RNA target [384, 385].

By NMR and X-ray crystallography , this domain is a symmetric homodimer that forms a six-helix chain fold. This structure is unique and differs from most dsRNA-binding domains. Dimeric NS1A binds to dsRNA duplexes with a 1:1 stoichiometry and a dissociation constant of approximately 1 μM. NS1A recognizes canonical A-form dsRNA, but does not bind to dsDNA or dsRNA-DNA hybrids.

In macrophages, influenza with a NS1 mutant that encodes the first 125 a.a. and lacks the C-terminal domain, induces significantly higher amounts of IFN beta, IL-6, TNF-alpha and CCL3 (MIP-1 alpha) when compared with the wild-type strain

[386]. Additionally, this mutant could inhibit IL-1 beta and IL-18 released from infected macrophages as efficiently as wild-type virus. Other NS1 mutants that lack or possess non-functional RNA-binding and dimerization domains induced 10-50 times more IL-1 beta and five times more IL-18 than wild-type virus or virus with an NS1 that lacks the C-terminal domain. These mutants induced apoptosis, which may be dependent on the enhanced activity of caspase-1. These data suggest that the N-terminal domain of NS1 may control caspase-1 activation and the initiation of apoptosis, as well as repress the maturation of pro-IL-1 beta and pro-IL-18.

NS1-deletion mutants are only capable of growing in cells that have defective IFN pathways. Interestingly, some cancers have defects in the IFN pathway, and reduced STAT1 expression may be a marker for these cell types. Initial work has shown that a NS1-deletion mutant virus lyses IFN-resistant tumor cells [387]. Further work in animal models is needed to determine whether modified influenza viruses could be used as tumor therapy. Additional studies in interferon (IFN)-deficient Vero cells have shown that an intact N-terminal RNA binding domain for NS1 was essential for viral growth [388]. It is possible to insert 275 amino acids into the NS1 reading frame to create a virus that expresses GFP and is capable of stable replication in Vero cells and PKR knockout mice.

Viruses with C-terminal deletions of the NS1 protein were temperature sensitive and showed small plaque size at the permissive temperature[389]. Analysis suggests that the NS1 protein plays a role in the control of transcription versus replication and also the expression of late genes.

i. Inhibition of Pre-mRNA Processing

One of the primary roles of the NS1 protein is that of down regulating host protein expression. NS1 achieves down regulation of cellular protein expression by inhibiting cellular pre-mRNA splicing, 3' end cleavage and polyadenylation, and mRNA nuclear

export [390].

NS1 inhibits pre-mRNA splicing by binding to the stem-bulge region in U6 small nuclear RNA (snRNA) [391]. This binding prevents the U6-U2 and U6-U4 snRNA interactions, which are required for splicing [391-393]. The NS1 protein may also inhibit splicing by binding to NS1-BP, which is a putative spliceosome factor [394]. Unspliced pre-mRNAs are retained in the nucleus possibly by interacting with the spliceosome itself; and this causes a down-regulation in cellular expression.

Interestingly, while influenza inhibits cellular splicing, it permits the cellular machinery to splice some of the NS and matrix transcripts. The majority of the NS transcripts are unspliced NS1 mRNA and only 5-10% are spliced NS2 transcripts. The low level of NS2 transcripts is the result of the action of the NS1 protein on the splicing machinery. NS1 was shown to be solely responsible, since inactivating the NS1 gene product, but not the NS2 gene product, results in the effective splicing of NS2 gene [395]. The exact mechanism by which some splicing of influenza genes occurs is not yet completely understood, but the splicing is thought to be controlled by cis-acting sequences in NS1 [396] and influenza may also control the access to the splice sites [375]. It has been reported that expression of the NS1 protein inhibits the splicing of matrix mRNA, resulting in a higher M1/M2 ratio [393]. However, others have reported that expression of NS1 protein does not affect the ratio of M1/M2 in Vero cells [397].

NS1 inhibits cellular pre-mRNA 3' end cleavage and polyadenylation by binding to two cellular factors involved in the process. Normally, cellular mRNA is processed in the following manner. The pre-mRNA contains a poly (A) addition site that is upstream of the site at which cleavage and polyadenylation occurs. This site is bound by the four-subunit cleavage and polyadenylation specificity factor (Cpsf). Downstream of the cleavage site is a U/GU-rich sequence that is bound by another protein called the cleavage stimulatory protein (Cstf). These two proteins recruit and stabilize cleavage factors I and II (CfI & CfII) to the cleavage site. Then poly (A) polymerase (Pap) binds

to the complex and cleavage occurs, followed by release of CfI & CfII, Cstf, and the downstream RNA cleavage product. The upstream pre-mRNA is still bound by Cpsf and Pap, which slowly adds 10-15 adenosine residues at the new 3'-OH terminus. Once enough adenosine residues has been added, the poly (A)-binding protein II (Pab II) binds to the short poly (A) sequence and acts as a cofactor for Pap to catalyzes the rapid and processive addition of adenosine residues until the chain is about 200 residues in length.

NS1 interrupts the cleavage and polyadenylation process at four different steps. First, NS1 binds to a 30 kD subunit of Cpsf [398] and prevents its binding to RNA substrates, which inhibits 3' end cleavage. Despite the binding of NS1 to the Cpsf, some 3' cleavage of cellular pre-mRNA occurs in virus infected cells, followed by the Pap-catalyzed addition of short poly (A) tails. The second stage at which NS1 interferes with mRNA processing is to inhibit the addition of a longer poly (A) tail. NS1 also binds to the PabII and prevents it from acting as cofactor for Pap [399]. The third stage at which NS1 interferes with mRNA processing is that PabII has been shown to shuttle between the nucleus and cytoplasm and is thought to play a role in nuclear export of cellular mRNAs. However, the binding of NS1 to PabII prevents it from shuttling out of the nucleus, providing yet another block in the processing and export of cellular mRNAs. Lastly, NS1 was found to bind poly (A) tails and inhibits the export of all cellular mRNAs tested [400]. The amino terminus of NS1 has been shown to bind to poly (A)-binding protein 1 (PABP1) [401]. It is unclear how NS1 selectively binds to cellular mRNA poly (A) tails to prevent their export without also preventing the export of influenza mRNAs, which also have poly (A) tails. Potentially, NS1 discriminates between cellular and viral mRNA poly (A) tails through the mechanism by which the tail is created. The viral mRNA poly (A) tails are produced by stuttering of the influenza polymerase, not by the host cell machinery.

It is important to note that the effect of NS1 on the export of cellular mRNA may also affect some of the viral transcripts. Surprisingly, NS1 was found to inhibit the

export of both NS1 and NS2 mRNA [402]. The retention signal was found to reside in sequences common to both transcripts and may be the poly (A) tail itself. How the NS transcripts make it to the cytoplasm for translation is not well understood. As expected, NS1 does not inhibit the nucleocytoplasmic export of PB1, NP, HA, M1, and M2 mRNAs. Further work is needed to elucidate the exact mechanism governing the retention or export of specific viral transcripts.

Due to NS1 inhibition of cellular pre-mRNA processing, the nucleus accumulates cellular pre-mRNAs that contain approximately 12-nucleotide-long poly (A) tails. These pre-mRNAs provide an abundance of cellular caps that are used by the influenza polymerase to transcribe its own genes.

ii. Effect on Translation

Not only does NS1 prevent the maturation of cellular transcripts, but NS1 also enhances translation of viral transcripts. NS1 expression has been shown to enhance the translation of NP and M1 protein from 5 to 100 fold, and this enhancement was specific to viral genes [403]. The enhancement is thought to be due an increase in the translation initiation rate, since the sizes of NP-specific polysomes were greater when NS1 and NP was co-transfected into cells, rather than NP alone. In another study, NS1 was shown to stimulate the synthesis of M1 protein [404]. Site-directed mutagenesis showed that the sequence GGUAGAUA upstream of the initiation codon on the matrix gene was required for stimulation. The NS1 protein is thought to enhance viral translation by binding eIF 4GI, which is part of the cap-binding portion of the ribosome. This binding recruits the 5' UTR of viral mRNA to the ribosome to allow preferential translation of virus messages [405]. This information supports the theory that the non-conserved non-translated region of the UTR modulates viral gene expression. However, the viral 5' UTR is not absolutely necessary for translation in the cytosol [406]. Another study has shown that NS1 protein expression results in enhancement of reporter gene activity from transfected plasmids [397], which appears to be mediated at the level of translation. This study also showed

that efficient viral protein synthesis in COS-7 cells required the NS1 protein.

Influenza takes over host protein expression to such an extent that at eight hours post-infection essentially only viral mRNAs are translated. It is important to note, that despite the fact that the NS1 protein prevents host cellular expression at several different steps, NS1-deficient viruses, in which the C-terminal effector domain has been deleted, continue to shut off host protein synthesis [407]. Similarly, another study examined del-NS1 viruses in Vero cells that lack IFN-α/β genes and found no major difference in host cell protein synthesis shutoff or in viral protein expression [397]. These data suggest the virus has a redundant mechanism to shut off host translation or that the N-terminus of NS1 is sufficient for shut off. The lack of host protein synthesis in NS1 minus infected cells could be due to specific degradation of host transcripts or preferential translation. There are some data to support the theory that cellular mRNAs are specifically degraded. In both wild-type and NS1-defective virus infected cells, mRNA transcripts are degraded starting at 6 hours post-infection, and actinomycin-D treatment suggests that the degradation is virus dependent [407]. This finding is supported by other studies that have observed that cellular mRNAs present in the cytoplasm are degraded during a viral infection [408, 409]. The destabilization of cellular mRNA may be due to the inhibition of their translation or the virus may selectively degrade cellular mRNA in a NS1-independent manner. The other possibility to explain the drop in host protein production is that viral mRNAs may be preferentially translated over cellular mRNA. There is some evidence to suggest that the translation of cellular mRNAs is inhibited at both the initiation and elongation steps [410]. The host translation may be inhibited due to alterations in the cell translation apparatus [411]. Such alterations may include the association of NS1 with the human homologue of staufen protein [412, 413] and the association of NS1 with eIF-4GI, which is a component of eIF-4F [405]. It has been shown that the shutdown of host protein synthesis is not dependent on PKR activity

[407].

iii. Inhibition of the Anti-Viral Response

NS1 is also involved in inhibiting the host anti-viral response [414]. The host antiviral response pathway is amplified by the induction of interferon alpha and beta (IFN-α/β). Expression of IFN-α/β upregulates effectors that help fight the viral infection. The importance of NS1 in the anti-IFN response was confirmed in a study that demonstrated that NS1-null mutant viruses can replicate in cellular systems deficient in the IFN pathway, but not in normal cells [415].

Surprisingly, the NS1 proteins of type A and B viruses do not inhibit the cellular anti-viral response pathway in the same manner. Both are involved in shutting down the IFN response pathway, but type A viruses prevent the initial expression of IFN, whereas type B viruses allow IFN to be expressed, which in turn allows for the induction of many other proteins. The manner in which type A viruses inhibit the host response is thought to be much more efficient since it is easier to prevent the activity of one anti-viral protein than many anti-viral proteins. In type B infections, one of the downstream proteins that are expressed in response to the high level of IFNs is the ubiquitin-like ISG15 protein. In contrast, in type A infections little or no ISG15 protein is produced during infection. The NS1B protein has been shown to block the action of ISG15 by preventing the covalent linkage to its target protein UBE1L [416]. NS1-B protein has been shown to efficiently inhibit virus-dependent activation of the IFN-beta promoter [417]. Surprisingly, NS1-B defective virus was attenuated in tissue culture cells that lack functional INF-alpha/beta genes, suggesting that the NS1-B protein is required for efficient viral replication for reasons unrelated to blocking the IFN response. More work is needed to help understand the manner in which type B viruses can efficiently suppress the host anti-viral response pathway once IFN has been activated.

The NS1 protein of type A viruses is involved in preventing the activation of the interferon response pathway. One of the key players in activating IFN-α/β is a

transcriptional regulator called interferon regulatory factor 3 (IRF3), which is a sentinel of the cell that is involved in activating the stress response-signaling pathway. IRF3 becomes activated by detecting dsRNA and quickly induces the expression of IFN-α/β expression. NS1 has also been shown to block the activation of IRF3, which curtails the expression of IFN-β and helps halt the anti-viral response [418]. Along these same lines, the NS1 protein also inhibits the dsRNA activated Jun N-terminal kinase (JNK) [414]. The amino terminal RNA-binding domain was shown to be necessary to prevent the IFN-β response [419]. JNK phosphorylates and activates AP-1 family transcription factors that cooperate with NF-κB and IRF to trans-activate IFN-β. In addition, the NS1 protein prevents the activation of NF-κB [420].

Microarray technology has been used to profile the changes in the level of 13,000 cellular transcripts due to influenza A virus infection of epithelial cells [421]. This study compared the following strains: A/PR/8/34, A/WSN/33, del-NS virus, and virus containing the sequence of the 1918 NS gene. The generated data confirm that the NS1 protein contributes to viral pathogenesis by disarming the host IFN defense system. Infection with PR/8 resulted in a significant induction of the genes involved in the IFN response, but deletion of NS gene increased the number and magnitude of cellular genes implicated in the IFN response. A virus with a C-terminal NS1 deletion had an intermediate phenotype. Interestingly, when a virus containing the 1918 NS segment was compared to A/WSN/33, the 1918 NS gene-containing virus more effectively blocked the IFN response pathway. These data contrast earlier evidence that the 1918 recombinant virus was attenuated in mice when compared to the isotype control, WSN [148].

Another anti-viral protein that detects a viral infection by monitoring the cell for dsRNA is dsRNA-dependent protein kinase (PKR). PKR is constitutively expressed and also up-regulated by the expression of IFN-α/β. Upon detecting dsRNA, PKR dimerizes and autophosphorylates to become active and subsequently shuts down the cellular machinery to prevent the viral infection from proceeding. PKR shuts down the

host machinery by phosphorylating the alpha subunit of eukaryotic translation initiation factor 2 (elF-2α), which decreases the ability of the ribosome to translate both cellular and viral transcripts. Influenza combats the activity of PKR by hiding its double stranded RNA by having the NS1 protein bind to the promoter, which is the only known region of the genome that is thought to be double stranded. Interestingly, NS1 seems to have a preference for influenza sequence and secondary structure since it binds to the promoter more strongly than to non-specific dsRNA [422]. The A bulge of the promoter may be specifically recognized by NS1. As a consequence of binding to the dsRNA regions of influenza, the NS1 protein blocks the activation of the dsRNA-activated protein kinase (PKR) [423, 424], which allows the host protein synthesis machinery to proceed as if no viral infection were underway. Influenza is also thought to repress PKR activity by recruiting $p58^{IPK}$, which binds and inactivates the kinase [425-427]. Other studies have suggested that NS1 binds PKR directly, but this interaction remains controversial [412, 428]. The effect of NS1 is strong enough so that delta NS1 viruses do not grow in cells unless PKR has been knocked out [429]. In $PKR^{-/-}$ mice, the interferon response pathway is unimpaired and the anti-viral response appears to be normal. The finding that del-NS1 virus can replicate in $PKR^{-/-}$ mice but not in wild-type mice, suggests that the role of PKR in inhibiting viral infection can not be easily substituted by the interferon response pathway. This finding also questions whether the role of NS1 in blocking the processing of pre-mRNAs and their export are essential for viral replication.

Since NS1 has been shown to be an IFN and PKR antagonists, influenza viruses that express altered NS1 proteins have been created for the purpose of vaccines. These live virus are still immunogenic, yet are unable to suppress the IFN response pathway and are cleared by host immunity. These altered live viruses have been used as a vaccine and shown to provide protection from challenge with wild-type viruses in mice [430].

The IFN response pathway is closely linked to apoptosis and influenza has been shown to induce apoptosis. The role NS1 plays in apoptosis and how it is induced is

not fully understood and controversial. NS1 has been shown to be both pro and anti-apoptotic. For a review of influenza and apoptosis, see Lowy *et al.* [431]. One study has suggested that the NS1 protein induces apoptosis when expressed by itself in MDCK and HeLa cells [432]. However, another study found that del-NS1 viruses were more lethal than wild-type viruses in chicken embryos and suggested that NS1 down-regulates apoptosis through an IFN-dependent mechanism [433].

## B. NS2 Protein

The NS2 protein is also called the nuclear export protein (NEP). The nuclear export signal (NES) of NS2 is located near the N-terminus and is highly conserved. For A/WSN/33, the NES has the sequence 12-ILMRMSKMQL-21, and is thought to be required for nuclear export of vRNPs mediated by a cellular export factor CRM1. The hydrophobicity of the NES is important and leptomycin B, which inhibits CRM1, prevents nuclear export. Minor alteration of the NES can cause delayed export and limited viral growth, making the NES an attractive target for the production of live-virus vaccines [434].

### i. Modulator of RNA Synthesis and NS2

In addition to the ability of NS proteins to affect the host anti-viral response, the NS and NP proteins have been considered candidates to affect whether the polymerase terminates RNA synthesis in the form of replication (no poly (A) tail) or transcription. This speculation has turned out to have little basis, since i*n vitro* analysis of the 3' end of plus-strand RNA by RPA showed no enhancement of read-through of the polyadenylation signal when NS 1 and NP were added [435]. As an additional side note, NS1 is a phosphoprotein and is phosphorylated by vRNPs, and NP was shown not to be responsible. The phosphorylation state of NS1 does not affect its ability to nonspecifically bind ssRNA [435], and it is unclear if it affects function.

Lastly, the role of the NS2 protein currently appears to be much simpler than that

of NS1. The NS2 protein was originally thought to be absent from the virion, but later experimentation suggests that 130 to 200 NS2 proteins are present in each virion [334, 346]. Due to its presence in virions, making it a structural protein, and the discovery that NS2 possesses a nuclear export sequence and is involved in the nuclear export of vRNPs, it has been suggested that the protein be renamed nuclear export protein (NEP) [436]. The NS2 protein has been shown to mediate vRNP nuclear export through a NES-independent interaction with hCRM1 [437]. Proteolytic cleavage of NEP defines an N-terminal domain that mediates Ran GTP-dependent binding to CRM-1 and a C-terminal domain that binds to the viral matrix protein, M1 [438]. The trafficking of RNA by influenza in and out of the nucleus has been compared to Thogoto and Borna disease viruses [439]. In addition, mutations in the NS2 protein have been found to lead to the generation of defective interfering particles specific to the PA segment [302], but it is not known how NS2 affects viral replication.

# Chapter 33. Promoter

Originally the promoter was thought to reside solely in the 3' vRNA end [440]. However, many of the studies that led to this conclusion utilized polymerase purified from micrococcal nuclease digested viral cores. These polymerases were later found to still possess parts of the 5' vRNA UTR that had been protected by the polymerase from micrococcal nuclease digestion, which was later shown to be indispensable for full promoter activity [441]. This hidden remnant of the 5' vRNA UTR was found to be sufficient to enable transcription from an exogenously added 3' vRNA template. Ultimately, it appears as though the polymerase can independently bind either arm of the vRNA (and cRNA), but that it binds to the 5' vRNA arm more tightly, and that binding the 5' vRNA arm improves binding of the 3' arm [442]. The current view is that the promoter is made up of the terminal thirteen nucleotides of the 5' vRNA end and the

terminal twelve nucleotides of the 3' vRNA end [440, 443]. The promoter nucleotides have been annotated in the following manner to clarify the location of nucleotides near the ends of the genome. The 3' vRNA strand is numbered 1, 2, 3... extending toward the middle of the gene, whereas the 5' vRNA strand is numbered with prime notation (1', 2', 3'...) extending toward the middle of the gene.

The two ends of the vRNA have partial complementarity and have been shown to be in close proximity by EM studies [444] and also exhibit some base pairing by mutagenesis studies (Intro. Fig. 3). The base pairing of the two ends has been modeled in three different ways: the panhandle model [444-446], the RNA-fork model [447], and the corkscrew model [448, 449]. These models are depicted in Intro. Fig. 4. The major difference between the panhandle model and the more recent RNA-fork and corkscrew models is that the later two predict that the terminal ends of the vRNA are 'melted' by the binding of the polymerase. It has been shown, that in the absence of proteins, the 5' and 3' ends base pair [450, 451]. And it has been suggested that the polymerase initially recognizes and binds to the proposed bulge region of double-stranded vRNA termini, followed by melting of the termini, generating the RNA fork or corkscrew structure in the open complex [450]. The terminal location of the polymerase is not a requirement for its activity, since an internally placed promoter will effectively initiate transcription and replication [452]. The "panhandle" has been shown to be extended by very short complementary sequences unique to each RNA [445, 453-455].

The activity of the polymerase is dependent on nucleotides 9-11 of 3'-vRNA (5'-CUG-3'), which may be the site of polymerase binding to the promoter [440, 456]. Nucleotides 1-4 are thought to be regulatory nucleotides that play a significant but less important role. Minor alterations or deletions in the U-rich spacer between theses two sites are tolerated. However, other studies of the promoter fail to confirm the importance of nucleotides 9-11 [457], and such discrepancies may be due to differences in methods used to prepare the polymerase. Other reports have dissected the cRNA promoter

and found that the first 11 nucleotides of the 3' cRNA end are minimally required for promoter activity and that the promoter does not work well if additional nucleotides are added to the end of the genome [458].

The vRNA and cRNA promoters have slight differences in sequence and structure that are thought to affect many of the functions of the polymerase, including packaging of the genome and the endonuclease activity of the polymerase [459]. The hairpin loop structure in the 3' arm of the influenza A virus vRNA promoter has been shown to be required for endonuclease activity [460]. In addition, sequences in the 5' arm of the vRNA promoter have been shown to be required for endonuclease activity [461].

## A. Promoter Structure

NMR has shown the promoter in the absence of protein to be double-stranded and helical in structure [450, 462]. The type A promoter has natural variation at the 4th position from the 3' vRNA end, which is either a uridine (U4) or cytosine (C4) residue. C4 promoters are invariably found in promoters of each of the three polymerase subunits, while most of the other viral genes carry a U residue [445, 454, 463, 464]. Two exceptions are the neuraminidase and matrix genes, in which both nucleotides have been observed at position 4. This difference in promoter sequence may be important for segment-specific regulation of transcription and replication [288, 465, 466]. In WSN/33, the C4 residue has been shown to down-regulate transcription and to activate genome replication [467]. This down-regulation may enable the virus to utilize the host resources more effectively, since the polymerase acts catalytically and is not needed in large amounts [468].

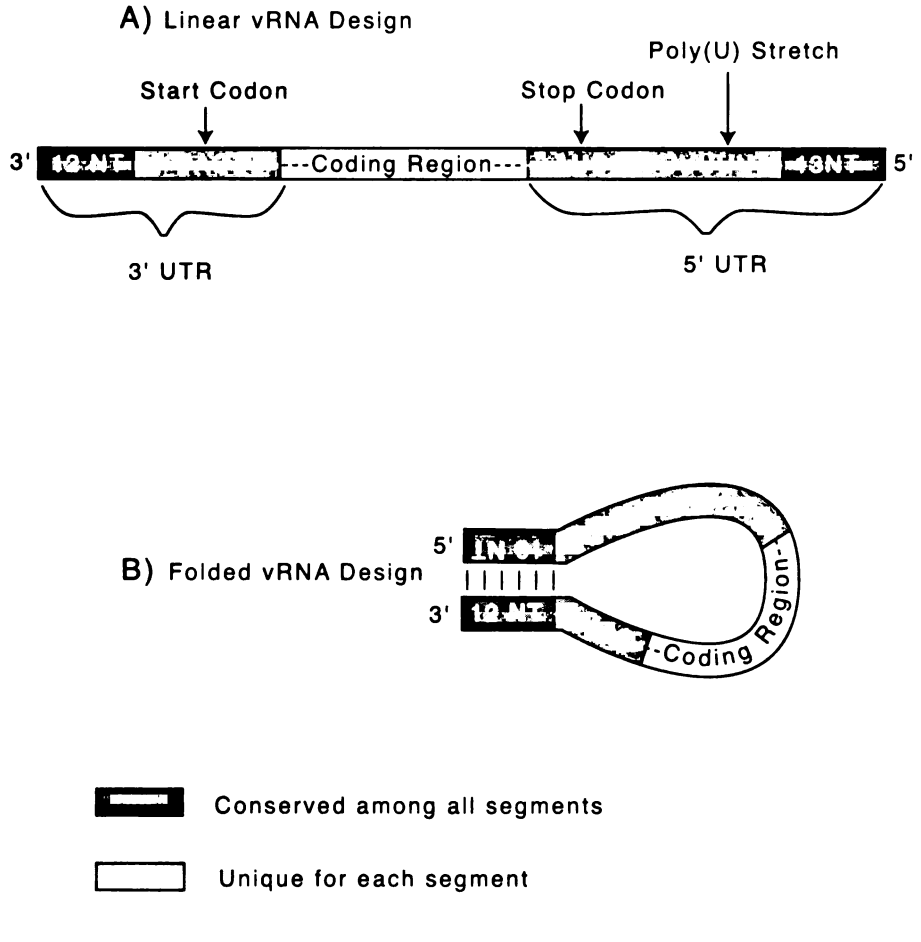
By NMR analysis, both the U4 promoter [462] and the C4 promoter [450] have a similar internal loop structure {(A10'-A11') •U10} that is unique to the vRNA and may be part of the packaging signal. The current polymerase binding model is that the heterotrimeric polymerase (also referred to as 3P) specifically recognizes and binds the

internal loop structure and then distinguishes between the U4 and C4 promoters. The two promoters are structurally different at the terminal stem of the promoter. The U4 promoter has a more stable terminal duplex, whereas the C4 promoter has a unique adenosine bulge (A4') structure and flexible base pairs. The stable duplex structure of the U4 promoter is thought to activate the endonuclease activity of the complex. In contrast, the more flexible terminal stem of the C4 promoter is thought to be in equilibrium between the single- and double-stranded structures, which may be more favorable for replication. In support of this theory, it has been shown that single nucleotide mutation (U4 to C4) of the influenza promoter results in slightly lower endonuclease activity [460].

Interior to the conserved terminal ends is a non-translated region whose sequence is unique to each segment and conserved among different strains. These regions vary in length from 7 to 45 bases and have been shown to modulate promoter activity differently [469]. This region may affect the timing of expression of the viral segments and may also potentially be involved in packaging.

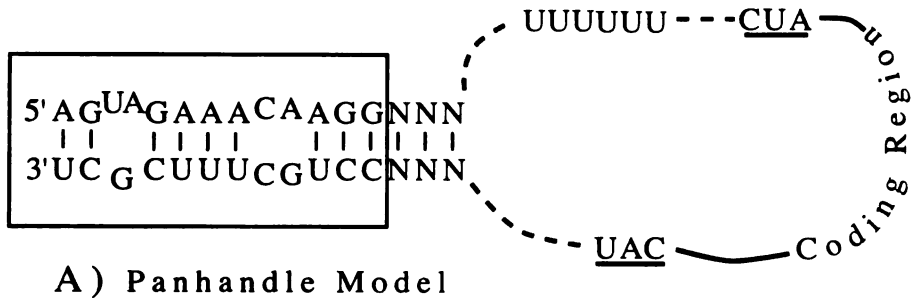
## B. Base Pairing Requirements of the Promoter

The promoter has been modeled as a panhandle, fork, and corkscrew. Many *in vitro* studies have been carried out that look into the basepairing requirements of the promoter. The study that led to the proposed RNA fork model, suggested that a crucial double-stranded region formed between complementary bases at positions 10-12 of the 3' terminus and positions 11'-13' of the 5' terminus, accompanied by the formation of 2 to 3 segment-specific base pairs [447]. Although some sequence restrictions exist, the duplex structure was found to be more important than the specific sequence for initiation of transcription. Positions 1-9 of the 3' terminus and positions 1'-10' of the 5' terminus were

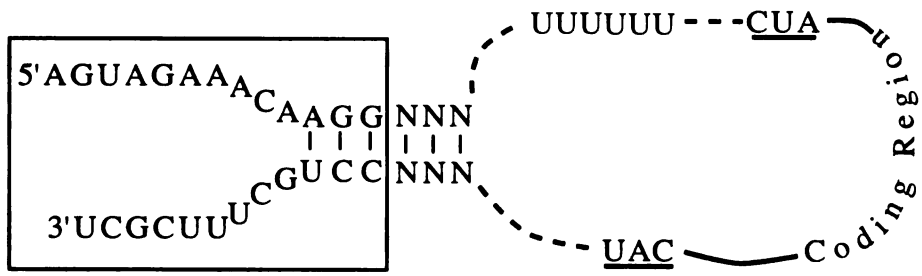



**Intro. Fig.3 Viral Untranslated Region**

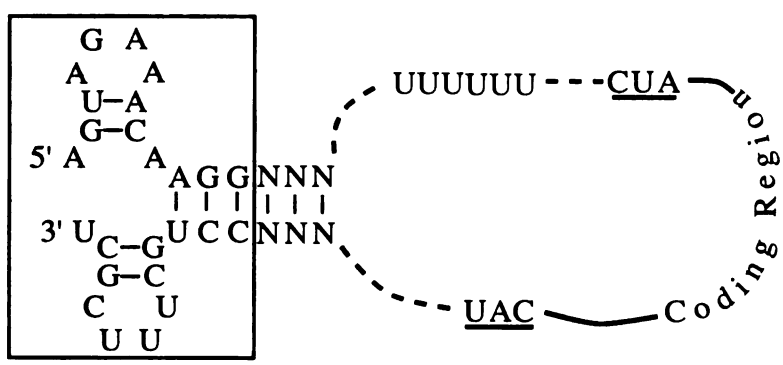
Schematic of vRNA. A) Linear depiction of the conserved and unique portion of the vRNA, and the location of the start and stop codons and poly(U) stretch. B) vRNA folded so the conserved ends of the UTR can interact to form the promoter.




A) Panhandle Model




B) Fork Model




C) Corkscrew Model

**Intro. Fig.4 Models of Promoter**

Models of promoter. The promoter may fold into multiple different conformations. Some of these conformations have been modeled as a panhandle (A), fork (B), or corkscrew (C).

found to be single stranded.

## C. Distinguishing Promoter Structures of Type A and B Genomes

The terminal noncoding regions of influenza A and B viruses have potentially similar panhandle structures and share common sequences. Differences are found in the panhandle duplex and the length of the uridine stretch juxtaposed to the RNA duplex, and such differences are thought to determine specificity between influenza A and B viruses. A transient ribonucleoprotein transfection method for the expression of a type B-NS-CAT reporter was used to elucidate the contributions of type specific signals in promoter recognition and transcription. The base pairing of the duplex was found to be important for both type A and type B polymerases. Type A, but not type B, was found to be sensitive to disruption of the 11-12' and 12-13' base pairs [470]. The non-conserved sequence of type B viruses can support formation of a duplex spanning a portion of the conserved and non-conserved regions of the UTR that is 9 base pairs in length, from 11'-19' of the 5' end and 10-18 of the 3' end. By making small mutations, this stretch of base pairs can be extended and type B polymerases exhibited the highest template activity when base pairing of the duplex is extended to 11 base pairs. In contrast, type A polymerases prefer a shorter 8 base pair duplex. Such modeling places 3 of the 5 uridines of the polyuridine stretch of the 5' vRNA end in the duplex region. In addition to tolerating different lengths of promoter base-pairing, the polymerases can tolerate different lengths of the polyuridine stretch. Type A virus polymerases can tolerate a 7 U stretch, whereas type B virus polymerases can tolerate 6 U stretches but not a 7 U stretch.

Another study found type A polymerase can efficiently replicate type A and C templates whereas type C polymerase can only utilize type C templates. In order for type C polymerases to recognize A-like templates, strong base-pair binding was required

between nucleotides 3' and 8' of the 5' end of the vRNA. This basepairing supports the corkscrew model. In contrast, type A polymerases can tolerate a weaker basepair interaction at these residues. In addition, residue 5 at the 3' vRNA end and residue 6' at the 5' vRNA end were found to contribute to type specificity of the polymerase to a template [471]. These data support the notion that both the nature of the nucleotides and the stability of the secondary structure at the extremities of the viral RNA are important determinants of type-specificity.

### D. Promoter Requirements for Transcription

In addition to examining the polymerase requirement for the ends of the vRNAs, work has been done looking at whether the polymerase can recognize an internal promoter. *In vitro*, the viral polymerase can recognize an RNA template that has five extra nucleotides at its 3' vRNA end and copy it, albeit at one-third the efficiency of wild-type [457, 458]. However, when these reporters were transfected into influenza infected cells, only the template with one additional nucleotide was functional, at 20% of wild-type [458]. RNA templates that contain 30 extra nucleotides are not functional *in vitro* or *in vivo* [472]. In transfected COS-1 cells, the influenza polymerase complex can recognize its promoter when located internally on the RNA template [473], however, the authentic 3' end of the reporter was required for CAT activity from a helper-virus-infected cell.

## Chapter 34. Transcription

The influenza polymerase is an RNA-dependent RNA polymerase made up of three subunits: polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acidic (PA). This polymerase complex is referred to as the 'polymerase' or 3P complex

which is 280 kD in size. The polymerase proteins are thought to be present at 30-60 copies per virion [474, 475]. In the virion, the 3P complex is associated with the terminal ends of the genome and upon infection the vRNP migrates into the nucleus after uncoating. While in the nucleus, the polymerase both transcribes and replicates the genome. Originally, these events were not thought to happen concurrently, as it was thought that the polymerase transcribes the genome prior to replicating it [288, 476-479]. However, recent experimentation suggests that transcription and replication may indeed happen concurrently (See Replication Section) [480]. The segmented nature of the genome suggests that transcription and replication is initiated on each segment independent of the other segments, which has been supported by UV irradiation experiments [288]. The events of transcription have been illustrated in Intro. Fig. 5 & 6.

While in the nucleus the vRNPs are thought to interact with nucleosomes [481]. The vRNP has been shown to bind to histone tails and M1 has been shown to bind to the globular domain of the histone octamer. The biological significance of the flu replication machinery binding to chromatin has not been determined.

The model for transcription has evolved over time. Originally, it was thought that the polymerase without 5' and 3' vRNA ends was transcriptionally inert. Binding to the 5' vRNA end allowed the complex to become activated for binding primer and the 3' vRNA end. A polymerase bound to the 5' vRNA end could bind and cleave a primer, but less efficiently than when the 3' vRNA is provided [482]. It was thought that sequential binding of the vRNA ends was required and the polymerase was unable to bind already base-paired 5' and 3' ends [483]. However, recent data have suggested that recognition of a pre-annealed duplex RNA of 5' and 3' vRNA ends enhances the polymerase's ability to bind cellular mRNA six fold over that observed when the polymerase is provided the 5' vRNA end prior to the 3' vRNA end [484]. In addition, polymerase bound to pre-annealed duplex has enhanced endonuclease activity when compared to polymerase that sequentially binds the promoter. Binding to 5' vRNA alone allows for weak primer

binding, and endonuclease activity is only slightly diminished without the 3' vRNA end. Later addition of the 3' end did not increase primer binding ability. The association and dissociation rates of primer were equal between polymerase bound to both ends and polymerase bound to only the 5' vRNA end.

The current model envisions the polymerase binding to a panhandle-duplex structure; however, since 850-2300 nucleotides separate the vRNA ends, it can be argued that the probability of the ends finding each other and forming a panhandle may be low [485]. Binding to the vRNA ends can occur in a cis fashion (same vRNA template) or a trans fashion (different vRNA templates), however cis binding is thought to be favored. It has been suggested that replication may occur in trans[456, 486], but it is unclear how the complex would coordinate initiation and termination such that uncapped polyadenylated or capped non-polyadenylated transcripts are made.

The activity of the complex also varies with the availability of magnesium. Magnesium was found to be required for primer cleavage, but not for primer binding although it enhances primer binding for both pre-annealed and sequentially provided promoters [484]. In the absence of magnesium the polymerase prefers to bind primers that are of the length normally generated by cleaving the cellular caps, 9-13 nucleotides in length. Cleaved products remain associated to the polymerase in the presence of magnesium, but will become dissociated if magnesium is removed. The polymerase is capable of holding onto the 5' vRNA and cycling through more than one 3' vRNA short end.

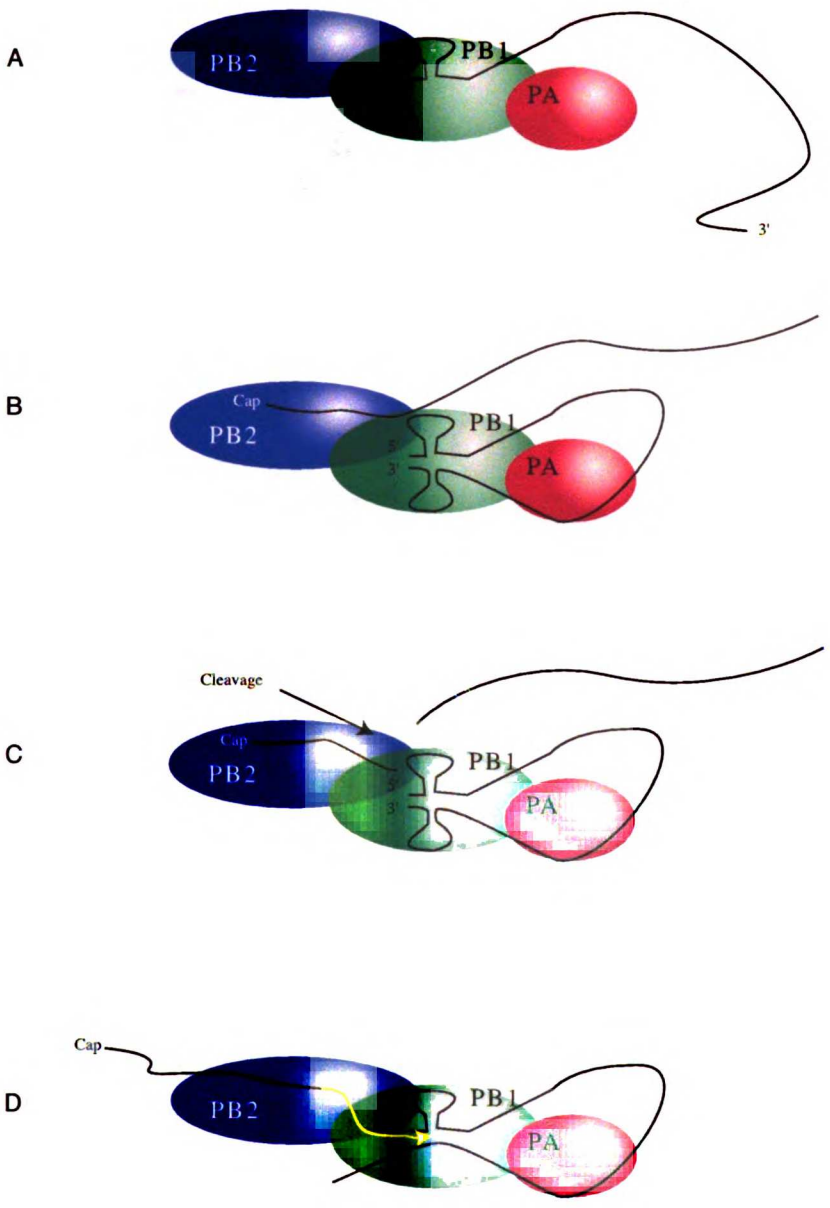
It is thought that the RNA structure of the promoter may differentially induce different activities to allow for coupling of cap binding with polyadenylation and cap-independent RNA synthesis with anti-termination. In order for the polymerase to have full polymerase activity, the promoter must be able to form a corkscrew structure [449, 461, 487].

The current model is that the polymerase must bind to one or both ends of the

influenza genome in order to bind cellular mRNA via the cap structure. The polymerase then cleaves cellular mRNAs downstream of the cap to create cellular primers that are extended by the viral polymerase during transcription of viral genes. The process of stealing cellular caps for viral use is termed 'cap snatching'. This phenomenon was discovered by the observation that α-amanatin treatment, which inhibits the ability of cellular RNA polymerase II to produce capped transcripts, prevents the transcription of the viral genome [488-492]. Work with globin and reovirus mRNAs determined that methylated caps, but not non-methylated caps, served as efficient primers for influenza transcription [493-496].

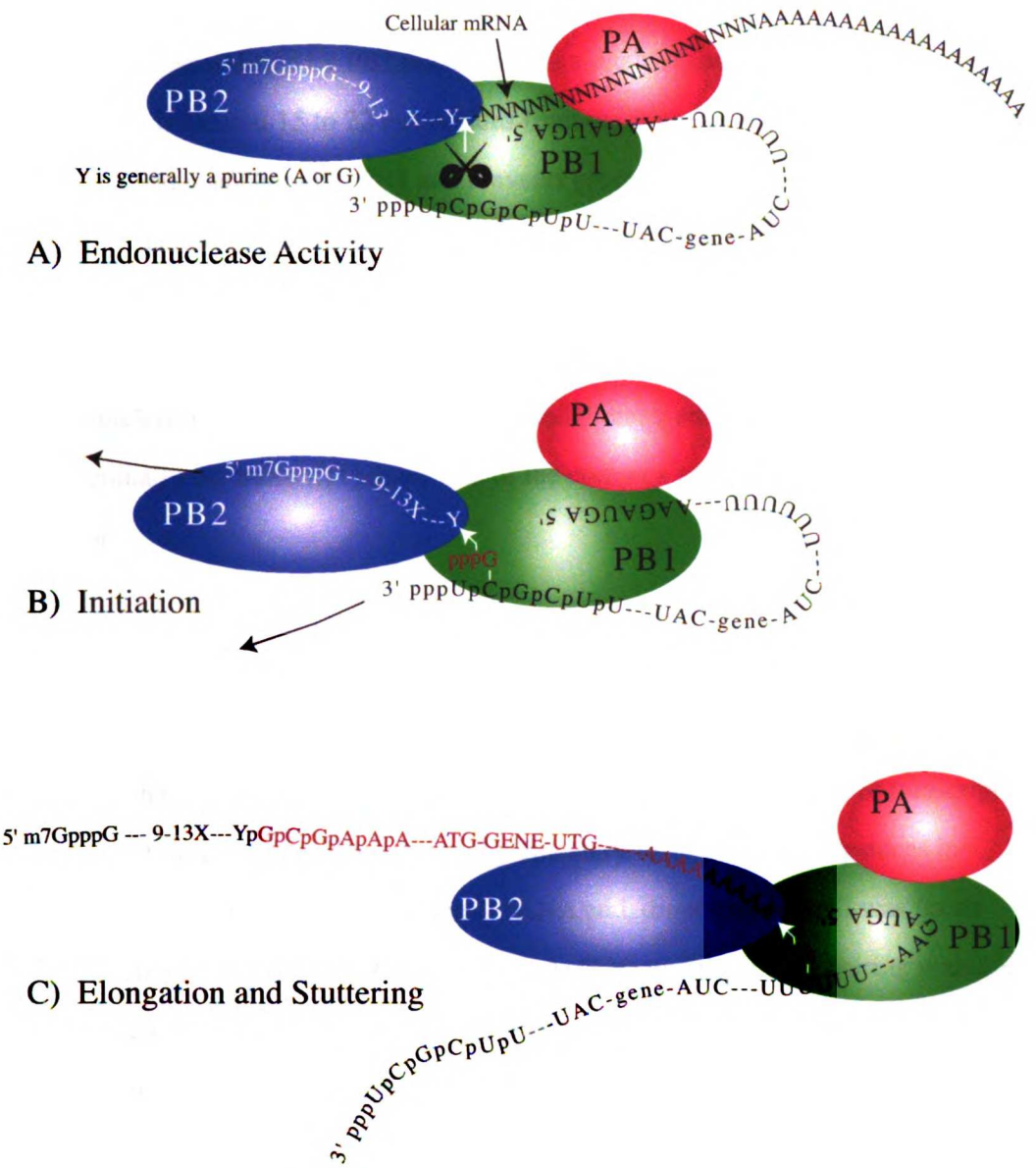
The polymerase binds to the cap via the PB2 subunit. The caps are cleaved 9-13 nucleotides from their 5' ends, preferentially after a purine residue [292, 456, 482, 496]. Initial data suggested that PB2 possesses the endonuclease activity [497, 498], but recent data strongly indicate that PB1 alone has the endonuclease activity [499]. The endonuclease site in PB1 is thought to comprise at least three critical acidic amino acids and has been shown to be dependent on divalent metal ions [500]. The endonuclease activity also requires hairpin loop structures in the 5' vRNA arm [461] and 3' vRNA arm [460].

Priming is not thought to require hydrogen bonding between the capped cellular primer fragment and the promoter of the viral genome [501]. Transcription is initiated by the incorporation of a G residue that is templated by the penultimate C residue on the 3' end of the vRNA [476, 488, 493, 495, 496, 502]. It has been postulated that during mRNA synthesis PB1 binds to and does not release the 5' vRNA end. Transcription is thought to occur by threading the 3' vRNA end through the catalytic site in a 3' to 5' direction. The chain is elongated by the action of PB1, which has been shown to possess four conserved sequence motifs that are frequently found in polymerases, including a SDD sequence found at amino acids 444 to 446 [503, 504]. Work with recombinant




**Intro. Fig.5 Events of Transcription**

Events for Transcription. A) 5' end of vRNA binds to PB1 and activates cap binding and 3' UTR binding, B) Cap and 3' UTR binds, C) upon binding of 3' UTR, endonuclease activity is activated and cap cleavage occurs, D) initiation and elongation occurs. The poly(A) tail is added by a stuttering mechanism over the poly(U) stretch near the 5' UTR (not shown).




**Intro. Fig.6 Events of Transcription**

Detailed look at transcription events. A) Endonuclease site, B) Initiation, C) Elongation and stuttering of the transcript.

polymerase complexes has identified the active site of PB1 [292, 499].

Nucleotide addition by PB1 occurs until a stretch of four to seven uridine residues is reached that is located 15 to 22 nucleotides from the 5' end of the vRNA. At this point, the 5'-vRNA end is anchored to PB1 and prevents processive copying through the uridine stretch. The anchoring may be facilitated by the presence of a hook in the 5' vRNA end that has been shown to be necessary for polyadenylation [487]. The retention of the terminal 5' vRNA arm by PB1 results in the catalytic site of PB1 to stutter over the poly-uridine stretch resulting in reiterative copying and thereby adding a poly (A) tail [505-508]. Eventually the transcript breaks free of the complex and is free to be exported to the cytosol.

## A. Initiation of Transcription

Later work on transcription focused on defining the position at which initiation begins. In a detailed study that examined the primer-dependent initiation of transcription using different dinucleotides that were complementary to all possible sequences in relation to the start of transcription at the 3' vRNA end, dinucleotide ApG was found to most effectively initiate transcription [509]. ApG is capable of base pairing with the first two nucleotides of the 3' vRNA terminus. Transcription was found to be initiated between positions 2 and 4, depending on the primer used, with position 3 being the most active. In contrast, capped primers initiate transcription preferentially at position 2 [488, 502], rather than position 3. Oligos GpG and ApGpC were also found to be active, yet heptanucleotides AGCGAAA and AGCAAAA, which base pair with either U4 or C4 promoters, were found to be inactive. In addition, only oligos that possessed sequence at the 3' end that would base pair with influenza sequence were found to be active, suggesting an essential role of base pairing immediately next to the first phosphodiester bond. This finding conflicts with data from early studies using cellular mRNAs as a primer source, which claimed hydrogen bonding was not required between the primer and

the viral promoter [501].

Much of the *in vitro* work on influenza polymerase activity has utilized the dinucleotide ApG to substitute for cellular mRNAs as a primer source. Transcripts initiated with ApG are still active for translation. However, translation of globin mRNA primed transcripts has been found to be three times more efficient than ApG primed transcripts [488]. In addition, globin mRNA is 3000-5000x more efficient at priming transcription than ApG [440]. In a comparison between B-globin mRNA and ApG, which was not performed with equal molarity, β-globin mRNA was found to stimulate transcription 75 fold and ApG 93 fold over unprimed transcription. In addition, α-globin mRNA was found to be 30% as effective per microgram as β-globin mRNA, and this difference is likely due to the presence of purines at the endonuclease cleavage site [488].

Studies using siRNA have recently suggested that newly synthesized NP and PA proteins are required for influenza virus transcription and replication [168].

## B. Secondary Transcription

Secondary transcription occurs after the polymerase has completed an initial round of transcription and these transcripts have been translated to provide additional viral proteins including the polymerase proteins that are then capable of further transcribing the original genome or newly synthesized genome. In contrast to primary transcription, where the polymerase is already bound to the genome, in secondary transcription, newly synthesized polymerase must find and bind genome prior to initiating secondary transcription. The first event that is thought to occur is that PB1 recognizes and binds the 5' vRNA end [456]. It is presumed that the complex is in the 3P format, although PB1-PA complexes have been shown to bind 5'-vRNA [443]. The binding of PB1 to the 5' vRNA sequence causes an allosteric alteration in the complex that activates two new functions, including: PB2 cap-binding activity [292, 456, 482, 499, 510] and PB1 3' vRNA binding activity [292]. Once the 3' vRNA arm is bound, the endonuclease

activity of the polymerase is activated, and PB1 can cleave the cap and initiate transcription.

## C. Temporal and Quantitative Control of Transcription

Many time course experiments have been carried out to ascertain when viral transcription, translation, and replication occur, and whether they occur equally between the different segments of the virus. These experiments have been carried out by treating infected cells with a variety of agents at different time points that affect the synthesis of RNA or protein. The agents used include: glutaraldehyde, which binds mRNA to polyribosomes, and has been used to monitor the time and level of viral mRNA expression [511], actinomycin D (1 $\mu$g/ml) [512] or $\alpha$-amanatin [488, 489], which inhibit RNA polymerase II from creating cellular caps and ultimately curtails influenza transcription, or cycloheximide (100 $\mu$g/ml) which inhibits protein synthesis but does not affect primary transcription [288, 512, 513].

Differential cycloheximide treatment has been valuable in studying primary transcription and secondary transcription. Cycloheximide treatment prior to infection is useful for studying primary transcription, which is not dependent on new protein synthesis. Because no new protein synthesis occurs, the results are not confounded by secondary transcription. Secondary transcription can be examined by treating infected cells with cycloheximide at two hours post infection, which allows enough time for the primary transcripts to be translated, and helps prevents any subsequent cellular response that may degrade the viral RNA. This type of treatment allows for the maximal mRNA synthesis from secondary transcription and the level of RNA is stable for six hours. Interestingly, cycloheximide treatment at the time of infection has been shown to inhibit 95% of vRNA synthesis, which suggests that vRNA synthesis is dependent on freshly translated proteins (potentially NP) [288, 512, 513].

There is some evidence to suggest that temporal control of mRNA transcription

occurs in influenza infected cells [514]. The genes that are preferentially transcribed and translated early are NP and NS, and to a lesser extent PB1 [512]. PB1 is also thought to be an early gene since the addition of actinomycin D at two hours post infection allows for the translation of PB1, NP, and NS, whereas addition at three hours post infection permits translation of all viral genes vRNAs. There are thought to be slight variations in the amount vRNA synthesis depending on the segment, and the relative levels of each RNA segment may change slightly over the course of infection.

Considering there is some preferential transcription that occurs after infection, the overall trend is that mRNA synthesis increases from 30 minutes to 2.5 hours post infection, and then gradually decreases over then next six hours [288, 505, 515]. In addition to mRNA, vRNA and cRNA synthesis is also thought to follow the same trend of synthesis, but the amplification is not as great. Competitive hybridization experiments that utilized S1 nuclease to digest away single stranded RNA have shown that the level of vRNA is less than 4% of mRNA levels [288]. In addition, 80% of the vRNA incorporated into virions was found to be synthesized in the cell within four hours of infection [288]. Lastly, there appear to be some differences in the ratio of mRNA to vRNA produced by different segments. This difference may be due to the difference in C4 versus U4 promoters. The polymerase genes invariably have C4 promoters and have a high vRNA:mRNA ratio when compared to segments with U4 promoters. This finding suggests that C4 promoter favors replication over transcription.

Other studies of viral mRNA transcripts have shown that transcripts undergo internal methylation at adenosine residues, which varies from one to six methylated nucleotides per transcript [494, 516]. The biological significance of methylated transcripts is unclear.

## D. Inhibition of Viral Transcription

Late in infection, the polymerase stops transcribing and replicating the genome,

and is exported out of the nucleus in the context of a vRNP. It is has been shown that the M1 protein migrates into the nucleus to inhibit transcription [341, 342].

The polymerase has also been shown to be sensitive to caps that are shorter than 12 nucleotides in length[517]. *In vitro* synthesized capped transcripts that were made to be a defined length by utilizing T7 RNA polymerase to extend cap-1 structures (m7GpppGm) or cap-1 analog structures (m7GpppGOH) using DNA oligonucleotides as templates. These synthetic caps were transfected with an *in vitro* transcribed NS-CAT reporter into a murine cell line that expresses each of the three polymerase proteins and NP under the control of dexamethasone. It was shown that 5'-capped RNA fragments that were 9-12 nucleotides in length inhibited transcription. Fragments of 9 nucleotides in length exhibited the greatest degree of inhibition that was concentration dependent and could reach 88% [517]. Analog cap structures had slightly lower inhibitory effect. The minimal RNA chain length required for priming activity was found to be 12 nucleotides long, and at 13 nucleotides the caps had little inhibitory effect. The authors suggested that PB2 recognizes and binds cap structures, and if the capped RNA is less than 12 nucleotides in length, the viral enzyme can bind the cap, but not effectively use it as a primer for transcription.

# Chapter 35. Replication

## A. Transcription versus Replication - a Switch?

The long standing model that the polymerase undergoes a "switch" to change from transcription to replication is now in doubt, due to the recent emergence of a challenging "stability model". The initial "switch model" contended that the level of soluble NP mediates the switch to replication. The exact mechanism by which NP regulates the switch is unclear, but NP has been shown to bind both PB2 and PB1 [518,

519]. The NP switching theory was postulated from data in infected cell extracts where the synthesis of full-length cRNA was found to be dependent upon the availability of NP [479, 520]. Supportive of this finding, several NP mutants have been isolated that affect genome replication, cRNA and vRNA, and mRNA transcription differentially [521-525]. In addition, *in vitro* lysis of purified virus releases transcriptionally competent RNPs that can synthesize capped and polyadenylated mRNAs when provided appropriate substrates [496, 526]. These same RNPs do not synthesize cRNA, except in the context of an infected cell and only after a round of mRNA transcription and subsequent protein expression [435, 515]. In addition, nuclear extracts from infected cells catalyze both viral mRNA synthesis and genome replication [527-529], however, depletion of these fractions of NP that is not already bound into RNPs abolishes both cRNA and vRNA synthesis [479, 520]. Possibly this is due to the fact that both vRNA and cRNA are encapsidated to form functional RNPs [479, 515, 530]. It has been proposed that increased levels of NP alter the structure of the RNA promoter causing a bias for the polymerase to initiate replication rather than transcription [441, 444, 485]. Other possibility is that NP binds to either PB1 and/or PB2 to allosterically change the polymerase [518, 519, 531].

However, this longstanding hypothesis that increasing concentrations of NP mediates the switch from transcription to replication was recently tested and the results suggest that this hypothesis is not true [532]. In both a plasmid based RNP reconstitution system and a transfection/infection system, titrating up the amount of NP did not promote higher levels of genome replication relative to transcription. In fact, the inverse was found to be true, replication (5-10% of positive sense transcripts) was highest at low NP concentrations (37 ng NP versus 250 ng polymerase components) and as the concentration of NP increased, the amount of replication decreased.

The plasmid-based system for reconstituting RNPs does not accurately represent a native viral infection, especially concerning the temporal control of viral transcription. In a system for reconstituting vRNPs via transfected plasmids, the amounts of all three

RNA species simply increase over time [532]. In contrast, a viral infection, for the matrix segment, vRNA levels will increase from 2.5 through 8 hrs., and both cRNA and mRNA are synthesized early after infection, peaking at 2 hrs p.i. followed by a rapid decline[532], All species are detectable at 1 hr, but some of the vRNA signal may be due to incoming vRNA rather than newly synthesized vRNA. Similar temporal control of the different RNA species has been described by others [288, 478, 515, 533]. Although cRNA levels are low, similar to a viral infection, the increase and lack of decline for both mRNA and cRNA does not accurately mimic a real viral infection. As a result, less confidence can be placed on data generated by plasmid generated vRNPs.

However, additional evidence now supports the argument against NP mediating a replication switch and contends there is no need for *de novo* protein synthesis for replication synthesis to occur, and in fact there is no switch at all, but rather the polymerase stochastically synthesizes both mRNA and cRNA early in infection [480]. These new data support at "stabilizing model" in RNA replication. This model contends that the cRNA synthesized early in infection is degraded by host nucleases and is hard to detect. Once primary transcription and translation has generated newly synthesized polymerase and NP, these cRNA transcripts are protected from the host nuclease. However, this protection can be mediated by pre-existing polymerase and NP, implying that newly synthesized proteins are not a requirement. Polymerase itself is sufficient to stabilize and protect the cRNA from host nucleases, but NP greatly enhances this protection, but NP itself is insufficient for cRNA protection. These data were generated by transfecting 293T cells with the polymerase components and NP 12-14 hrs prior to infection with A/WSN virus. The polymerase components were mutated to inactivate their ability to transcribe or replicate a genome, but they still retained ability to bind the viral promoter. At the time of infection, cells were incubated with 100 $\mu$g/mL of cycloheximide, which inhibits new protein synthesis. In these cells, which already have polymerase present, the synthesized cRNA is stabilized early in infection and

accumulates to detectable levels. In contrast, if cells are not transfected with polymerase prior to treatment with cycloheximide, cRNA synthesis is barely detectable [533]. Polymerase mutants with reduced binding capability had reduced ability to stabilize cRNA. All three components of the polymerase were found to be necessary to protect the cRNA from host nucleases. Host nucleases do not degrade viral mRNA due to the presence of a host cap and polyadenylated tail.

Therefore, the new "stabilization model" suggests that when a virus infects a cell, the incoming polymerase is necessary and sufficient for cRNA synthesis. Although early cRNA is unprotected and degraded, later cRNA is protected by newly synthesized 3P and NP. The authors of the model suggest that newly synthesized polymerase binds to the 5' and 3' ends of the cRNA, as previously shown [534], and this binding may serve as a nucleation point for binding of free newly synthesized NP, leading to the formation of active and stable cRNPs suitable for replicative vRNA synthesis [480]. These data suggest that the polymerase does not "switch" but is regulated stochastically. This model provides an explanation for why mutations in NP [479, 518, 519, 525], PB2, [535] or PA, [536] which may interfere with cRNP complex formation, can prevent the detection of cRNA. Vreede *et. al.*, admit that their data do not exclude the possibility that a host factor [537] or forms of a higher-order polymerase complex or lattice [538] may regulate the replication of the virus, but they support the notion that subtle conformational differences in the structure of the polymerase [539, 540] allow for stochastic regulation of transcription/replication initiation. This model is also supported by data from my own studies, generated in a five plasmid transfection system, in which cRNA becomes detectable at the same time as mRNA (see Chapter 44, F).

Aside from the unclear role NP plays in switching from transcription to replication, NS1 and NS2 have also been implicated in the process [541, 542]. However, subsequent work is in disagreement with the role of the nonstructural proteins in the switching process [543]. The polymerase's choice between transcription and replication

and the sequence of these species is illustrated in Intro. Fig. 7.

Initially, NP was thought to be involved in antitermination [527]. But, recent work suggests that NP and NS1 have no role in antitermination [435].

## B. Dogma of Replication

In contrast to transcription, replication is not dependent on a primer. Polymerase purified from virions has been used in *in vitro* transcription assays and the 3'-end RNA (+) products have been analyzed RNase protection and found to only synthesize mRNA [435]. Interestingly, the addition of regulatory proteins NP and NS1 did not permit the complex to make cRNA. In a study that utilized fowl plague virus (avian influenza strain) infected chick embryos, it was determined that an initial round of viral mRNA synthesis and translation of viral proteins must occur in order for synthesis of cRNA to begin [515].

Replication is a two-step process. First, a full-length copy of the vRNA must be synthesized to make complementary RNA (cRNA). Then, the cRNA serves as a template for the second step in replication, which is to amplify the vRNA genome [492, 515, 544]. It is thought that cRNA and vRNA are initiated *de novo*, meaning without the aid of a primer. This primer-independent initiation mechanism was postulated from analysis of 5' ends of both cRNA and vRNA, which were demonstrated to possess terminal 5' adenosine triphosphate groups [494, 545-547]. Once unprimed initiation has occurred, it is necessary to bypass the poly-uridine stretch. This process of skipping polyadenylation is called anti-termination [505, 515, 546]. As previously mentioned, the role of NP in mediating anti-termination by causing an allosteric change in the polymerase is controversial. The supposed allosteric change in the polymerase causes the release of the 5' vRNA end from PB1 and permits processive copying of the vRNA through the poly-uridine stretch to the end of the genome [520].

The synthesis of vRNA has been shown to be independent of primer [545]. In

addition, free NP is thought to be required for the synthesis of vRNA [479].

## C. Polymerase Modification

Many viruses have polymerases that are modified or regulated during the lifecycle of the virus by either host or viral factors. For influenza, host factors have been claimed to be involved in replication [533, 546, 548-554]. To date, no cellular factors have been definitively shown to be required for completion of the replication cycle.

## D. Minimal Requirements of the Polymerase

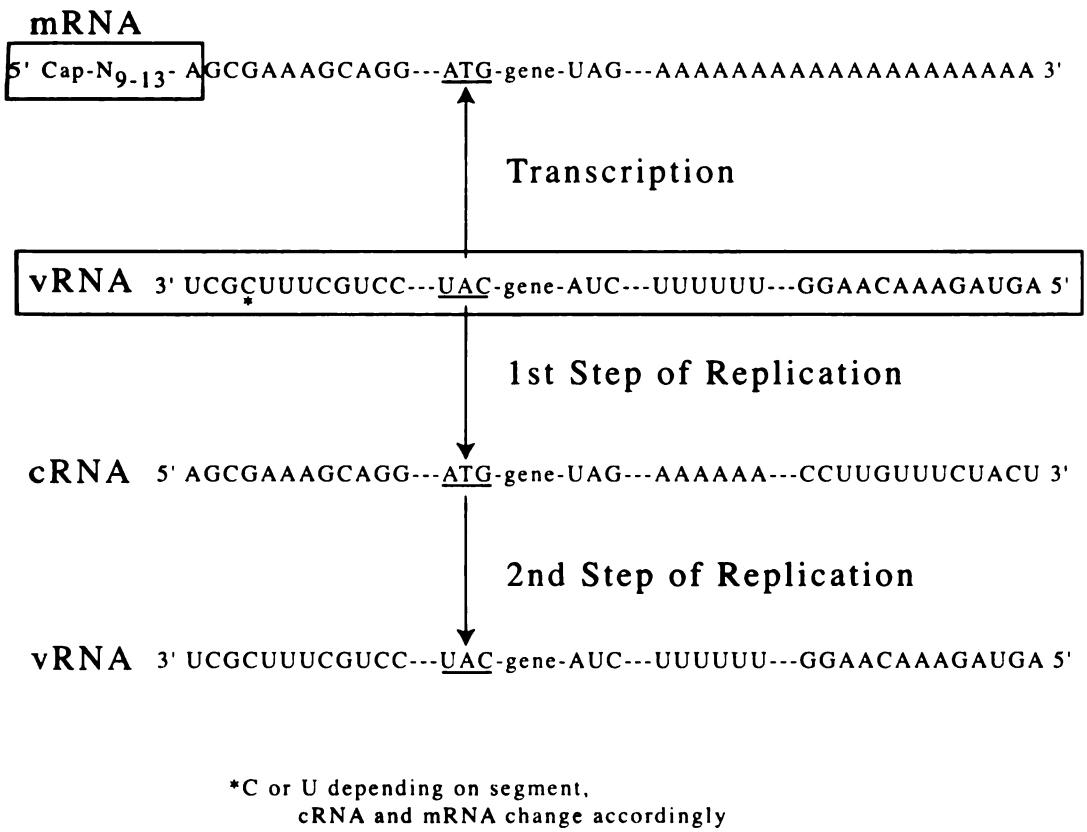
Attempts to isolate 2P from infected cells has shown that most of the polymerase complexes are in a stable heterotrimeric complex, and very few 2P complexes exist [555], although in insect cells PB2-PB1 complexes form [556]. Nonetheless, work has been carried out to help identify the minimal protein subunit requirements for transcription and/or replication. The issue of whether different forms of the polymerase carry out different functions is an important issue that is biochemically a challenge to address due to the apparent low quantity of 2P present in infected cells. Although much research has been done in the field of defining the minimal components necessary for each phase of transcription and replication, much controversy still exists, necessitating further work to resolve the issue.

The dogma that 3P and NP are required and sufficient for efficient transcription and replication activities has been established from work by many laboratories [443, 473, 474, 543, 557-559]. Some of this work is briefly described below. First, in the context of a viral infection, the transcriptive complexes isolated were found to contain each of the three polymerases [474]. Secondly, vRNPs reconstituted *in vitro* are only active when transfected into cells that have previously been infected with recombinant vaccinia viruses that express each of the three polymerases and NP [543]. Cell lines

tested include: mouse fibroblast C127 cells, MDBK, HeLa, 293, and L cells. Another study reported that successful replication occurred only when an NS-CAT reporter incubated with *E. coli* purified NP was transfected into a stable cell line expressing each of the influenza polymerase subunits and NP under the control of dexamethasone [557]. Studies have shown *in vitro* transcribed reporters are replicated as long as the polymerase and NP is present [558, 559]. These components can either be purified from virions and transfected into cells or expressed inside cells via recombinant SV40 viruses that express the necessary influenza proteins [558]. Alternatively, cells can be infected with a recombinant vaccinia virus expressing the T7 RNA polymerase (vTF7-3) and transfected with plasmids for the expression of each polymerase subunit and nucleoprotein [443, 559]. Another method has utilized the nuclear extracts of insect cells that were infected with recombinant baculoviruses that express each of the polymerase subunits [560, 561].

The polymerase complex can be separated from extracts using vRNA-ends as bait. Essentially, 5'-vRNA-ends are conjugated to biotin and added to the extracts. After binding, the extracts are mixed with streptavidin coated magnetic beads to separate the polymerase from the milieu [443]. Transcription can then be initiated by incubating the complexes with 3'-vRNA ends in transcription buffer. Using this system, the 3P complex has been shown to be capable of unprimed initiation that was confirmed by analysis of the 5' end groups, whereas no activity was demonstrated with PB1-PA complexes [443]. These results demonstrated the necessity of PB2 for unprimed replication and the necessity of PA in enhancing the binding of PB1 to the 5'-vRNA end, since PB1-PB2 did not bind to detectable levels. In this study, NP was not required for the initiation of unprimed transcription.

The requirement of the three polymerase subunits and NP for polymerase activity has recently been challenged [443, 560-564]. First, PB2 has been claimed to be required for transcription but not replication [562]. This study was performed in a cell line expressing PB1, PA, and NP under the control of dexamethasone. NS-CATc and NS-




**Intro. Fig.7 Transcription versus Replication**

Steps of transcription and replication. The vRNA can either be transcribed into mRNA or replicated into cRNA. The cRNA serves as a template of vRNA production.

CATv were both found to be synthesized into the complementary strand RNA, supporting the claim that PB2 is not required for replication. In addition, poly (A)+ CAT RNA was detectable, but the transcript was inert unless capped using purified yeast mRNA capping enzyme (mRNA guanylyltransferase), suggesting that PB2 is required for capping viral transcripts but not for polyadenylation. In a second report by the same group, the PB1 subunit was found to be able to catalyze cRNA synthesis alone, but the addition of PA was required for vRNA synthesis [563]. In that report, PB1 and NP were expressed conditionally in a cell line under control of dexamethasone and it was determined that cRNA and poly (A) RNA synthesis were functional, but vRNA synthesis was not. The authors claimed that PA acts as an allosteric modulator to cause a conformational change in PB1 to allow for vRNA synthesis from a cRNA template. In another report, the nuclear extracts of insect cells, infected with a recombinant baculovirus that expresses PB1, were found to be capable of dinucleotide primed transcription, supporting the theory that PB1 alone can transcribe templates [560, 561]. The finding that PB1-PA complexes are inactive [443] was refuted by another study that utilized HIS-tagged polymerases to affinity purify complexes from insect cells infected with recombinant baculoviruses [564]. In that system, the PA-PB1 complex performed the function of a replicase by preferentially catalyzing *de novo* initiation of RNA synthesis, but it was unable to bind capped RNA. The 3P and PB1-PB2 complexes were shown to possess all the functions of a transcriptase, including cap binding and cleavage, model vRNA binding, mRNA synthesis, and polyadenylation. The authors proposed that the catalytic specificity of PB1 is modulated to the transcriptase by binding PB2 or to the replicase by binding PA.

Each of the above systems has pros and cons that must be taken into account when interpreting the validity and biological relevance of the reported findings. The following points should not be overlooked when examining the data: 1) the use of recombinant viruses to express polymerase subunits introduces many unknown factors, 2) insect cells are not natural hosts of influenza infection, 3) HIS-tagged polymerases may

compromise function, 4) purification utilizing only 5' vRNA ends may exclude functional but weakly-binding polymerase complexes, and 5) reporters which have coding sequences that are not of influenza origin or are greatly truncated do not aptly imitate viral genes. In addition, purification of the polymerase from some systems such as the yeast *Pichia pastoris* has shown that it is difficult to remove trace amounts of cellular nucleases, which may confound the results [565].

Many questions still remain regarding influenza transcription and replication. For example, it is not known whether the polymerase completely dissociates from the vRNA once it has made a transcript or if it is capable of repositioning itself on the vRNA without ever releasing the genome. Similarly, it is not known if the cRNA and vRNA are synthesized during physically separate events or if the two processes are linked. Nor is it known what percentage of transcription initiation events end in polyadenylation, nor what percentage of unprimed replication initiation events end in anti-termination. Nor is it clear if the polymerase that is pre-assembled and enters upon viral infection is biochemically the same as the polymerase synthesized later for secondary transcription. Each of these issues needs further study.

# Chapter 36. Viral Translation

Influenza virus proteins are synthesized in two phases, early and late. The NS1, NP, and PB1 proteins are synthesized early, whereas the other proteins are synthesized late. The switch between these two phases appears to be regulated at the level of mRNA export from the nucleus [478]. The regulation of viral translation is mediated by NS1, which inhibits the export of late protein transcripts from the nucleus until late in infection. The exact mechanism by which this regulation occurs is unclear, although NS1 has been shown to bind poly(A) tails of all RNA molecules tested [390, 400], the specificity of regulation remains an enigma. It is thought that the activity of NS1 for

the late transcripts must be inactivated by post-translational modifications, since protein kinase and methyltransferase inhibitors block export of late protein transcripts [336, 566, 567].

Many eukaryotic viruses are able to inhibit cellular protein synthesis [568-571]. Both adenovirus and poliovirus assure selective translation of viral mRNAs by utilizing cap-independent mechanisms for translation initiation. Poliovirus degrades a component of the cap-binding protein complex, eIF4F1, preventing cap dependent translation, but permitting cap independent translation. Whereas, adenovirus dephosphorylates eIF4E to permit the selective translation of viral mRNAs that have a reduced requirements for eIF4E.

In contrast to poliovirus and adenovirus, influenza translation is dependent on cellular caps. Influenza relies on several strategies to ensure the selective and efficient translation of its mRNAs over the translation of cellular mRNA. Those strategies include: 1) stealing cellular caps and preventing the processing of cellular pre-mRNAs so that few cellular mRNA are exported to the cytosol [572]; 2) inhibiting cellular mRNA translation at both the initiation and elongation stages [410], 3) inactivating PKR [569], and 4) preferentially targeting viral mRNA to the translation machinery by NS1 as previously mentioned.

The sequence and structure of influenza viral mRNAs has been shown to be critical for selective viral protein synthesis [403, 404, 514, 568, 573, 574]. This was determined by transfecting cDNA chimeras containing the noncoding and coding regions of cellular and viral mRNAs into previously infected cells. Two cellular proteins that have been shown to interact specifically with the viral 5'-UTR include the La autoantigen [575] and the guanine-rich sequence factor 1 (GRSF-1) [576]. These proteins are thought

recruit some viral mRNAs to polyribosomes for enhanced translation [577].

# Chapter 37. Heterotrimeric Polymerase and vRNP

## A. Polymerase Subunit Contact Sites

Each polymerase subunit contains a nuclear localization signal and can migrate independently to the nucleus [578-580]. The issue of whether the polymerase subunits migrate into the nucleus as a complex or individually is still debated. Pulse-chase analysis of fowl plague virus-infected MDCK cells suggests that PB1 and PB2 migrate rapidly into the nucleus together with NP and NS1, whereas PA remains largely in the cytoplasm [581]. In support of this view, it has been shown that, at 3 hrs p.i., PB1 and PB2 are present mainly in the nucleus, whereas PA is present in both the nucleus and cytoplasm [578, 582]. Despite those findings, there are other studies which suggest that PB1 and PA stimulate each others' nuclear accumulation, and that this interaction is dependent on the N-terminal one-third of PA [582]. Although the exact kinetics of the polymerase subunits entering the nucleus are still unclear, it is clear that by 9 hrs p.i. all three polymerase subunits are found in the nucleus.

The three polymerase proteins form a complex in the cytoplasm and nucleus of infected cells that is largely resistant to disruption by normal immuno-precipitation buffers, and this complex sediments on sucrose gradients at 11S to 22S [555]. Upon simultaneously expression of the polymerase subunits in *Xenopus* oocytes, the 3P complex forms in the absence of vRNA and can be immuno-precipitated. Immuno-precipitation experiments demonstrated that PB1 is the central subunit that binds both PA and PB2, which are not thought to interact with one another [583]. Much progress has been made in refining the regions of the polymerase subunits that are thought to be involved in binding. Fine mapping of the 3P contact sites has revealed that the N-

terminal 12 amino acids of PB1 bind near the C-terminus of PA (at residues 668-692 [584-590]), and that the C-terminal 600-757 amino acids of PB1 bind residues 51-259 of PB2 [584, 591]. These studies have been carried out using antibody epitope mapping and mutagenesis with immuno-precipitation.

The low-resolution 3D structure of the influenza virus polymerase complex has been modeled based upon electron microscopy and image processing of recombinant ribonucleoproteins (RNPs) [592]. The polymerase structure is very compact with no discernable boundaries among subunits. The positions of the PB1, PB2, and PA subunits were determined by 3D reconstruction of either RNP-mAb complexes or tagged RNPs.

## B. Polymerase Subunit RNA Contact Sites

In addition to the protein-protein contact sites of the polymerase being mapped, each subunit has been examined for its ability to bind RNA. Some data suggest that the vRNA ends bind to the polymerase in single-stranded format, not hydrogen-bonded to one another as suggested by the "panhandle model" [444, 446]. This finding has been confirmed by others who used gel shift, modification interference, and density gradient techniques, which each suggest that PB1 binds terminal vRNA ends only if they are not in double-stranded format [456]. This implies that the viral polymerase must melt the panhandle structure in order to access the 3' vRNA end for transcription and replication, or that the polymerase possesses helicase activity. To date, no helicase activity has been associated with the polymerase. The lack of helicase activity is further supported by work with vaccinia virus-derived polymerase complexes that were shown to be unable to activate cap-binding or endonuclease activity when provided a panhandle-style promoter [456]. This finding has spurred the development of alternative hypotheses including the "RNA-fork" model (Intro. Fig. 4), in which the terminal 5' and 3' bases are not hydrogen bonded to each other [447]. This theory has been supported by biochemical experiments that have indicated that the 5' and 3' vRNA ends are not juxtaposed unless the polymerase

is present [485]. Ultraviolet (UV) light-induced cross-linking experiments have shed light on the subunits responsible for binding RNA. PB2 is not thought to bind the vRNA, but has been shown to bind the cap of cellular mRNAs [593-595] and this finding is supported by analysis of temperature-sensitive PB2 mutants [596]. The tryptophan-rich domain of PB2 has been implicated in the binding of cap structures. In addition, PA has been shown to UV crosslink to vRNA, but whether this binding is functional, or just a matter of proximity has not been determined [441, 597].

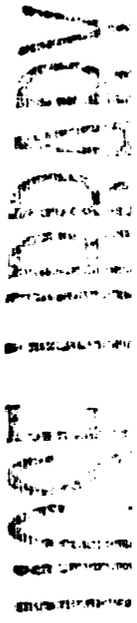
The polymerase complex mediates most of its binding to the vRNA template through PB1. UV cross-linking studies have been used to identify the active site of PB1 [594], as well as the 3' and 5' vRNA binding sites. The amino acids responsible for binding PB1 to the vRNA and cRNA are in dispute. UV cross-linking of thio U-substituted RNA was used to identify R571 and R572 of PB1 as the residues required to bind the 5' vRNA arm; and a ribonucleoprotein 1 (RNP1)-like motif at residues 249-256, which contains two phenylalanines, is required to bind the 3' vRNA arm [292]. Mutagenesis of these residues confirmed their requirement for RNA binding. However, another study used Northwestern analysis and competition experiments with labeled model RNA bound to deletion mutants of PB1, and found the 5' vRNA and 3' cRNA arms were shown to share common binding sites in the N-terminal 139 amino acids of PB1, whereas the 5' cRNA arm binds to residues 267-493, and the 3' vRNA arm binds to residues C-terminal to 493 [293, 442]. The Kd's of PB1 to vRNA and cRNA have been determined to be approximately $2x10^{-8}$ M and $7x10^{-8}$ M, respectively. Interestingly, PB1 bound the arms of cRNA equally well, whereas the 5' arm of vRNA bound much more tightly than the 3' arm of vRNA when presented alone. Optimal binding of vRNA to PB1 required both arms. Another study examined vRNA binding to PB1 by incorporating radioactive 8-azido GTP (8-N3 GTP) or oxidized GTP into RNA that was then photo-cross-linked to PB1. Binding domains were identified after V8 protease cleavage and N-terminal sequencing of cleavage products. The RNA binding domains of PB1 were

determined to be located around residue 298 and C-terminal to residue 458 [598, 599].

Other work has suggested that the polymerase may bind to the non-conserved noncoding region and perhaps to the coding region, but any specific nucleotides and residues involved have not yet been defined [303].

# Chapter 38. Nucleoprotein

Nucleoprotein (NP) is one of the type-specific antigens of influenza, in that NP from type A, B, and C are antigenically distinct from one another. The phylogenic tree of NP has two major branches, one containing all avian strains and the other containing all human strains with a side branch of the classical swine viruses [600]. NP is the second most abundant protein in the virion and is a major target of cross-reactive cytotoxic T lymphocytes [601]. NP is a multifunctional protein. One of the functions of NP is to bind RNA. Binding is thought to be facilitated through its many arginine residues [602]. The lack of clusters of basic residues implies NP may have many regions that contribute to RNA binding. Each NP binds about 20 nucleotides of RNA [603], however the RNA remains sensitive to digestion by RNase [604]. The binding of NP to RNA is thought to improve the processivity of the polymerase and NP has been shown to be necessary for chain elongation *in vitro* [605]. In particular, it has been shown that nucleoprotein (NP) is required for the anti-termination step of replication [520]. The properties of temperature sensitive mutants of NP, which continue to bind the polymerase and to self-associate but fail to bind RNA, suggest that NP-RNA interaction is ordinarily required for transcription [519]. However, NP is not required for transcription of templates less than 200 nucleotides long [605, 606]. It has been claimed that host factors are required for NP to bind newly replicated RNA [479]. The ability of NP to self-associate may facilitate its binding of vRNA and cRNA. In addition, NP can bind PB2 and PB1, which may stimulate a putative switch from transcription to replication [518, 519, 607]. *In vivo*, NP



has been shown to be essential for influenza gene expression [543].

In addition to its roles in RNA binding and polymerase activity in the nucleus, NP has been shown to shuttle between the nucleus and cytoplasm. The function of nuclear-cytoplasmic transport is unclear, but it is achieved through the action of two nuclear localization signals [608-610] and a cytoplasmic retention signal [611] that was originally thought to be an NLS [612]. Residues 327-345 of NP were shown to be responsible for nuclear accumulation [612, 613]. To facilitate entry into the nucleus, NP may interact with several members of the importin family [614, 615].

It has been proposed that regulation of transport in and out of the nucleus may be mediated through the phosphorylation state of the protein. NP itself is a phosphorylated protein that has been shown to be able to phosphorylate NS1 [435]. NP has also been shown to bind filamentous actin in the cytosol, and it has been proposed that this binding causes cytoplasmic retention of vRNPs [611]. Late in infection, NP from influenza A undergoes cleavage by a cellular caspase but it is unclear if this is a byproduct of apoptosis or a specific component of the viral lifecycle [616].

NP is detectable in newly infected cells by indirect immuno-fluorescence within one hour post infection. NP is initially localized predominantly in the nucleus [344]. At 2.5 hours post infection, the level of NP in the cytosol begins to rise, and after 10 hours, when the concentration of NP has peaked, NP becomes predominantly localized to the cytsol. Newly synthesized late proteins (M1, M2, NA, and HA) become detectable 2.5 hours after infection, and this timing coincides with the shift of NP from the nucleus to the cytosol [344]. Using a protein kinase inhibitor (H7), that has been shown to selectively block the synthesis of late influenza proteins without preventing viral RNA transcription or replication [250, 566, 617], NP was confined exclusively to the nucleus at 10 hours post-infection. In addition, microinjection of anti-M1 antibodies into infected cells prevented import of M1 into nucleus after synthesis, and also resulted in confinement of NP to the nucleus. In contrast to other work, pulse-chase experiments

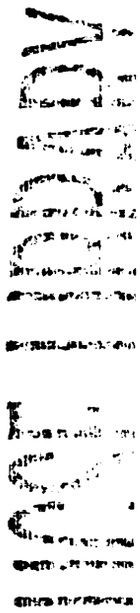
looking at NP in the virion suggest that vRNPs are assembled in the cytosol prior to assembly [344, 618], not in the nucleus as suggested by other studies.

NP has been shown to form homo-oligomers *in vivo*. Treatment with DTT has suggested that NP undergoes an oxidative maturation process [619]. Such studies suggest that NP undergoes a transient disulfide bond formation that links NPs together. This transient state allows for proper conversion into a disulfide-free NP, which forms non-covalently stabilized NP-oligomers. *In vitro*, in the absence of RNA, purified NP forms vRNP-like structures that are indistinguishable from intact influenza vRNP by electron microscopy [620].

Intracellular NP oligomers have been found to be noncovalently stabilized, and although nascent NPs have disulfide linkages, these are reduced in NP subunits of NP oligomers, and this likely contributes to their stability and compactness [619, 621, 622]. NP oligomers are thermostable and resistant to dissociation by SDS, urea, and high ionic strength. NP oligomers can be completely dissociated at pH below 5. The stability of NP oligomers is thought to arise from strong hydrophobic bonds and electrostatic interactions.

When NP is expressed by itself, it localizes to both the nucleus and cytoplasm, though at higher expression levels there is a bias toward cytoplasmic accumulation [611, 623]. This same dose-dependent localization pattern of NP was observed in cells transfected with the polymerase subunits and a reporter [532].

NP is clearly required for replicative RNA synthesis. Its possible role in mediating a switch from transcription to replication is unclear, but NP is required to stablize nascent cRNA and vRNA species. In the absence of NP and the polymerase, replicative RNA species are degraded by host nucleases [480]. In addition, when infected cell extracts are depleted of NP, short vRNA products of heterogeneous length are produced [479]. In addition, *ts* NP mutants have been created that are unable to bind



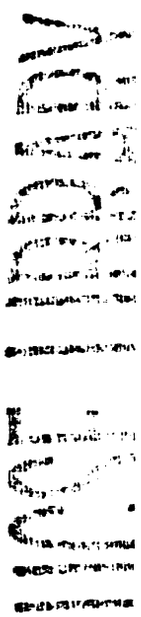
RNA at elevated temperatures and are specifically defective in replication [519].

# Chapter 39. PB1

PB1 plays a central role in the transcription and replication of the virus. Most of the details of importance have been mentioned above. Analysis of the PB1 subunit using ATP analogs capable of cross-linking protein, has suggested that residues 179-297 of the PB1 subunit face the 5'-end of the primer nucleotide, which can be extended upon addition of other nucleotides [624]. Of additional interest, in infected cells, antisera against PB1 have revealed three different isoforms, including 94 kD full-length protein and two smaller isoforms of 85 kD and 70 kD [625]. Limited proteolysis and peptide mapping confirmed the identity of these isoforms and suggest that they may be translated independently of their full-length counterpart. The smaller isoforms were not detected in purified virions, but were found to be associated with vRNP in infected cells. Whether these isoforms play a functional role in the biology of the virus is unclear.

## A. PB1-F2

Some, but not all viral strains have a PB1 gene that is capable of generating two products. Those that can generate two gene products synthesize an abundant amount of an 87-residue protein called PB1-F2. This protein is generated by translation in the +1 reading frame [181, 626]. The function of this protein is unknown, but the current theory is that PB1-F2 is secreted from infected cells to kill host immune cells that respond to the infection. The protein has been shown to be toxic since exposure of synthetic PB1-F2 to cells induces apoptosis. The protein is immunogenic and is recognized by $CD8^+$ T cells. Interestingly, the PB1-F2 protein is absent from swine influenza, exhibits variable expression in individual infected cells, is rapidly degraded by the proteosome, and



localizes to mitochondria.

Recent work has shown that PB1-F2 targets the inner mitochondrial membrane and disrupts mitochondrial function [627]. Residues 46 to 75 have been shown to be both necessary and sufficient for mitochondrial targeting and localization [628].

# Chapter 40. PB2

The main function of PB2 is to bind cellular caps, as described above. Additional work has shown that mutations in the N-terminal region of PB2 affect viral replication but not transcription [536]. Additionally, Hsp90 interacts with the PB2 subunit and has been shown to act as a stimulatory host factor involved in RNA synthesis [537]. Lastly, the sequence of PB2 may influence the range of temperature in which the polymerase will successfully function. This is of importance since human influenza replicates in the upper respiratory tract at a temperature of about 33°C, whereas avian influenza replicates in the intestinal tract at a temperature of approximately 41°C. Many avian viruses replicate poorly at the colder temperature of the human respiratory tract, which provides a thermal barrier to cross-species infection. Much of this cold sensitivity has been attributed to residue 627 of PB2 [629].

A recent study has identified two regions of PB2 that bind NP and also a novel PB1 binding site [531]. The NP and PB1 binding sites show considerable overlap, PB1 being able to out-compete NP for binding at high concentrations. Such competitive binding may contribute to the postulated regulatory effect of NP on transcription and

replication.

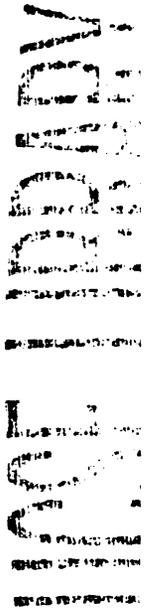
# Chapter 41. PA

In 1989, PA was cloned and sequenced [630]. The PA protein is encoded by segment 3, which is 2,233 nucleotides in length (in strain A/WSN/33). The full-length protein is 716 amino acids long, and has a calculated molecular weight of 82,530 Daltons. The protein derives its name (protein acidic) from its having more acidic residues than basic residues (114 and 109, respectively), so that at pH 6.5 its charge is -13.5, and its isoelectric point at pH 5.41.

There are five major lineages of PA including human, swine, equine, gull, and all other birds [631]. In 1989, a comparison of the predicted amino acid sequences of these lineages revealed ten amino acid residues in the PA proteins that distinguish all avian and H3N2 swine viruses from human viruses. However, the current database includes human viruses that have residues that were previously thought to be solely of avian origin.

Within each type of PA, the length of the protein seems remarkably conserved. As of the year 2000, the influenza database indicated that 27 of 27 type-A isolates were 716 residues in length, 24 of 25 type-B isolates were 726 residues in length, and the two completely sequenced type-C isolates were 708 and 709 in length, respectively.

By BLAST, PA shares little similarity to other proteins. The most similar proteins include yellow jacket venom, 28% identity and 44% similarity between residues 324-429, and rat liver dipeptidyl peptidase III, 30% identical and 51% similar between residues 231-303. Mutations within these spans M4 (M249A, S250A), M5 (E351A, E352A), M6 (K362A, T363A), and mutations made by Fodor *et al.* [632] , W406A, E410A, and L425A had little effect on the virus, suggesting PA does not share functional similarity to either venom or proteases. In addition, PA has "weak relatedness" to several nucleotide-



binding proteins including various DNA and RNA polymerases, E-coli translation initiation factor IF-2, and a sodium channel protein [630].

Analysis of PA in MotifFinder [633] indicates that PA has no motifs recognized by either the Prosite Pattern or Prosite Profile Databases. However, MotifFinder also identifies blocks. Blocks are multi-aligned ungapped segments corresponding to the most highly conserved regions of related protein groups. Each block has a position-specific matrix that can be compared to any query sequence to determine relatedness. The probability of a relationship is indicated by a score, and scores over 1000 are considered to be of interest. PA has 55 blocks of interest. Some of these blocks are found in the following: ribosomal proteins, recA bacterial DNA recombination protein, microtubule associated tau protein, clathrin adaptor complexes medium chain, hsp90 proteins, tubulin family proteins, and dynein light chain proteins. MotifFinder also lists PA as having a PRINT of the rhodopsin-like GPCR superfamily signature.

Although no structure for PA has been determined, computer programs can mathematically fold the protein into a hypothetical structure. The 3D-pssm Fold Recognition Server [634] compares this theoretical fold to other proteins of known structure and function. The superfamilies most related to the theoretical shape of PA include the ribosomal protein S7 and the fen-1 protein. Fen-1 protein is an endonuclease/ exonuclease that interacts with PCNA and functions in base excision repair and has been shown to be necessary for Okazaki fragment processing. From pure structure/sequence analysis, PA seems to be most related to proteins involved in translation and nucleic acid repair.

Early work on influenza from the mid 1970's to the early 1980's utilized temperature sensitive mutants to provide insight to the functions of the influenza proteins [522, 524, 635-641]. In 1975, Sugiura *et. al.*, used 5-fluorouracil to mutate the segments and isolated a temperature sesistive mutant, *ts*53 [635]. Based upon complementation mixed infections, it was surmised that the *ts*53 mutation resided in the third segment (the

PA segment) [642]. In 2005, this inference was proven correct by sequencing [643].

The *ts*53 mutant exhibits a reduced level of vRNA synthesis at the non-permissive temperature, which suggests that PA plays a role in vRNA synthesis (replication) [522]. Subsequent work has shown the *ts* phenotype to be caused by a L226P mutation, and reverse genetics has confirmed this mutation is sufficient to generate the *ts* phenotype [643]. Analysis of transcription and replication activities of this mutant confirmed that *ts*53 synthesizes mRNA at the non-permissive temperature (39.5° C), and is less able to synthesize vRNA. However, the defect in vRNA synthesis may be due to a decreased ability to synthesize cRNA [643]. Additional work on the *ts*53 mutant has suggested that PA is involved in assembling functional polymerase complexes from inactive intermediates [643].

In a natural influenza infection, PA localizes completely to the nucleus within several hours after infection. The localization of PA has been studied utilizing SV40-recombinant viruses that express PA [644]. When COS-1 cells were infected with a recombinant virus that expresses PA, the localization of its accumulation was found to be variable. In some cells, PA accumulated in the nucleus, whereas in others, PA accumulated in the cytoplasm or was distributed throughout the cell. Co-infection of SV40-PA with SV40-PB1 or SV40-NS1 restored nuclear localization, suggesting that these proteins may be involved in the nuclear targeting or nuclear retention of PA. Despite the apparent requirement of PB1 or NS1 to facilitate nuclear localization, work has been carried out to determine if PA itself has a nuclear localization signal. The nuclear localization signal (NLS) of PA has been identified and is comprised of two independent regions [645]. Region I spans residues 124 - 139 and is similar to the nucleoplasmin NLS and region II is larger and spans residue 186 - 247. No consensus targeting sequence has been identified for region II. Interestingly, small mutations in either region prevented nuclear accumulation, whereas large deletions of either region, but not both, permitted nuclear accumulation, suggesting that either region alone can

serve as an NLS. Of particular interest is that point mutant T154A, which maps between these regions, completely eliminates nuclear transport.

As mentioned earlier, PA contacts PB1, which binds the vRNA, but it is not known whether PA itself functionally interacts with any of the RNA species involved in the viral lifecycle. However, PA has been shown to UV crosslink to vRNA, suggesting that it is at least in close proximity to the genome [441, 597]. Although PA has not been definitively shown to bind RNA, a putative nucleotide-binding motif exists between amino acids 502-509 [630].

Since 1995, 17 manuscripts have been published on work related to PA, which has begun to shed light on this protein. Initial work by Sanz-Ezquerro *et al.*, indicated that PA was capable of inducing general proteolysis [646]. This finding was based from data generated from an infection/transfection system in which COS-1 cells are first infected with a recombinant vaccinia virus that expresses the bacteriophage T7 RNA polymerase, and then transfected with a vector that expresses PA under the control of a T7 promoter. In this system, PA's ability to induce general proteolysis was mapped to the N-terminal 247 residues of the protein [647]. This region of PA contains the NLS sequences, which have been shown to be indispensable for the induction of proteolysis.

Additional work on PA and its ability to induce proteolysis found mutants with different phenotypes. PA mutants E154G and T157A accumulate in transfected cells and do not induce general proteolysis [647, 648]. Other mutants Y157E and Y162A were impaired for induction of proteolysis, and were unable to replicate [648]. These negative data suggest that replication may be dependent on the ability to induce proteolysis.

Another group investigated the induction of proteolysis in a system independent of vaccinia and agreed that induction of proteolysis appeared to be a general characteristic of PA proteins from type A influenza strains [649]. The level of proteolysis appears to be variable and dependent on the source of PA. Avian PA generally induces more proteolysis than human PA. Also, the level of proteolysis is affected by the host cell

type in which is it expressed [649].

In the course of the experiments described in this thesis using PA from A/WSN/33, A/Victoria, and A/PR8 in 293T cells, MDCK cells, and rabbit reticulocyte lysates; no obvious signs of proteolysis was observed (Chapter 44, L).

The relationship between PA-induced proteolysis and replication has been examined. Naffakh *et. al.*, created mutant C241A that was found to induce proteolysis to 35%, but expressed a CAT reporter to wild-type levels [649]. In addition, proteolysis was shown to be severely defective at 33°C compared to 37°C, but the level of polymerase activity was not affected, supporting the claim that proteolysis induction is independent of replication, and casts doubt on its biological relevance.

Other work on PA has focused on the potential phosphorylation sites of the protein. The sequence of the Victoria strain suggests the following potential sites exist: 12 CKII motifs, 13 PKC motifs, 2 cAMP-dependent protein kinase motifs, and 2 tyrosine phosphorylation motifs. When the phosphorylation status of PA was examined in infected cells, PA was found to be phosphorylated at both serine and threonine residues, but not tyrosine residues [650]. When examined *in vitro*, PA can be phosphorylated by casein kinase II. Five of the potential CKII phosphorylation sites in the N-terminal portion of the PA have been mutated and T157 has been shown to be phosphorylated *in vivo*. The T157 residue has also been shown to affect vRNA to cRNA synthesis but not transcription [535, 648].

PA has proven to be very sensitive to mutation. In one study, all deletion mutants tested for polymerase activity were found to be inactive [473]. Interestingly, PAS509, bound to PB1 with wild-type affinity, but was inactive for polymerase activity. This mutant was shown in a competition experiment to be a dominant negative [590].

By yeast two-hybrid, PA has been shown to bind cellular hCLE, which has homology to a family of transcription activators [651]. Whether this cellular protein is functionally relevant for the viral lifecycle, has not yet been determined. Recently,

Fodor *et al.*, systemically made single alanine mutations within conserved regions of the C-terminal half of PA [632]. Mutant H510A was capable of replication, but unable to make mRNA due to a defect in the endonuclease activity of the polymerase. Fodor *et al.* also mutated serine 624, to investigate whether this residue was important for the viral lifecycle since Hara *et al.*, had shown that PA is a serine protease and serine 624 was the catalytic residue [652]. However, no significant defect in viral replication was observed when this serine was substitute for an alanine [632].

Ser 624 has been shown not to be essential for growth in cells and mice, but required for maximal viral growth [653]. Another study demonstrated that both virion purified M1 and hexa-histidine-tagged M1 bound to PA in a salt-dependent manner, suggesting an electrostatic interaction. In addition, M1 binding was shown to inhibit the ability of PA to cleave a Suc-Leu-Leu-Val-Tyr-MCA substrate [654].

In addition, Fodor *et al.*, determined that mutation R638A results in an attenuated virus that produces pin-sized plaques. Further study determined that this mutant produced and packaged defective interfering genomes [655]. Mutation C453R compensated for this phenotype suggesting distant portions of the protein comprise a domain that is necessary for the stability of the polymerase on a template.

In infected cells, antisera against PA have identified three different isoforms. In addition to full-length protein of 87 kDa, two smaller isoforms of 62 kDa and 60 kDa exist [625]. Limited proteolysis peptide mapping confirmed the identity of these isoforms and pulse-chase data suggests the smaller isoforms may be translated independently of their full-length counterparts. The smaller isoforms were not detected in purified virions, but were found associated with vRNP in infected cells. Similar observations regarding different isoforms of the polymerase were observed by Fodor *et. al.*, [597]. Whether these isoforms play a functional role in the biology of the virus is unclear.

PA is UV cross-linked to the 5' vRNA molecule [441]. PA has a chaperone effect on PB1 or increases the ability of PB1 to bind 5' vRNA [443]. A suppressor mutation of a

PB2 temperature-sensitive mutant was found in the PA gene [656].

# Chapter 42. Reconstitution Systems

## A. *In Vitro* Reconstition Systems

Valuable data on the functionality of the influenza polymerase and the description of its promoter has been generated from procedures that allow the *in vitro* reconstitution of a virus-like ribonucleoprotein complex (vRNP) [440, 457, 657]. Such reconstituted vRNP are often made with a reporter that facilitates analysis. A frequently used reporter is chloramphenicol acetyltransferase (CAT) that is cloned in the anti-sense orientation with an influenza UTR, such as NA or NS. Such reporters can be *in vitro* transcribed by T3 or T7 RNA polymerase and added to purified NP and 3P complexes. These protein complexes can be expressed and purified from virions, transfected cell lines, or cells infected with viruses such as vaccinia or SV40 that have been engineered to express influenza genes. If the 3P has been isolated from virions it is necessary to incubate the 3P with micrococcal nuclease to digest away the endogenous viral genome. However, micrococcal nuclease digestion generally does not digest away the native 5' UTR, which makes such polymerase preparations difficult to use for studying the promoter. The "*in vitro*" reconstituted vRNP can then be assayed for the production of reporter in a rabbit reticulocyte lysate or a buffered system containing the necessary rNTPs plus a source of caps or dinucleotide ApG to prime transcription.

## B. *In Vivo* Reconstitution Systems

Alternatively, vRNPs can be reconstituted *in vivo*. The following systems have furthered the ability to dissect the cis-acting RNA regions involved in transcription and replication and have also have proved helpful for identifying the viral proteins

required for RNA synthesis. The *in vivo* reconstitution systems have evolved over the years. Early attempts included assembling a vRNP *in vitro* as described above for transfection into cells [457]. Later, the vRNP were assembled *in vivo* by a variety of methods. Initially, the reporter could either be transcribed *in vitro* and transfected in as either naked or NP encapsidated RNA, or the reporter could be transcribed *in vivo* [657]. *In vivo* transcription of the reporter was accomplished by bacterial T3 or T7 RNA polymerase that had been provided by transfection or infection with an engineered virus such as vaccinia or SV40. The reporter was then assembled inside cells into a vRNP by providing the polymerase proteins and NP *in trans* using either a helper virus, recombinant vaccinia, or recombinant SV40 virus that was engineered to express the necessary viral proteins [296, 473, 543, 558]. These systems demonstrated that an *in vitro* transcribed recombinant CAT RNA can be introduced into a cell with additional influenza components, and that this segment can then be amplified, expressed, and packaged into virus particles [296]. These data indicate that the noncoding termini of the influenza segments are at least minimally sufficient to provide the signals for RNA transcription, RNA replication, and packaging of the RNA into particles.

Recently, systems have been created that rely entirely on plasmids for the generation of vRNPs as well as the generation of viable virus. These systems are called 'reverse genetics systems' since they allow for easy site-directed mutagenesis of influenza genes that are encoded on plasmids; these plasmids can then be used to generate virus-like particles, where the effects of mutations can quickly be determined.

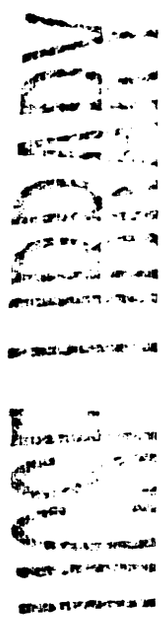
The first generation of the *in vivo* influenza reverse genetics system allowed for the synthesis of a flu chromosome by utilizing a truncated human polymerase I promoter and a delta hepatitis C ribozyme sequence to generate the desired 3' end by autocatalytic cleavage [658-660]. The addition of polymerase II driven plasmids that express viral PB2, PB1, PA, and NP allowed for complete plasmid-based transfection to generate vRNP *in vivo*, which can be used to study cis- and trans-acting signals of

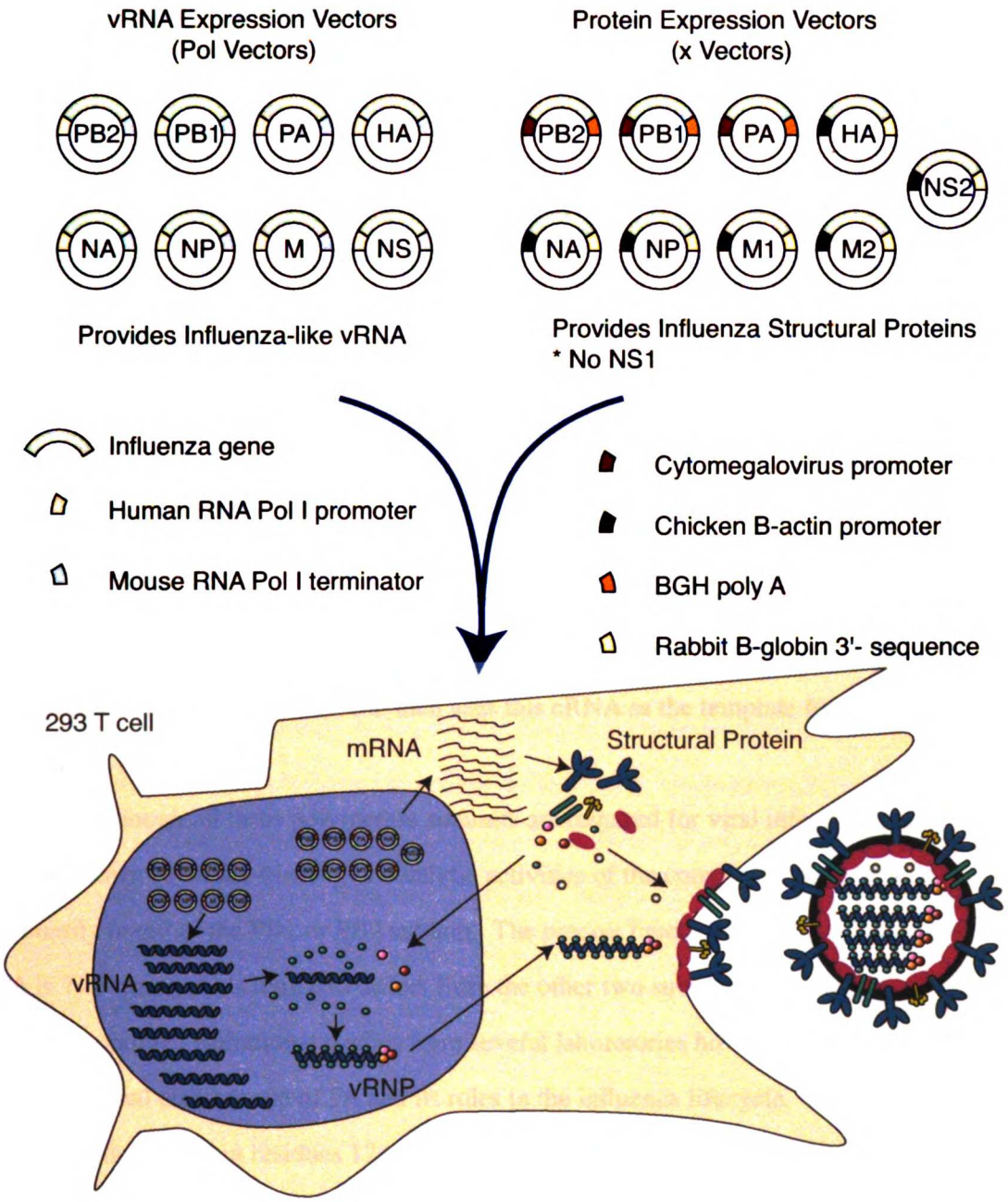
viral transcription and replication. In addition, these *in vivo* generated vRNPs could be incorporated into a virus by infecting with a helper virus.

The establishment of a plasmid that would drive the expression of a flu-like chromosome, which is non-capped and non-polyadenylated, led to a 17-plasmid system (Intro. Fig. 8) and an 8-plasmid system that enabled the generation of a virus, without the using recombinant SV40 or vaccinia viruses, or helper viruses [661, 662]. These systems utilize a combination of the following promoters and terminators: human RNA polymerase I promoter, mouse RNA polymerase I terminator, cytomegalovirus promoter, chicken β-actin promoter, and a bovine polyadenylation signal. For the 17-plasmid system, in order to get infectious virions, one 293T cell must receive the eight vRNA expression vectors and protein expression vectors for PB2, PB1, PA, and NP. Protein expression vectors for the other structural proteins, including HA, NA, M1, M2, and NS2 are not essential, but do improve the viral titer. In contrast, all eight plasmids are required to generate infectious particles for the 8-plasmid system, which has each of the eight chromosomes on plasmids with opposing polymerase promoters.

The ability to create viable virus-like particles (VLP) from a plasmid based transfection system has greatly facilitated the study of influenza with minimal exogenous factors. A reverse genetics system has also been created in African green monkey kidney (Vero) cells that can generate high-yielding human and animal virus-like particles that may help pave the way for a cell-based method for vaccine generation [663, 664].

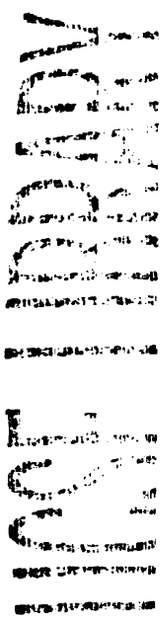
The reverse genetics system used in this study utilizes human RNA polymerase I promoters, mouse RNA polymerase I terminators, cytomegalovirus promoters, and chicken β-actin promoters. Each of these promoters and terminators can be recognized by mammalian cellular machinery.






**Intro. Fig.8 17 Plasmid Transfection**

A reverse genetics system that utilizes 17 plasmids. Optimally, seventeen plasmids are transfected into 293T cells and 48 hours later, the supernatant is harvested and contains approximately one million plaque forming units per milliliter.



# Chapter 43. Published Work



## A. Introduction

The influenza A virus encodes a heterotrimeric RNA-dependent RNA polymerase whose subunits are designated PB1, PB2, and PA. Localized in the nuclei of infected cells, this trimeric complex is essential for transcribing all virally encoded mRNAs and for replicating the eight negative-sense single-stranded RNA segments (vRNAs) that make up the viral genome. To carry out transcription, the polymerase endonucleolytically cleaves short, 5'-capped oligonucleotides from cellular mRNAs to be used as primers, which are then elongated using all but a few nucleotides from any given vRNA as a template. Replication, by contrast, is achieved through a primer-independent, two-step process in which the polymerase first copies a vRNA template to produce a full-length complementary RNA (cRNA), and then uses this cRNA as the template for new vRNA synthesis.

Although all three polymerase subunits are required for viral infectivity, each of the known substrate-binding or catalytic activities of the complex appears to reside primarily in either the PB1 or PB2 subunit. The precise functions of PA are less certain. PA is 716 amino acids long and differs from the other two subunits in having an overall negative charge. Mutational studies from several laboratories have begun to delineate the functional architecture of PA and its roles in the influenza lifecycle. Sequences near its N terminus (within residues 124-246) have been found to target PA into nuclei [645], whereas C-terminal residues 668-692 mediate its incorporation into the polymerase trimer by interacting specifically with PB1 [584, 586]. Early studies of a temperature-sensitive mutation, later mapped to residue 226, indicated that PA might be specifically required for replication but not for transcription [522, 643]. Mutation of residue 510, on the other hand, impairs transcription by decreasing cap-endonuclease activity, and point substitutions at a variety of other locations can diminish or eliminate synthesis of all three

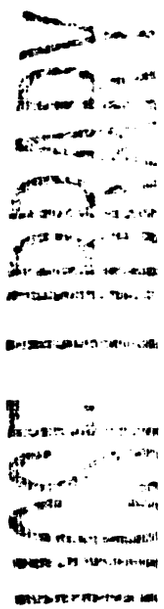
classes of viral RNA [632]. Mutating residue 638 has been found to promote synthesis and packaging of truncated, defective-interfering RNAs [655], perhaps reflecting impaired stability or processivity of the mutant polymerase complex. Collectively, these and other results suggest that PA may modulate or regulate diverse aspects of polymerase function. In addition, some reports have suggested that PA either possesses intrinsic proteolytic activity [652] or can induce generalized activation of cellular proteases [535, 647, 648], but contradictory findings have been reported [632, 649] and the possible relationship of proteolysis to polymerase function or viral growth remains unclear.

Here we report additional systematic mutagenesis aimed at better characterizing the roles of PA in influenza biology. We have constructed 16 novel PA mutants containing paired alanine substitutions that target conserved, polar residues along the entire length of the protein. Each of these mutants has been characterized with respect to its ability to support viral infectivity and the expression of viral RNAs and protein. Our results confirm and extend earlier work in indicating that PA mutations can yield a spectrum of effects on polymerase function. Additionally, however, we identify a novel mutation in PA that supports robust expression of all three classes of influenza RNAs, yet completely abolishes production of infectious viral particles. Our results are the first to suggest that PA plays an indispensable role in influenza virion assembly that is independent of RNA polymerase activity.

## B. Materials and Methods

**Plasmid Construction and Mutagenesis.**

The 17-plasmid influenza reconstitution system of Kawaoka and colleagues was used as described [661], except that the PA protein-expression vector was modified to express coding sequences from the A/WSN/33 strain, identical to those in the PA vRNA vector. The substitution mutations listed in Pub. Table 1 were first introduced into the PA vRNA vector through PCR-based mutagenesis, then subcloned into the PA protein vector



using unique ClaI and ApaI restriction-enzyme sites and verified by sequencing. This strategy inserted a truncated human RNA polymerase I promoter at the C-terminal end of every mutant or wild-type PA protein vector used in this study.

To create luciferase-encoding reporters, the luciferase gene from pGL3 (Promega) was amplified by polymerase chain reaction (PCR) using primers that added the 3' or 5' untranslated region from the neuraminidase (NA) vRNA or cRNA onto each end, flanked by BsmBI sites; the products were then inserted into the BsmBI site of the vector pHH21 [661, 662]. The PB2-GFP reporter vector, encoding a fusion of 151 N-terminal residues from PB2 to the N terminus of green fluorescent protein (GFP) has been described [665]. The NS-GFP reporter, which encoded 79 N-terminal residues of influenza NS1 protein fused to the N terminus of GFP, was created by replacing a unique NcoI-MfeI fragment from the coding region in the NS protein-expression vector with a GFP cassette.

**Cells and Antibodies.**

293T and MDCK cells were maintained as described [297, 661]. Anti-PA polyclonal antibodies were obtained by immunizing rabbits against a recombinant protein comprising 204 N-terminal residues of PA from strain WSN/33 with a 6xHIS C-terminal tag. Other primary antibodies included mouse anti-M1 (Serotec), mouse anti-nucleoprotein (-NP) (Serotec), rabbit anti-GFP, mouse anti-mitochondria (Ab-2, Lab Vision), and rabbit anti-histone H3 (Novus Biologicals). Secondary antibodies used for Western blot were goat anti-mouse IgG and donkey anti-rabbit IgG, both peroxidase conjugated (ImmunoPure®, Pierce Endogen).

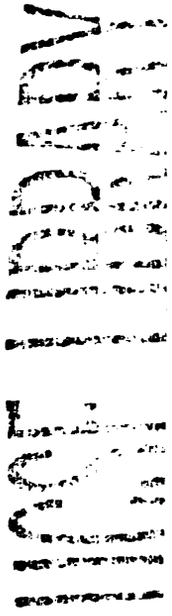
**Transfections and Expression Assays.**

293T cells were transfected transiently on 35-mm plates using TransIT-LT1 (Mirus). For VLP production by 17-plasmid transfection, cells were transfected as described [661], with vector additions or substitutions as indicated. To assay polymerase

function by 5-plasmid transfection, cells were transfected with 1 μg of each of an indicated reporter and of the protein-expression vectors encoding PB2, PB1, PA, and NP. A β-galactosidase (β-gal) expression plasmid pCH110 (Pharmacia) was included in some studies as an internal control for normalizing transfection efficiency. At indicated times, cells were harvested either for luciferase assay or for isolation of total cellular RNA using TRIzol (Invitrogen). Luciferase assay was conducted according to manufacturer's protocol (Promega) using a Turner Designs 20/20 luminometer, after normalizing to β-gal activity.

**Primer-Extension Assays.**

Primers were 32P end-labeled with T4 polynucleotide kinase (Invitrogen) and designed to bind near the 5' ends of RNA species. First-strand cDNA synthesis was carried out as described by Fodor et al. [632] and the products were analyzed by electrophoresis on a 6% PAGE-urea gel. The predicted sizes of the 32P-labeled cDNA products are listed below, in parentheses, after each primer sequence.
When detecting RNA generated from pol NA-Luc reporters the following primers were used:
vLuc75 5'-GAGAGATCCTCATAAAGGC-3' (vRNA 75 nts) and
cLuc84 5'-GCGGTTCCATCTTCCAGCGG-3' (cRNA 84 nts and mRNA 93-96 nts).
When detecting RNA generated from the pol NS 238-GFP-374 reporter, the following primers were used:
vNS145 5'-TGAGACACAGACTGAAGATAACAGA-3' (vRNA 145 nts) and
cNS105 5'-GTTCTTGGTCTGCAACTCTTTTGCG-3' (cRNA 105 nts and mRNA 114-118 nts). To detect native NA viral RNA species, the following primers were used:
vNA155 5'-GGGGCTACCTGAGGAGGACGCA-3' (vRNA 155 nts) and
cNA125 5'-GGCTAATCCATATTGAGATTATATT-3' (cRNA 125 nts and mRNA 134-138



nts).

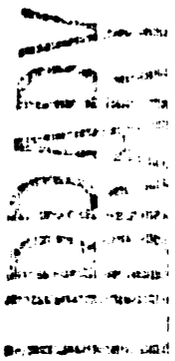
**Isolation of Virus-Like Particles (VLPs) and Infection of MDCK cells.**

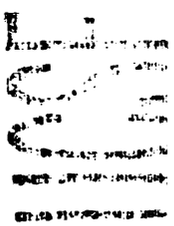
Transfection supernatants were clarified of debris by centrifugation at 2000 rpm for 10 min, then further purified by ultracentrifugation at 27,700 rpm for 3 h through a 20% sucrose cushion. Pellets were resuspended in phosphate-buffered saline (PBS) and assayed for viral M1 protein by Western blot. Aliquots containing equivalent amounts of M1 were then used to infect MDCK cells; the approximate multiplicity-of-infection for wild-type VLPs was 2.2. Plaque-forming titer was estimated by serial 10-fold dilutions on MDCK monolayers as a measure of infectivity.

**Quantitative RT-PCR.**

First-strand cDNA was synthesized from RNA samples using Superscript™ III RNase H- Reverse Transcriptase (Invitrogen) and then quantified with the specific forward (F) and reverse (R) primer pairs listed below. Quantitative PCR was performed using an ABI Prism® 7700 Sequence Detector (Applied Biosystems).

The primer pairs and probes used to amplify and quantify viral sequences included: PB2-F 5'-ACGTGGTGTTGGTAATGAAACG-3', PB2-R 5'-TGGCCATCCGAATTCTTTTG-3', cPB2-FAM 6FAM-CGGAACTCTAGCAT ACTTACTGACAGCCAGACA-TAMRA; PB1-F 5'-GAATCTGGAAGGATAA AGAAAGAGGA-3', PB1-R 5'-CACTATTTTTGCCGTCTGAGCTC-3', cPB1-VIC VIC-TTCACTGAGATCATGAAGATCTGTTCCACCA-TAMRA; PA-F 5'-TTAATGATCCCTGGGTTTTGCT-3', PA-R 5'-TTGCCACAACTATCTCAATGCAT-3', cPA-FAM 6FAM-AATGCTTCTTGGTTCAACTCCTTCCTCACA-TAMRA; HA-F 5'-TCAGATTCTGGCGATCTACTCAACT-3', HA-R 5'-TAGAACACATCCAGAAACT GATTGC-3', cHA-VIC VIC-TCACTGGTGCTTTTGGTCTCCCTGG-TAMRA; NA-F 5'-TGTCAATGGTGAACGGCAACT-3', NA-R 5'-TCTTTTTGTGGTGTGAATAGTGA





TACTG-3', vNA-FAM 6FAM-AGCACCGTCTGGCCAAGACCAATC-TAMRA; NP-F 5'-CGGACGAAAAGGCAACGA-3', NP-R 5'-CATTGTCTCCGAAGAAATAAGATCCT -3', cNP-VIC VIC-CGATCGTGCCCTCCTTTGACATGAGT-TAMRA; M-F 5'-CTATG TTGACAAAATGACCATCGTC-3', M-R 5'-TGCCAGAGTCTATGAGGGAAGAAT-3', vM-FAM 6FAM-CCACAGCATTCTGCTGTTCCTTTCGA-TAMRA; NS-F 5'-CAATA GTTGTAAGGCTTGCATAAATGTT-3', NS-R 5'-GAAGAAATAAGATGGTTGATTGA AGAAG-3', vNS-VIC VIC-TTGCTCAAAACTATTCTCTGTTATCTTCAGTCTGTGTC TC-TAMRA (Invitrogen). Viral RNAs in cellular extracts were assayed after normalizing for expression of the cellular 5.8S ribosomal RNA, which was detected using 5.8S-F 5'-TAGCCCCGGGAGGAACC-3', 5.8S-R 5'-AGCGCTAGCTGCGAGAATTAA-3', and v5.8S-VIC VIC-TGTCGATGATCAATGTGTCCTGCAATTCAC-TAMRA.

**FACS Analysis.**

Two-color flow-cytometric analysis of transfected 293T cells or infected MDCK cells was conducted as described by Liang et al. [665]. Sorting of cells was performed using a MoFlo instrument (Dako Cytomation).

**Isolation of Nuclear and Cytoplasmic Fractions.**

293T cells were lysed for 5 minutes on ice in a hypotonic sucrose buffer (320 mM sucrose, 5 mM MgCl2 10 mM HEPES, pH 7.4) supplemented with 1% (v/v) Triton X-100. Nuclei were separated from cytoplasm by centrifugation at 2000 x g for 1 min, then were washed twice in the sucrose buffer minus Triton X-100 and resuspended in the sucrose buffer.

**Gradient Centrifugation.**

VLPs were filtered through 0.45-$\mu$m polyethersulfone membrane filters (Whatman) and then pelleted through a 30% glycerol cushion by centrifugation at 25,000

rpm for 3 hrs in an SW-41Ti rotor at 4 °C. Pellets were resuspended and layered over 30 - 50% continuous glycerol gradients and again centrifuged in the same rotor at 25,000 rpm for 2 hrs at 4 °C. Fractions were collected from the bottom, assayed for plaque-forming titer, and then concentrated by centrifugation through a 30% cushion as before. Pellets were resuspended in PBS.

**Agglutination of Chicken Erythrocytes.**

VLPs were incubated with 200 μL of glutaraldehyde-stabilized chicken red blood cells (Research Diagnostics, Inc.) with agitation for 1 hr at 4 °C. The cells were washed three times with Opti-MEM buffer supplemented with 0.3% (w/v) BSA and 0.01% (v/v) fetal bovine serum, then treated with 600 units of MCN for 1 hr at 37 °C. RNA was harvested from the samples with QIAamp Viral RNA mini kit (Qiagen) and assayed by quantitative RT-PCR and primer-extension assays.

## C. Results

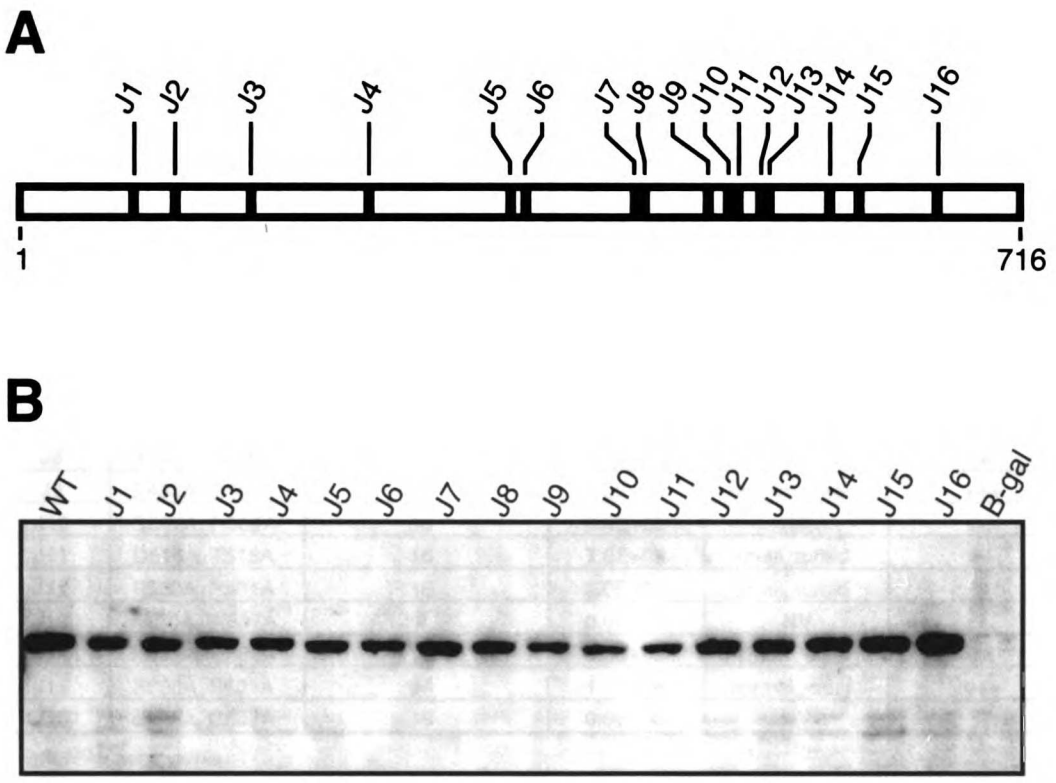
These studies utilized a reverse-genetic system [661] comprising 17 plasmid expression vectors that together encode the eight wild-type vRNAs and all essential viral proteins of influenza A. When transfected transiently into 293T cells, these 17 plasmids direct the assembly and release of infectious influenza particles that can subsequently be passaged indefinitely. In this report, we refer to the products of an initial transfection as virus-like particles (VLPs), whereas any particles that have been passaged one or more times are termed virions, regardless of their origin. The 17-plasmid system used here includes two vectors that encode the PA protein and PA vRNA, respectively, each derived from the A/WSN/33 strain. We created mutations, designated J1-J16, that each replaced two consecutive codons in the PA coding sequence with alanine codons; identical mutations were introduced into both the vRNA- and protein-expression vectors. These mutations, shown schematically in Pub. Fig. 1A and listed in Pub. Table 1, generally

targeted residues that were hydrophilic, likely to be exposed on the protein surface, and evolutionarily conserved.

The wild-type and mutant PA protein expression vectors, along with a β-galactosidase (β-gal) expression vector as internal control, were each transfected into 293T cells, which were then lysed 22 hr later and assayed for PA protein by Western blot. As shown in Pub. Fig. 1B, all 16 mutants were of the expected relative mobility and were expressed at steady-state levels approximating that of the wild-type protein when normalized to β-gal activity.

We initially surveyed the functional effects of these mutations in the 17-plasmid reconstitution system. In these first experiments, 293T cells were transfected with a mutant PA vRNA together with wild-type forms of all other essential viral components, including wild-type PA protein; thus, wild-type PA was expressed in the transfectants, but any resulting VLPs could transduce only the mutant vRNA. Supernatants were harvested 48 hr after transfection and were assayed for plaque-forming activity on MDCK cells. As summarized in Pub. Table 1, six of the mutants (J5, J6, J9, J11, J14, and J15) had titers above 105 pfu/ml, comparable to that seen with wild-type PA though with varying plaque morphologies. Mutants J2, J3, J7, J10, J13, and J16, by contrast, produced no plaques, while the four remaining mutants (J1, J4, J8, and J12) yielded markedly reduced but measurable titers (102-104 pfu/ml) and abnormally small plaques. In each instance where plaques were obtained, we harvested RNA from plaque-purified virus, amplified PA coding sequences by RT-PCR, and verified that the expected mutation was indeed present. Curiously, the J1 mutant reproducibly yielded plaques from the initial transfection supernatants but could not be plaque-purified.

To estimate the production and release of viral structural proteins, supernatants from the 17-plasmid transfections were also assayed for their ability to agglutinate chicken erythrocytes. The hemagglutination titers of the mutants, listed in Pub. Table 1, were generally 2 to 4 fold lower than that of the wild-type, and tended to be lowest




**Pub. Fig.1 Mutations in PA**

Structure and expression of PA mutants. (A) Schematic depiction of mutations studied here, which were introduced into the PA protein of strain A/WSN/33. (B) Western blot detection of wild-type (WT) and mutant PA proteins in lysates of 293T cells transfected with the PA expression vector and a β-gal control vector. Samples were normalized by β-gal activity and analyzed using a polyclonal anti-PA antibody. β-gal = cells transfected with the control vector alone.

| | Mutation | Hemagglutination Titer | Plaque Titer | Plaque Morphology [a] | Relative Infectivity [b] |
|---|---|---|---|---|---|
| WT | --------- | 64 | 1.0E+07 | normal, clear | +++ |
| J1 | G81A, R82A | 16 | 2.2E+03 | small, turbid | + |
| J2 | D111A, Y112A | 16 | 0.0E+00 | NV | - |
| J3 | E165A, E166A | 16 | 0.0E+00 | NV | - |
| J4 | M249A, S250A | 16 | 5.0E+04 | small, clear | + |
| J5 | E351A, E352A | 32 | 1.0E+07 | normal, clear | +++ |
| J6 | K362A, T363A | 16 | 8.0E+05 | normal, clear | ++ |
| J7 | R442A, R443A | 16 | 0.0E+00 | NV | - |
| J8 | Y445A, F446A | 16 | 6.5E+02 | small, clear | + |
| J9 | E493A, G494A | 16 | 5.0E+06 | normal, clear | +++ |
| J10 | G507A, R508A | 16 | 0.0E+00 | NV | - |
| J11 | D514A, T515A | 16 | 7.0E+05 | small, turbid | ++ |
| J12 | P530A, R531A | 16 | 3.0E+04 | small, turbid | + |
| J13 | K536A, W537A | 8 | 0.0E+00 | NV | - |
| J14 | M579A, E580A | 16 | 4.0E+05 | small , clear | ++ |
| J15 | S600A, S601A | 64 | 1.0E+06 | normal, clear | +++ |
| J16 | E656A, G657A | 16 | 0.0E+00 | NV | - |

[a] NV = none visible.

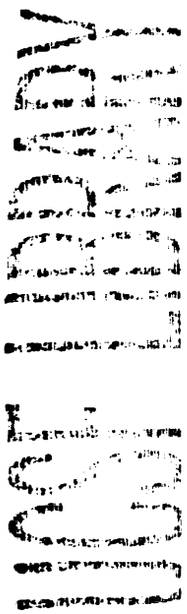
[b] plaque-forming titers expressed as +++ equals > $10^6$ pfu/ml, ++ equals $10^5$-$10^6$ pfu/ml, + equals < $10^5$ pfu/ml, or - equals no plaques visible.

**Pub. Table.1 Viability Table**

Properties of influenza virus-like particles containing wild type (WT) or mutant PA proteins.

for mutants that had very low plaque-forming activity. These modestly reduced hemagglutination titers likely reflect an inability of the most severely defective mutant polymerases to amplify viral protein expression by transcribing new viral mRNAs in the transfected cells (see below). Nevertheless, the observed differences in hemagglutination titer were not sufficient to account for the effects on pfu titer, which ranged over at least 7 orders of magnitude.

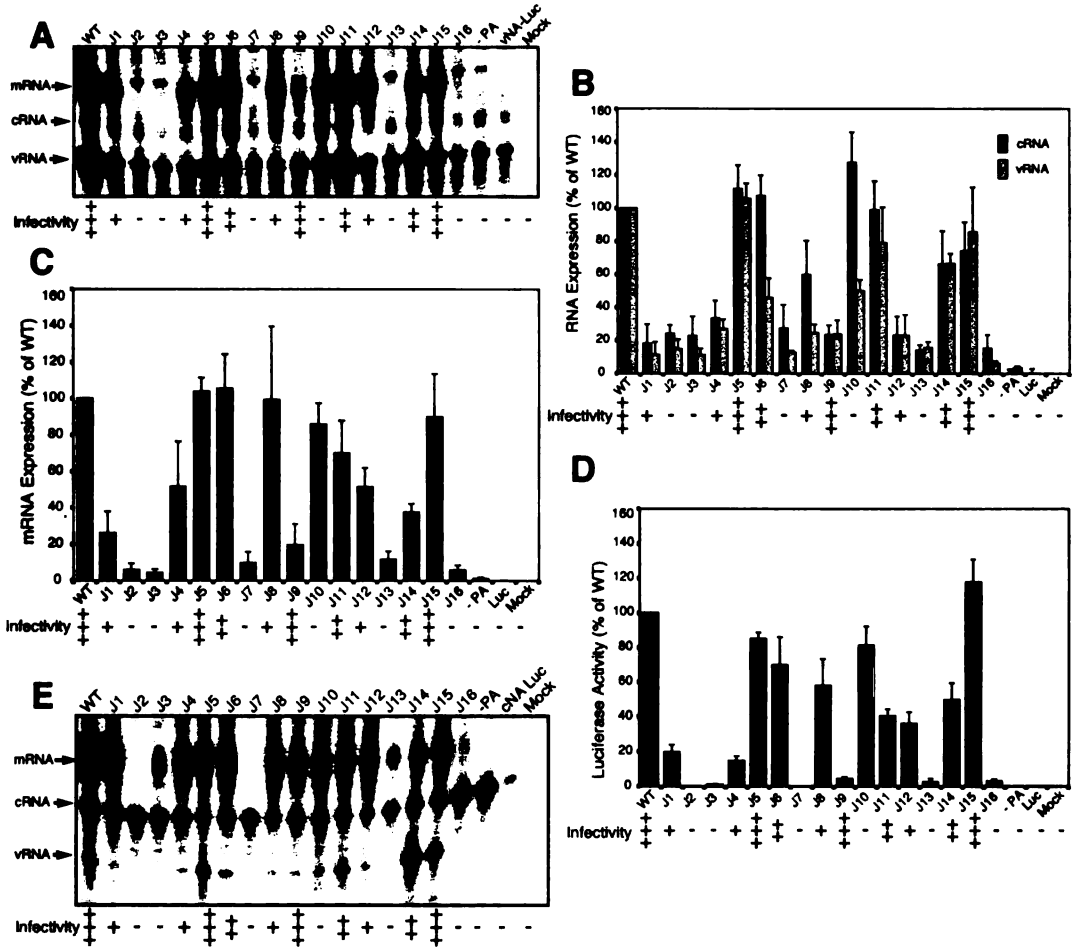
We next evaluated the ability of polymerases containing the various PA mutants to support viral RNA expression in cells. In these experiments, each transfection included only 5 viral expression plasmids. One was a reporter encoding a modified influenza neuraminidase (NA) vRNA in which a luciferase cassette (in negative sense) replaced the native NA coding sequences. The other four directed expression of the viral PA, PB1, PB2, and nucleoprotein (NP) proteins, respectively, which together are known to be sufficient to support both replication and transcription from a vRNA template [457]. We transfected 293T cells with those 5 plasmids, along with the β-gal internal control vector, harvested RNA 36 hr later, and quantified reporter-specific mRNA, cRNA, and vRNA sequences simultaneously using a primer-extension assay. Representative results are depicted in Pub. Fig. 2A, and quantitative data from triplicate experiments are summarized for the individual RNA species in Pub. Figs. 2B and 2C. As expected, transfecting the reporter either alone (Luc), or in combination with only the PB1, PB2, and NP plasmids (-PA) revealed low-level background expression of reporter-derived vRNA that was undetectable in sham-treated cells (Mock). None of the latter control transfections yielded appreciable amounts of reporter cRNA or mRNA, however, confirming that PA is required for polymerase activity in this assay. By contrast, inclusion of the wild-type PA vector (WT) led to the synthesis of all three reporter-derived RNA species, with vRNA and mRNA predominating, indicating the formation of a catalytically active polymerase. Through similar transfections in which various mutants replaced wild-type PA, we found that five mutants (J2, J3, J7, J13, and J16) yielded



nonfunctional polymerases (Pub. Fig. 2A-C), a finding that accords with their complete inability to support viral infectivity (Pub. Table 1). Not surprisingly, five other mutants (J5, J6, J11, J14, and J15) that had previously demonstrated moderate or high infectivity each expressed at least 50% of the wild-type levels of vRNA, cRNA, and mRNA. Of note, J9 appeared less active, generating levels of all three RNA classes that were only 20-30% of wild-type, though it had yielded high-titer plaque-forming VLPs (Pub. Table 1). This implies that even mutants which function comparatively poorly in this 5-plasmid assay may be compatible with full infectivity.

To verify that the mRNAs produced by these mutants were functional, extracts of cells from the 5-plasmid transfections were also tested for luciferase activity using a luminescence assay (Pub. Fig. 2D). Comparison with Pub. Figure 2C reveals that luciferase activity roughly corresponded to the level of reporter mRNA expressed by each mutant, implying that these mRNAs were biologically active. As a further test of polymerase integrity, we also created a positive-sense (cRNA) form of the NA-based luciferase reporter and tested the ability of each mutant to act on these cRNAs as substrates, using the 5-plasmid assay. As shown in Pub. Figure 2E, only modest steady-state yields of vRNA were obtained, generally from the highest-titer mutants (c.f. Pub. Figure 2A); nevertheless, the ability of mutants J1, J4-J6, J8-J12, J14, and J15 to produce abundant mRNA under these conditions implied production of a functional vRNA intermediate.

The most remarkable phenotype was that of J10, which supported robust expression of all three classes of reporter RNA (Pub. Fig. 2) yet consistently failed to produce plaque-forming virus (Pub. Table 1). For this mutant, vRNA, cRNA, and mRNA expression were 50±7%, 128±18%, and 86±12%, respectively, of the wild-type levels in the 5-plasmid luciferase vRNA reporter assay (Pub. Figure 2B). Indeed, the expression levels of all three RNAs by J10 equaled or exceeded those obtained with other mutants (J4, J6, J8, J9, and J12) that produced infectious VLPs (Pub. Figs. 2B-2D). Uniquely




## Pub. Fig.2 Activity of Mutants

Reporter RNA expression by mutant influenza polymerase in cells. 293T cells underwent 5-plasmid transfections that included either the wild-type (WT) PA protein vector or the indicated mutants, along with a luciferase reporter vector representing either the vRNA (panels A-D) or cRNA (panel E) product of the influenza NA gene. Total RNA was harvested 36 hr (for the vRNA reporter) or 44 hr (for the cRNA reporter) after transfection and was probed for NA-specific vRNA, cRNA, and mRNA by primer-extension assay. (A) Representative products from the vRNA reporter. Expression of each RNA type was quantified by phosphorimaging from three independent transfections;

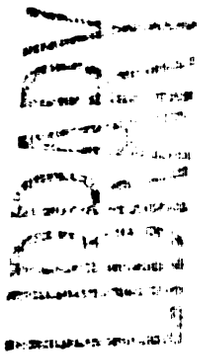
**Pub. Fig.2 cont.**

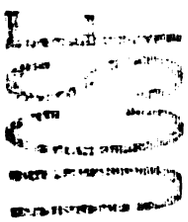
the relative amounts of cRNA (black bars) and vRNA (gray bars) are presented in (B) and those of mRNA are shown in (C), each relative to the corresponding WT. Luciferase expression from triplicate transfections is depicted in (D). Representative products from the cRNA reporter are shown in (E). Mock = sham-transfected cells. Luc = reporter plasmid (vRNA or cRNA) only. –PA = PA vector omitted. Relative infectivities of the PA mutants (from Pub. Table 1) are indicated at bottom of each panel.

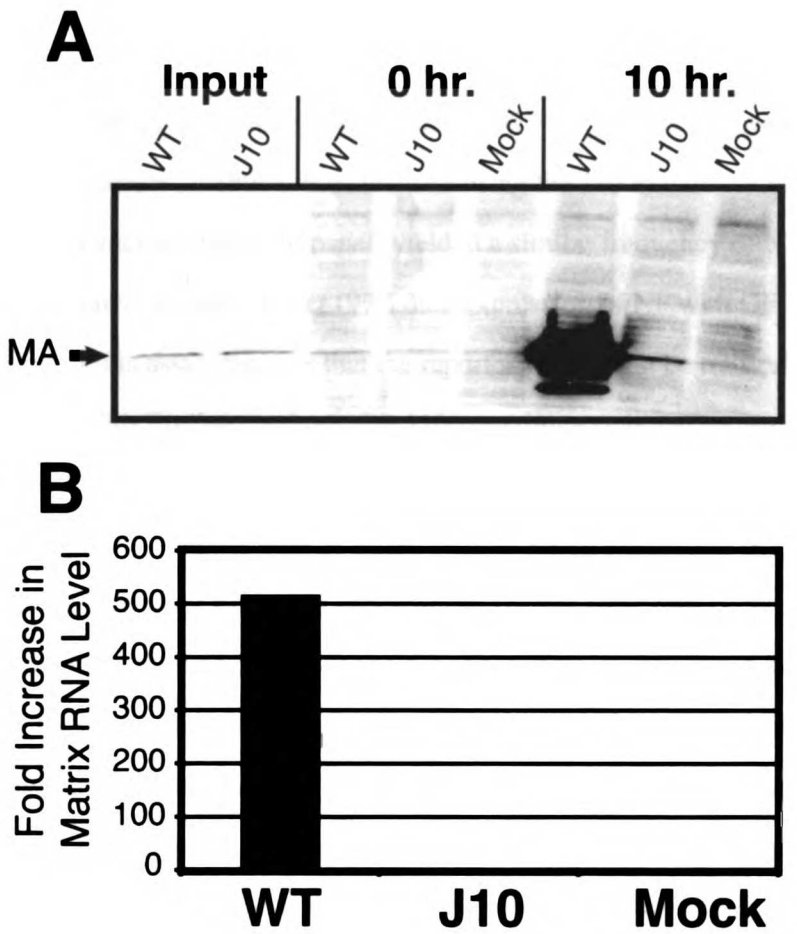
among our mutants, J10 thus appeared unable to support detectable viral infectivity despite generating a polymerase complex that was competent for both RNA replication and transcription. We therefore characterized the J10 mutant in greater detail.

We first used the 17-plasmid expression system to determine whether the failure of J10 supernatants to form plaques (Pub. Table 1) reflected a lack of viral RNA or protein synthesis in the MDCK target cells. In these and all subsequent experiments, transfected 293T cells received either wild-type or J10 forms of both the PA protein and vRNA vectors, and so expressed exclusively wild-type or mutant PA. Supernatants from the transfectants were harvested after 48 hr and used to infect MDCK cells, after normalizing for viral matrix (M1) protein, a marker for virion particles which we found was roughly 7 fold more abundant in wild-type as compared to J10 supernatants (data not shown). Extracts of the MDCK cells were then probed for M1 protein by Western blotting and for its corresponding RNAs by quantitative RT-PCR. We found that M1 protein was undetectable in cells immediately after exposure to wild-type VLPs but had accumulated in large amounts 10 hr later, implying new synthesis (Pub. Figure 3A). This was accompanied by a 500-fold accumulation of M1-specific RNA within 6 hr after infection (Pub. Figure 3B). Exposure to supernatants from J10-transfectants, by contrast, yielded little or no detectable synthesis of the viral M1 protein or its RNA.

Those results were extended by using a flow-cytometric assay to count and characterize individual infected cells [665]. In this assay, 293T cells were transfected with the 17-plasmid expression system along with a reporter derived from the PB2 vRNA that had been modified to encode green fluorescence protein (GFP) in antisense orientation. Supernatants were collected after 48 hr and aliquots were used to infect MDCK cells, which were harvested soon (15 hr) after inoculation to avoid subsequent rounds of infection. Both the 293T producer and MDCK target cells were then fixed, permeabilized, and indirectly immunostained for viral NP protein, and 20,000 cells from each population were analyzed using two-color flow cytometry to detect GFP

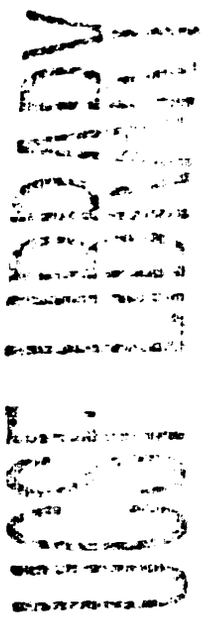






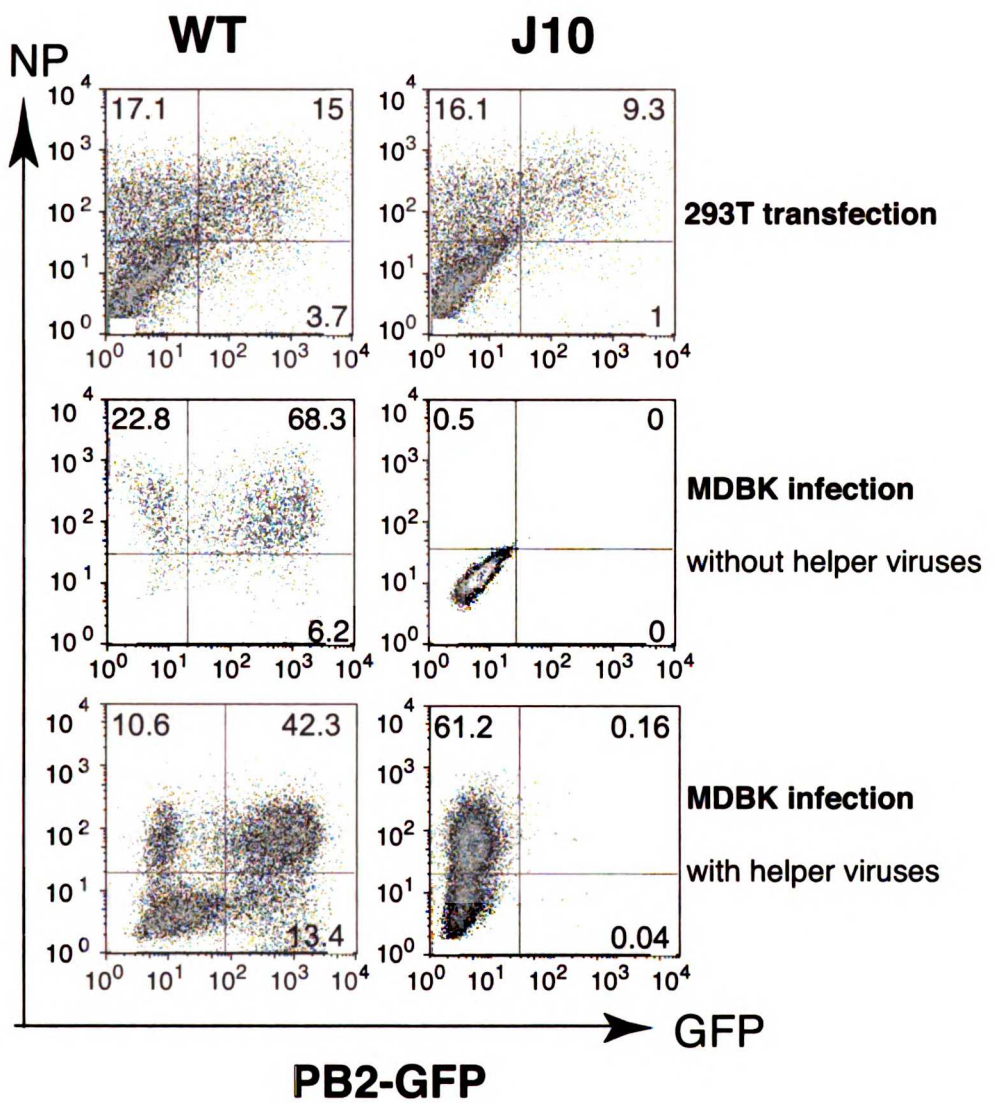

**Pub. Fig.3 Infected MDCK Cells**

Protein and RNA expression in infected MDCK cells. Aliquots of 293T supernatants from 17-plasmid transfections with wild-type (WT) or J10 PA vectors, were normalized for influenza matrix (M1) protein expression and used to infect MDCK cells. (A) Western blot detection of M1 protein in 293T supernatants (Input) and in the MDCK cells at 0 hr and 10 hr post-infection. (B) Expression of M1-specific RNA as determined using quantitative RT-PCR, normalized to 5.8S ribosomal RNA, indicated as the fold increase at 5.5 hr as compared to 0 hr post-infection. Mock = sham-infected MDCK cells.



(green) and NP (red) protein expression. As shown in Pub. Fig. 4 (top left panel), roughly 17% of 293T cells transfected with all 17 wild-type vectors along with the reporter expressed NP protein alone, and an additional 15% of cells expressed both NP and GFP. By comparison, identical transfections using the J10 mutants of both the PA protein and vRNA vectors (top right panel) yielded a similar frequency of NP expression alone (16%), and only modestly lower (9%) dual expression of NP with GFP. Because GFP expression in this assay requires that the reporter vRNA first be transcribed into functional mRNA, this finding confirms that J10-containing polymerase is competent to transcribe vRNA. Moreover, when wild-type supernatants were used to infect MDCK cells (center left panel), roughly 90% of target cells acquired expression of NP, GFP, or both. Identical infections using J10 supernatants (center right panel), by contrast, yielded virtually no cells expressing NP or GFP. Thus, despite supporting substantial polymerase activity in transfected 293T cells, the J10 mutant fails to generate VLPs that can detectably transduce native viral (NP) or reporter (GFP) vRNAs into other cells. Even when the J10 supernatant was supplemented with excess wild-type helper virions as a source of functional polymerase and NP, GFP expression remained undetectable (lower right panel), confirming the absence of transduction-competent VLPs.

One hypothetical explanation for this failure to transduce was that the vRNAs produced in J10-transfected cells might be inappropriately localized and hence unavailable at sites of virion assembly in the cytoplasm. To address this possibility, we transfected 293T cells with a vRNA reporter and the 4 protein vectors (PA, PB1, PB2, and NP) needed for polymerase function, and then examined the distributions of selected RNAs and proteins in the nuclear and cytoplasmic compartments. The reporter was derived from the NS vRNA and encoded an in-frame fusion of the N-terminal 79 residues of influenza NS1 protein to the N terminus of GFP. Cells were harvested 30 hr after transfection, the WT and J10 populations were each physically sorted into GFP-expressing (GFP+) and nonexpressing (GFP-) subpopulations by fluorescence-




**Pub. Fig.4 Facs Analysis**

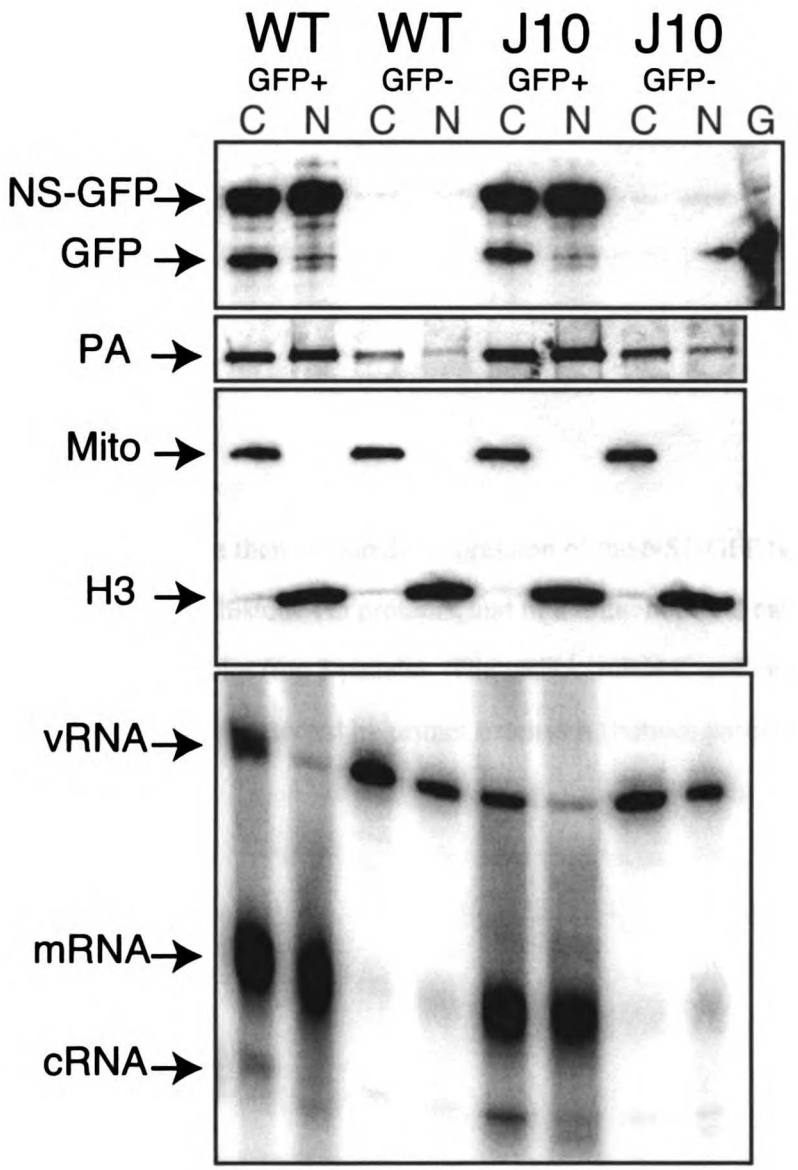
Expression and transduction of influenza NP and of a vRNA-derived reporter in 293T producer and MDCK target cells. 293T cells underwent 17-plasmid transfections that included wild-type (WT) or J10 mutant PA vectors and a PB2-derived vRNA reporter vector that encoded GFP. Supernatants harvested after 48 hr were used to infect MDCK

**Pub. Fig.4 cont.**

cells. Two-color flow cytometry was used to score expression of immunoreactive NP protein and of GFP fluorescence in the 293T producer cells at 48 hr post-transfection (top) and in the MDCK target cells at 15 hr after inoculation with supernatant either alone (center) or together with authentic influenza helper virions at a multiplicity-of-infection of approximately 1.5 (bottom). Percentages of cells expressing NP only, GFP only, and both markers together are indicated in the upper left, lower right, and upper right quadrants of each plot, based on counting 20,000 cells from each population. As expression of the GFP reporter is assumed to require NP, the small percentage of cells expressing GFP alone was disregarded in our analysis. NP expression in the bottom right panel presumably results from infection with the helper virus.

activated cell sorting. We then prepared nuclear and cytoplasmic fractions from these four subpopulations, and assayed selected proteins and RNAs in each (Pub. Figure 5). Purity of the fractions was verified by Western blot detection of mitochondrial (mito) and histone (H3) proteins that serve as markers of cytoplasm and nucleus, respectively. As expected, Western blotting revealed immunoreactive GFP only in the two GFP+ subpopulations, and so validated the sorting procedure. The NS-GFP fusion protein, which includes a nuclear localization signal from NS1 [380, 381], was distributed equally between nuclear and cytoplasmic compartments, whereas unfused GFP (resulting from translational initiation at the internal GFP start codon) was largely confined to the cytoplasm. Wild-type PA protein and the J10 mutant, detected using an antibody that recognizes both, were expressed at comparable levels in the nucleus and cytoplasm of GFP+ cells, and also (at somewhat lower abundance) in GFP- cells, a proportion of which presumably expressed PA protein without the reporter or other polymerase subunits needed for GFP expression. Similarly, primer-extension assay (bottom panel) revealed reporter-derived vRNA in the two GFP- populations, with little or no accompanying mRNA or cRNA products to indicate polymerase activity. The GFP+ cells, by contrast, showed abundant reporter mRNA in both cellular compartments, along with substantial vRNA and traces of cRNA that were each predominantly cytoplasmic. These localization patterns may differ somewhat from those seen in infected cells, owing to overexpression, absence of other viral proteins, or other attributes of the 5-plasmid expression system. Nevertheless, it is clear from these results that expression and localization of all three classes of viral RNA, and of the PA protein itself, are comparable in cells transfected with either wild-type PA or J10. In particular, both forms of PA yielded similar amounts of PA protein and reporter vRNA in the cytoplasm, where virion assembly occurs.

To examine VLP formation, we analyzed supernatants from 17-plasmid 293T transfectants using density-gradient centrifugation. Authentic influenza A/WSN/33 virions were analyzed in parallel for comparison. Each was fractionated through a




**Pub. Fig.5 Localization of Viral Components**

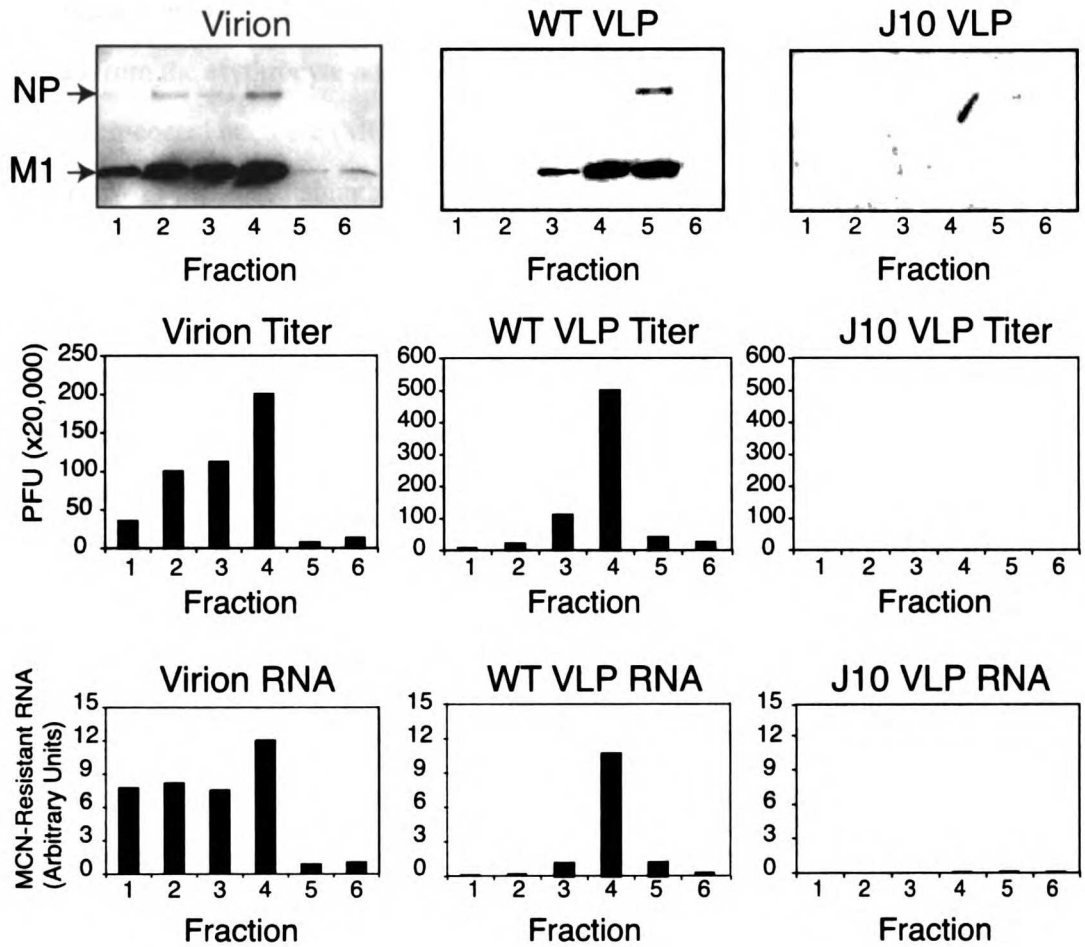
Subcellular localization of viral RNAs and PA protein in 293T transfectants. 293T cells underwent 5-plasmid transfection that included either the wild-type (WT) or J10 form of the PA protein vector, along with an NS-derived vRNA reporter encoding an NS1-GFP fusion protein. At 31 hr post-transfection, cells were sorted into GFP-positive (GFP+) and GFP-negative (GFP-) subpopulations, from which nuclear (N) and

**Pub. Fig.5 cont.**

cytoplasmic (C) extracts were then prepared. Expression of the NS1-GFP fusion (NS-GFP), unfused GFP, PA, and histone H3 proteins, and of a mitochondrial antigen (mito), was determined by Western blot (top 3 panels). The vRNA, mRNA, and cRNA products of the NS vRNA reporter were detected by primer extension (bottom panel).

linear glycerol gradient, and fractions were then assayed by Western blotting for the viral M1 and NP proteins (Pub. Figure 6, top), by plaque-forming assay (middle), and by quantifying micrococcal nuclease-resistant viral RNA, a hallmark of virion particles (bottom). Authentic virions, containing both M1 and NP protein, sedimented in fractions 2-4, which also exhibited high levels of plaque-forming activity and of nuclease-resistant RNA. Supernatants from wild-type transfections likewise contained abundant VLPs with peak infectivity and RNA content localized in fraction 4, though M1 and NP immunoreactivities peaked at somewhat lower buoyant density in fraction 5, perhaps representing polymeric aggregates of those proteins without associated viral RNA [270, 620]. Corresponding fractions from the J10 supernatants, by contrast, contained only traces of NP without associated M1-immunoreactivity or nuclease-resistant RNA, and lacked any detectable plaque-forming activity. J10-transfected cells thus fail to generate VLPs whose density resembles that of wild-type VLPs or genuine influenza virions.

These gradient-fractionation results did not, however, exclude the existence of J10-containing VLPs that were heterodisperse or anomalous in size, shape, and density. Expression of the viral M1, NA, and hemagglutinin (HA) proteins from their respective plasmid vectors should alone be sufficient to generate hemagglutinating VLPs even in the absence of polymerase function [271, 660], and this, together with cytolytic debris, could account for the high hemagglutinating titer we observed in J10 supernatants (Pub. Table 1). As an alternative approach to enriching such VLPs, we therefore incubated 293T supernatants with chicken erythrocytes, which were then pelleted, washed, and probed for influenza proteins and vRNAs. Western blots (Pub. Figure 7A) confirmed that the erythrocyte-adsorbed material from authentic virions (Vir) or from supernatants of cells transfected with the full complement of 17 wild-type (WT) vectors contained abundant M1 and PA proteins, but neither of those proteins was detectable in supernatants from J10 transfectants (J10) or when PA vectors were omitted from the transfection (-PA). As shown in Pub. Figure 7B, a primer-extension assay was then used to probe for



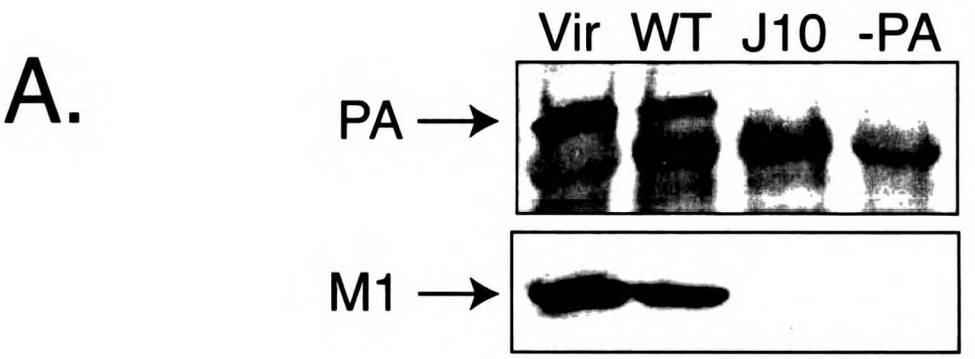

**Pub. Fig.6 Gradient Separation of VLPs**

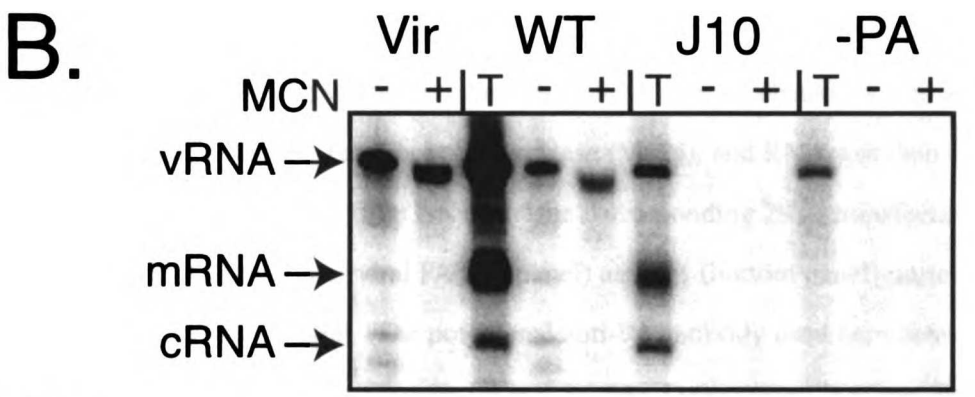
Density-gradient fractionation of virions and virus-like particles (VLPs). Supernatants from 293T cells, collected 48 hr after 17-plasmid transfection were used as a source of wild-type (WT) or J10 VLPs. Supernatants from infected MDCK cells were used as a source of authentic influenza A/WSN/33 virions. These supernatants were fractionated by centrifugation through continuous glycerol density gradients, and corresponding fractions were analyzed for viral NP and matrix (MA) proteins by Western blot (top panel), for plaque-forming activity (middle panel), and for micrococcal nuclease- (MCN)-resistant PB1-specific viral RNA by quantitative RT-PCR (bottom panel).

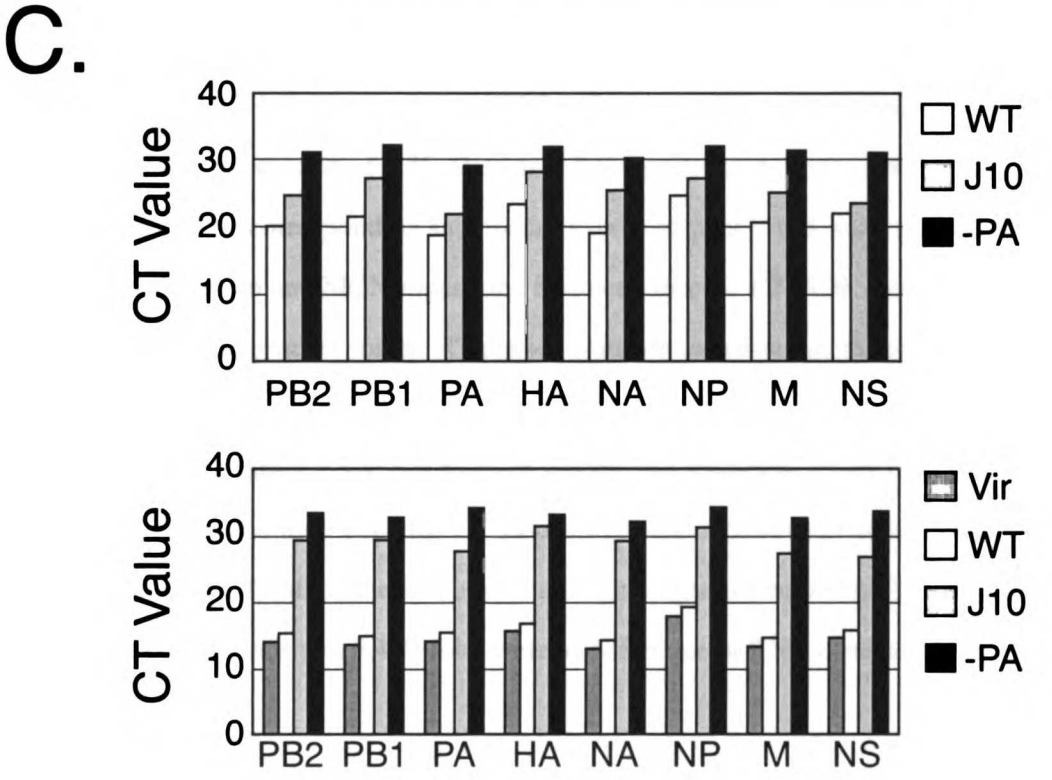
sequences from the viral NA gene in RNA extracted from the transfected cells (T), as well as from the erythrocyte-adsorbed supernatants both before (-) and after (+) digestion with micrococcal nuclease (MCN). Although cells transfected with all 17 wild-type vectors (WT) expressed abundant NA-specific vRNA, mRNA, and cRNA, the adsorbed material from their supernatants contained almost exclusively vRNA that was nuclease-resistant and indistinguishable from that found in similarly-enriched authentic virions (Vir), implying that it had been packaged selectively into intact VLPs. When PA vectors were omitted (-PA), only the background of vector-derived NA vRNA was expressed in the cells, and none was detected in erythrocyte-adsorbed supernatant. As expected, lysates from J10-transfected cells contained all three forms of viral RNA, confirming that a J10-containing polymerase can both replicate and transcribe a native NA vRNA. Strikingly, however, erythrocyte-adsorption failed to recover any detectable NA-specific vRNA. We extended this finding by using a highly sensitive, quantitative RT-PCR assay to search for RNA products of all eight viral segments individually. As shown in Pub. Figure 7C (top panel), an average of four fewer PCR cycles were required to reach critical threshold for any segment in wild-type- than in J10-transfected 293T cells (i.e., the CT value was lower by 4), indicating that wild-type transfectants expressed roughly 16 fold more of the cognate RNA. Parallel assays of hemagglutinated, nuclease-treated supernatants from those cells (Pub. Figure 7C, bottom panel), in comparison, revealed that products of all eight segments were 2,000 to 30,000 fold more abundant in wild-type than in J10 supernatants, and were only modestly (i.e., less than 100 fold) more abundant in J10 supernatants than when PA vectors were omitted entirely. Together, these results demonstrate that any hemagglutinating VLPs that may be present in J10 supernatants are essentially devoid of viral RNA.

## D. Discussion

The results of this study add to a growing body of mutational data on the roles of


A.
Vir WT J10 -PA
PA
M1


B.
Vir WT J10 -PA
MCN - + T - + T - + T - +
vRNA
mRNA
cRNA


C.
CT Value
40
30
20
10
0
PB2 PB1 PA HA NA NP M NS
WT
J10
-PA
CT Value
40
30
20
10
0
PB2 PB1 PA HA NA NP M NS
Vir
WT
J10
-PA

## Pub. Fig.7 cRBC Aggultination of VLPs

Enrichment of hemagglutinating particles by adsorption to chicken erythrocytes. Supernatants from infected MDCK cells were incubated with chicken red blood cells to adsorb authentic influenza virions (Vir) and other hemagglutinating material. Similar adsorption was performed using supernatants from 17-plasmid 293T transfections that included wild-type (WT) or mutant (J10 ) PA vectors, or from which PA vectors had been omitted (-PA). Erythrocytes were pelleted, washed, and then lysed for Western blot analysis. Alternatively, the washed erythrocyte pellets were incubated for 1 hr at 37 °C either with (+) or without (-) micrococcal nuclease (MCN), and RNA was then extracted for analysis, along with RNA from lysates of the corresponding 293T transfectants (T). (A) Western blot detection of viral PA (top panel) and M1 (bottom panel) proteins in erythrocyte-adsorbed material. The polyclonal anti-PA antibody used here detects J10 protein when present (see Pub. Figures 1B and 5). (B) Detection of NA-specific RNA species by primer-extension assay. (C) Detection of viral RNAs by quantitative RT-PCR. Aliquots of RNA from transfected 293T cells (top panel) were treated with DNase and normalized to expression of 5.8S ribosomal RNA prior to analysis. RNA from equal volumes of the corresponding supernatants (bottom panel) was analyzed following erythrocyte-adsorption and MCN-digestion. Segment-specific RT-PCR was performed using vRNA-specific primers for the RT phase. Data are expressed as the number of PCR cycles required to reach critical threshold (CT), which is inversely proportional to concentration of the target RNA; RNAs from virions and wild-type VLPs were diluted 50 fold for analysis and their depicted CT values were adjusted accordingly. The mean difference in CT values shown here for all eight segments between J10 and WT supernatants corresponds to a 9,400-fold average difference in RNA concentration.

PA in the influenza life cycle. In agreement with earlier studies, our analysis of 16 novel substitution mutations in PA reveals a spectrum of effects on polymerase function (Pub. Figure 2). Certain mutations (e.g., J2, J3, J7, J13, and J16) impair the synthesis of all three classes of influenza RNA, confirming that PA is essential for polymerase function and for viral infectivity. Some in the latter group (e.g., J3 and J16) map in or near regions that mediate nuclear localization [645] or PB1 binding [584, 586], which likely accounts for their effects. Other mutations (e.g., J8) have subtler consequences, in some cases promoting the accumulation of specific RNA classes at the expense of others. Additional study will be needed to unravel the mechanisms underlying those phenomena, which might reflect activities intrinsic to PA or indirect effects on the function, stability, or localization of the polymerase holoenzyme or of its RNA products [480].

The present study focused principally on the novel phenotype of one mutant, J10, that suggests a previously unrecognized role for PA in influenza assembly. In our initial screening, this mutant was found to be unique in its complete failure to produce infectious virus despite its ability to support relatively high-level expression of all three RNA classes. The catalytic integrity of J10-containing polymerase was evident in transfected 293T cells expressing all of the proteins and RNAs necessary for virion assembly (Pub. Figures 4 and 7C), as well as in those expressing only the four viral proteins (PA, PB1, PB2, and NP) minimally required for polymerase function (Pub. Figure 2). During the course of our study, J10-containing polymerases proved capable of utilizing both vRNA and cRNA templates as well as a variety of vRNA reporters. Although we cannot formally rule out a defect in expressing a subset of viral genes, our results demonstrate that this mutant polymerase can efficiently act on substrate RNAs from the NA (Pub. Figures 2 and 7B), PB2 (Pub. Figure 4), and NS1 genes (Pub. Figure 5). This functionality implies that J10 does not interfere with polymerase trimer assembly or substrate binding, though experiments to test this directly were not carried out. The various RNA products of J10 polymerase also appeared normal in physical

assays such as primer extension (Pub. Figures 2A, 5, and 7B) and by functional criteria. In particular, the ability of J10-containing polymerase to amplify vRNAs (Pub. Figures 2A, 2B, 2E, and 7) implies that it synthesizes functional cRNA intermediates, and our studies demonstrate that mRNAs it produces can be translated to yield luciferase (Pub. Figure 2D) or GFP (Pub. Figures 4 and 5). The levels of J10-dependent protein or RNA expression we measured were generally at least 50% of wild-type, and exceeded those observed with certain other PA mutants (e.g., J4 and J12) that supported viral growth and plaque formation. Thus, we found no enzymatic defect in J10-containing polymerase that could account for its profound defect in generating plaque-forming virions.

Instead, the replicative defect of J10 appears to reflect a failure to assemble viable virions. Notwithstanding high-level expression of all requisite virion constituents, J10-transfected 293T cells fail to produce detectable VLPs whose buoyant density approximates that of wild-type influenza virions; the relevant glycerol density fractions contained virtually no viral RNA, M1 protein, or plaque-forming activity (Pub. Figure 6). Moreover, although the supernatants from J10 transfectants exhibit substantial hemagglutinating activity (Pub. Table 1), implying the release of particles that can bind chicken erythrocytes, we found that enriching for such particles in J10 supernatants yields no detectable nuclease-resistant vRNA, a hallmark of intact virions (Pub. Figure 7).

The J10 mutation alters a potentially surface-exposed glycine-arginine pair at positions 507 and 508 in PA. The functional architecture of this region of PA is unknown, but an earlier study found that single-codon mutations at a cluster of nearby residues (positions 502, 510, 524, and 539) each abolished infectivity [632]. The reported effects of those mutations on viral RNA expression varied widely, however, and none recapitulated the phenotype of J10. Interestingly, the normal sequence of residues 502-509 resembles a motif called the P-loop ([G/A]XXXXGK[S/T]), which functions in other proteins as an ATP- or GTP-binding site [630, 666, 667], though the biological significance of this resemblance remains speculative. Insertion of a serine residue after

position 509 has been reported to yield a dominant negative mutant that inhibits the activity of wild-type PA in trans [590].

Our inability to detect production of RNA-containing particles by J10 transfectants is particularly striking in that the influenza M1, HA, and NA proteins alone can direct formation and release of hemagglutinating VLPs [271, 660]. This raises the hypothesis that the J10 mutant either interferes with the production of such VLPs or prevents them from incorporating vRNAs and, perhaps, other key components. Though the potential involvement of PA in influenza virion assembly has not been extensively explored, polymerase trimers are known to be contained in the virion core, bound stably to the 3' and 5' ends of each vRNA. Electron micrographs of influenza cores suggest that the vRNAs within them are packed in a densely stacked array [285, 615] and, while the nature of this array and the factors that give rise to it have not yet been determined, polymerase may conceivably play a structural role in core formation that might be perturbed by the J10 mutation. Alternatively, by virtue of binding to vRNAs, the polymerase might collaborate with other viral or cellular proteins that direct the intracellular trafficking of vRNAs to sites of viral assembly. Indeed, polymerase has been speculated to play a role in the differential cytoplasmic accumulation of vRNA as opposed to cRNA [294]. Although our data indicate that both J10 and wild-type PA support cytoplasmic vRNA accumulation (Pub. Figure 5), we cannot rule out subtler localization defects that might result from this mutation. Finally, assuming that all components are present at the assembly sites, one may speculate that PA provides signals that target a polymerase complex into nascent virions, bringing with it a vRNA bound through its PB1 and PB2 subunits. Precedent for this model may be found in the hepatitis B virus, whose polymerase mediates packaging in trans when bound to its genomic operator site [668]. The loss of PA-associated packaging signals might thus account for the apparent failure of our J10 mutant to incorporate viral RNA into VLPs. If correct, this model would implicate PA as a critical trans-acting factor in the pathway of influenza

vRNA packaging, whose mechanism is presently unknown.

# Chapter 44. Unpublished Work

## A. Titration of PA and NP

During the course of this study, a trend was observed that suggested J10 has a tendency to accumulate more cRNA in the primer extension assay than does WT. As there has been some speculation that PA may serve as a modulator that is capable of shifting the polymerase from transcription to replication, we examined the possibility that the J10 mutation creates an imbalance in the modulator switch and biases the polymerase toward replication. To address this hypothesis, we designed an experiment that would "push" the polymerase toward either transcription or replication. This was achieved by titrating the amounts of NP, PA, and reporter included in the transfection. (Please see the chapter on "Transcription versus Replication - a Switch?" for more information regarding the role of NP in affecting the activities of the polymerase).

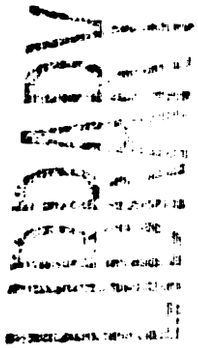
The results from this experiment suggest that NP levels have a modest effect on the levels of accumulated RNA species (Unpub. Fig. 1). At the highest levels of NP (1.0 $\mu$g/well - lanes 1 and 10), both WT and J10 promoted a higher level of cRNA accumulation than seen with lesser amounts of NP. For WT, as the level of NP decreased, the level of mRNA increased slightly. The level of accumulated mRNA in J10 samples was not markedly affected by different levels of NP.

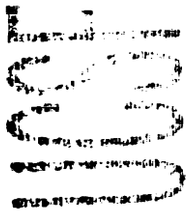
In contrast, the level of PA included in the transfection had a significant effect on the level of accumulated RNA. At its highest concentration (1.0 $\mu$g/well), WT polymerase accumulated relatively small amounts of RNA (lane 1). However, as the level of PA was decreased, the level of accumulated viral mRNA increased. This trend held true down to the lowest concentration of PA tested, which was only 0.01 $\mu$g of PA

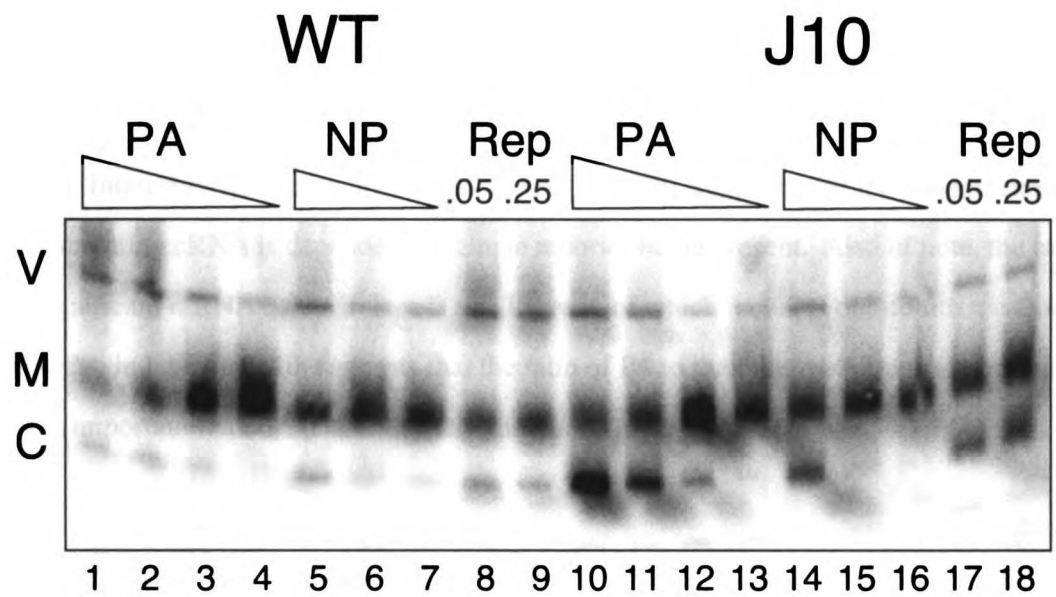
protein expression vector per 35-mm well (lane 4). These data suggest that at higher concentrations of WT PA, less viral RNA accumulates by the 48 hour time-point.

Similarly to WT, J10 also produced highest level of mRNA at a relatively low concentration of PA protein expression vector (0.05 $\mu$g/ 35-mm well – lane 12). However, in contrast to WT, at higher levels of expression, J10 skewed the accumulation of RNA in favor of cRNA. In fact, at 1.0 $\mu$g/ 35-mm well, J10 accumulated more cRNA than mRNA. These data show that varying the expression level of J10 has a qualitative effect on the RNA species that accumulate in the transfected cells, which was not as prominent in WT transfected cells. These results, in turn, that PA may be a modulator for the polymerase in regards to RNA synthesis or the stability of synthesized RNA.

The observation that lower quantities of RNAs are present in samples with high concentrations of WT PA is consistent with any of the following possibilities. First, as the primer extension assay measures the level of RNA at the time of harvest and does not provide any information as to the rate of RNA synthesis or degradation, it is important to run time-course experiments and possibly pulse-chase experiments for each concentration of WT PA. It is possible that at higher concentrations of PA, more viral RNA was present at earlier times, and either the viral RNA or the viral protein product (NA) initiated a cellular response, such as apoptosis or activation of host endonucleases, which resulted in the degradation of the viral RNA. However, one time-course experiment has been performed and the data argue against this hypothesis (see Unpubl. Fig. 5). There is also the possibility that J10 is better at protecting transcripts, particularly cRNA, from host endonucleases. Alternatively, WT PA may be more toxic to cells at higher levels of expression; however, this is unlikely since WT PA successfully synthesized RNA and protein from a pol NA-Luc reporter (See Pub. Fig. 2). Lastly, it is possible that at higher levels of WT PA expression the transfection efficiency drops, but again, this is unlikely in light of our work using a high level of WT PA to transcribe pol NA-Luc reporters (See





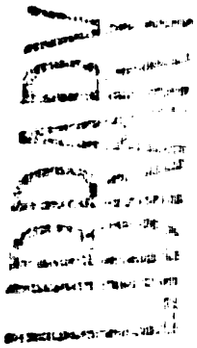


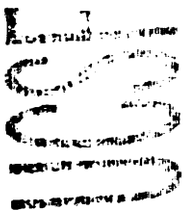

**UnPub. Fig.1 Titration of PA and NP**

293T cells were transfected with varying amounts of PA, NP, and native pol-NA reporter, and RNA was harvested with Trizol at 48 hours post-transfection for analysis by primer extension. Reverse transcription was carried out as previously described, using 5 $\mu$g of total RNA with $^{32}$P-end labeled primers NA-vRNA-155 and NA-cRNA-125, which have the sequence

5'-GGGGCTACCTGAGGAGGACGCA-3' and

5'-GGCTAATCCATATTGAGATTATATT-3', respectively. The standard transfection included 1.0 $\mu$g of each xPB2, xPB1, xPA, xNP, and pol NA. These values were kept constant, with the exception of xPA, xNP and pol NA, which were individually included at lesser amounts in the indicated lanes. xPA was included at 1.0, 0.25, 0.05, and 0.01 $\mu$g/ well for WT (lanes 1, 2, 3, and 4) and J10 (lanes 10, 11, 12, and 13). xNP was included at 1.0, 0.25, 0.05, and 0.01 $\mu$g/well for WT (lanes 1, 5, 6, and 7) and J10 (lanes 10, 14, 15, and 16). pol vNA was included at 1.0 and 0.05 $\mu$g/well for lanes 1 and 8 (WT) and lanes 10 and 17 (J10), respectively. Lanes 9 and 18 were transfected with the each of the 5-plasmids at 0.25 $\mu$g/well, therefore the same DNA used in lanes 1 and 10, respectively, but at 1/4 the amount.





Pub. Fig. 2).

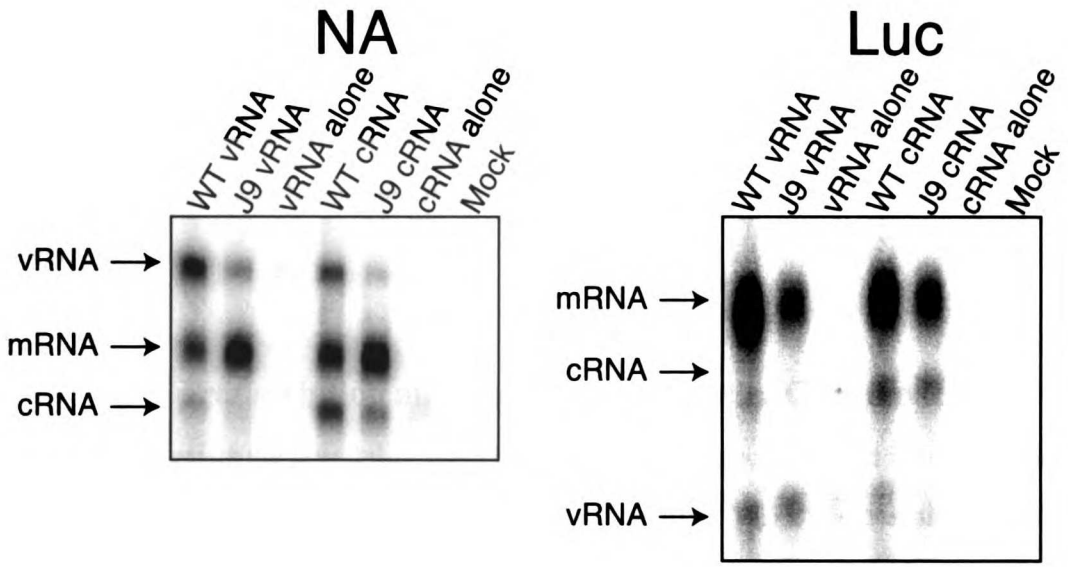
Comparison of lane 8 to lane 1 (WT), suggest that decreasing the amount of reporter to 0.05 $\mu$g/ 35-mm well, does not significantly affect the level of accumulated RNA. Interestingly, comparing lane 17 to lane 10 (J10), suggests that the phenomenon of accumulating cRNA is dependent on ample reporter being present. Also of note, the lack of significant mRNA bands in lane 9 and 18, each of which includes one-fourth the DNA included in lanes 1 or 10, suggests that the ratio of PA to the other polymerase subunits is more important than the amount of DNA included in the transfection.

## B. J9 Transcription and Replication

According to our published results, the level of polymerase activity in the 5-plasmid transfection system generally correlates well with viral titer. The two exceptions to this rule are the J10 mutant, which we have shown to have a defect in viral assembly, and the J9 mutant. Our published data suggest that J9 is a high-titer virus that generates relatively little mRNA (20-30% of WT) when provided a pol vNA-Luc reporter. To address this discordance, we repeated the 5-plasmid transfection assay, using native (pol NA) and hybrid (pol NA-Luc) reporters that expressed either vRNA- or cRNA-sense transcripts.

These results demonstrate that indeed J9 synthesizes significantly less mRNA than WT when given a hybrid reporter (vNA-Luc), but the opposite trend is observed when J9 transcribes a native reporter (pol NA) (Unpub. Fig. 2). Furthermore, when J9 is provided a cRNA reporter, the same reporter-dependent trend is observed, in which J9 is more active on a native neuraminidase reporter than a hybrid reporter. These data re-affirm the J9 phenotype described in our published work (See Pub. Fig. 2).

These data also suggest that J9 is an active polymerase that discriminates between the neuraminidase and luciferase coding regions. If further experimentation confirms that this finding is true, J9 would be the first mutatn to suggest that PA is involved in




**UnPub. Fig.2 J9 Transcription and Replication**

WT and J9 5-plasmid transfections of 293T cells with native (pol vNA and pol cNA) and hybrid (pol vNA-Luc and pol cNA-Luc) reporters. The cells were transfected with 1.0 $\mu$g of each xPB2, xPB1, xPA, xNP, and the indicated reporter. The RNA was harvested at 46 hours post-transfection with Trizol and analyzed by primer extension. Reverse transcription was carried out as previously described, primers used include vNA-155, cNA-125, vLuc -75, and cLuc-84 primers, whose sequences have previously been provided in our published report.

recognizing the coding region of influenza genomes.

Alternatively, it is important to note that the 5-plasmid transfection system likely results in a higher concentration of PA being expressed in cells than in the 17-plasmid system, which is used to generate VLPs. We have shown that over-expression of WT PA in the 5-plasmid system, with a native reporter, reduces the accumulation of mRNA (Unpub. Fig. 1). Nonetheless, the J9 mutant needs further investigation to clarify its phenotype.

## C. Transcription and Replication of Entire Genome

There exists the possibility that J10 has a segment-specific defect in transcription and/or replication that leads to the failure of J10 to generate VLPs that contain RNA. To address this possibility, we performed 5-plasmid transfections that included all 8 native flu reporters and the hybrid pol vNA-Luc reporter that was used in our published work. The primers used for the experiment included: cPB2-99 5'-GGTGGTTTTTGTGAGTATC TCGCGA-3', vPB2-134 5'-GCAAGGAGACGTGGTGTTGGTA-3', cPB1-90 5'-GAAAG TTGTGCTTATAGCATTTTGT-3', vPB1-120 5'-GAAGGATAAAGAAAGAGGAGTT-3', cPA-120 5'-TGTTTCGATTTTCAGGTCCTCTCCA-3', vPA-150 5'-TATGAAGCAATT GAGGAGTGCCTGAT-3', cHA-105 5'-GGTAGCCTATACATATTGTGTCTGC-3', vHA-145 5'-CTCAACTGTCGCCAGTTCACTGGTG-3', cNA-125 5'-GGCTAATCCATATTG AGATTATATT-3', vNA-155 5'-GGGGCTACCTGAGGAGGACGCA-3', cNP-110 5'-GC ATTCTGGCGTTCTCCATCAGTCT-3', vNP-140 5'-CGGGGAGTCTTCGAGCTCTCG GACG-3', cM-120 5'-AAGACATCTTCAAGTCTCTGTGCGA-3', vM-150 5'-AAATAC GGTTTGAAAAGAGGGCCTT-3', cNS-105 5'-GTTCTTGGTCTGCAACTCTTTTGCG -3', vNS-145 5'-TGAGACACAGACTGAAGATAACAGA-3', cLuc-100 5'-ATGCAGTT GCTCTCCAGCGGTTCCA-3', and vLuc-141 5'-GAGGAGTTGTGTTTGTGGACGA-3'.

The length of the expected cDNA product is listed in the name of the primer, and

the 'v' and 'c' denote the RNA species from which the cDNA was amplified (vRNA and cRNA, respectively). The cDNA bands generated from the 'c' primers that represent the mRNA species are generally 9-12 nucleotides longer than the band representing cRNA species.

The primer extension assay yielded two minor differences between WT and J10 polymerases (Unpub. Fig 3). First, the neuraminidase gene seemed to generate a unique pattern, in that WT accumulated mostly vRNA, whereas J10 accumulated mostly mRNA and cRNA. This experiment was repeated and J10 was shown to accumulate more mRNA and cRNA than did WT for all of the segments. The significance of J10 accumulating more mRNA and cRNA than WT is unclear, especially considering the results obtained in Unpub. Fig.1. Further experimentation under careful titration of PA is needed to determine whether the tendency for J10 to accumulate more cRNA is genuine and whether this may contribute to the defect in generating VLPs.

The second discrepancy between WT and J10 is that J10 accumulated noticeably less vRNA than WT for several segments, but most prominently for HA, NP, and NA. This observation suggests that J10 either fails to stabilize vRNA and it is degraded, or there is a defect in cRNA to vRNA synthesis that is segment specific. This possibility is explored in the following figure (Unpub. Fig 4).

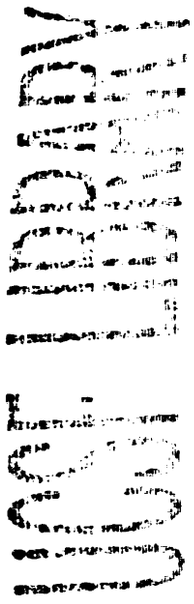
## D. J10 vRNA Synthesis

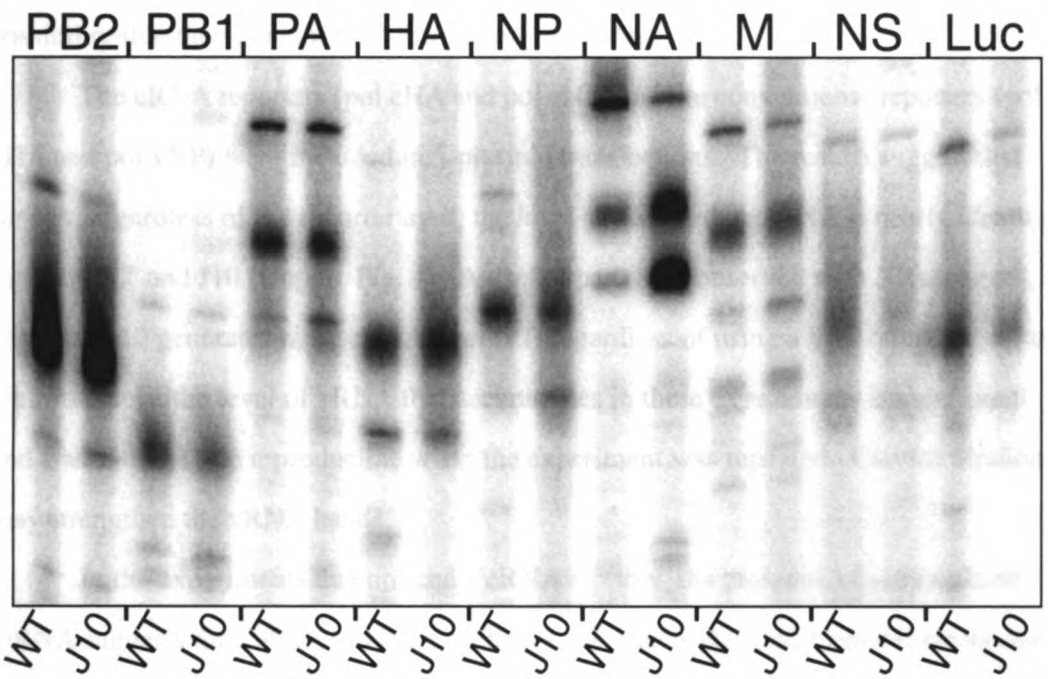
To examine whether J10 has a specific defect in cRNA-to-vRNA synthesis, HA and NP reporters were cloned that generate cRNA-like transcripts (pol cHA and pol cNP). These clones were generated by PCR amplifying the entire HA and NP genes with the following primers:

cHA-up 5'-GCGGCGGCGTCTCGGGGAGTAGAAACAAGGGTGTTTTTCCT-3', and

cHA-low 5'-GCGGCGGCGTCTCGTATTAGCAAAAGCAGGGGAAAATAAAAAC-3',

and cNP-up 5'-GCGGCGGCGTCTCGGGGAGTAGAAACAAGGGTATTTTTCTTTAA






**UnPub. Fig.3 Transcription and Replication of Entire Genome**

WT and J10 5-plasmid transfections of 293T cells included each of the eight native reporters and a hybrid reporter (pol vNA-Luc). The cells were transfected with 1.0 $\mu$g of each xPB2, xPB1, xPA, xNP, and the indicated reporter, and the RNA was harvested at 48 hours post-transfection with Trizol and analyzed by primer extension. Each reverse transcription reaction 5 $\mu$g of total RNA with the appropriate $^{32}$P-end labeled primers.

TTG-3', and

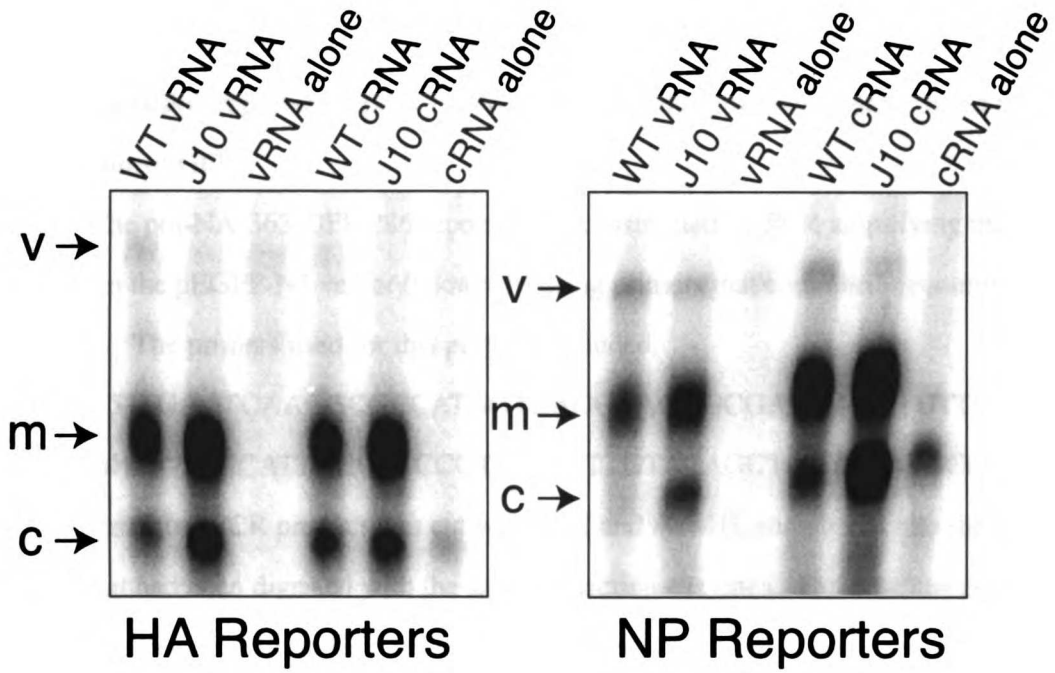
cNP-low 5'-GCGGCGGCGTCCTCGTATTAGCAAAAGCAGGGTAGATAATCAC-3'. PCR products were cut with *Bsm*BI and cloned into pHH21 that had been linearized with *Bsm*BI. Neither of these clones were sequenced or used to generate VLPs, so there is the possibility that they contain PCR introduced mutations.

The cRNA reporters (pol cHA and pol cNP) and the conventional reporters (pol vHA and pol vNP) were included in 5-plasmid transfections. The results suggest that for HA, regardless of the reporter used, the level of accumulated RNA is nearly identical for both WT and J10 (Unpub. Fig. 4). A similar pattern is observed with NP reporters, however, J10 generates more cRNA than WT, regardless of using a vNP or cNP reporter. Unfortunately, the level of vRNA that accumulates in these experiments is quite small, and was shown to be reproducible when the experiment was repeated. Careful titration may strengthen the vRNA bands.

In the experiments that utilized a cRNA reporter, the presence of accumulated mRNA suggests that cRNA to vRNA synthesis was successful, and from the synthesized vRNA, mRNA was synthesized. Although the evidence for efficient cRNA to vRNA synthesis is indirect, it is clear that J10 is capable of performing the second step of the replication process. However, these data do not prove that J10 is capable of successfully stabilizing vRNA to create vRNPs that are eventually packaged.

## E. J10 Transcription and Translation

To examine the possibility that one of the J10-generated transcripts are not translated, we examined transcription and translation of flu-generated transcripts. Ideally, we would examine native influenza gene products, however, we only have one antibody (anti-matrix), which can be used for this purpose. To circumvent this problem, we created influenza-GFP fusion products, so that we could detect the quantity of fusion protein synthesized with one antibody (anti-GFP). To this end, we created HA-GFP, NA-




**UnPub. Fig.4 J10 vRNA Synthesis**

WT and J10 5-plasmid transfections of 293T cells included cRNA and vRNA reporters for HA and NP gene segments. The cells were transfected with 1.0 $\mu$g of each xPB2, xPB1, xPA, xNP, and the indicated reporter at 3 $\mu$g/ 33 mm well. The RNA was harvested at 49 hours post-transfection with Trizol and analyzed by primer extension.

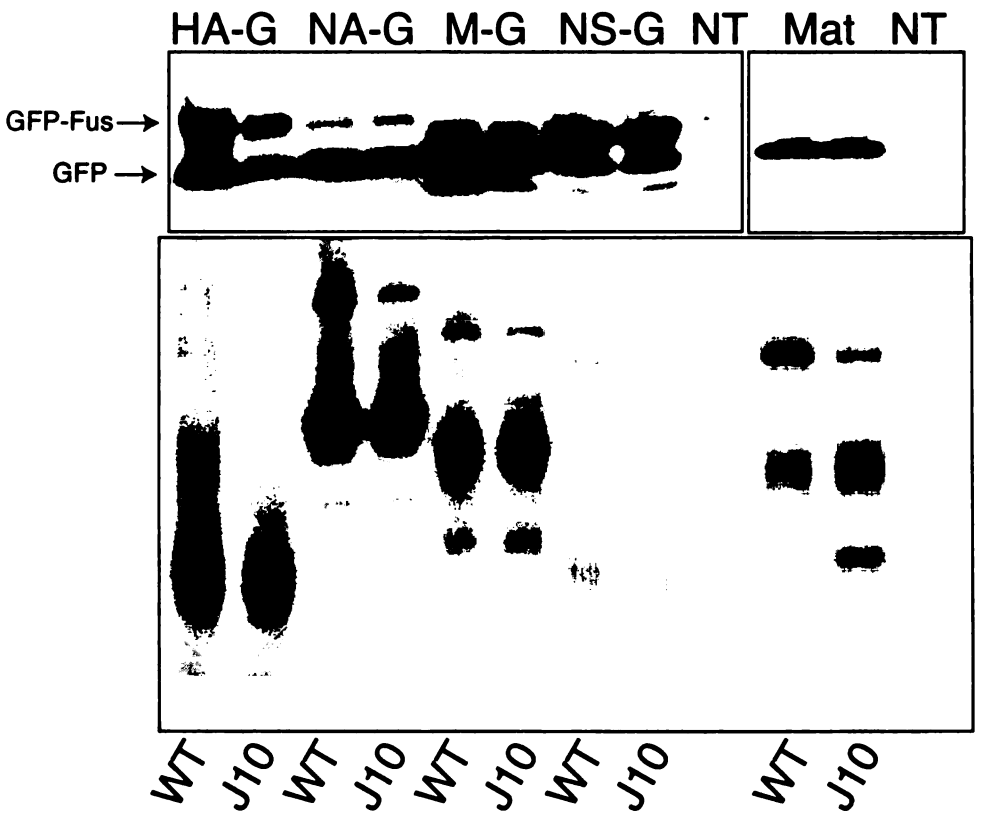
GFP, M-GFP, and NS-GFP fusion products, each of which contain a significant amount of influenza coding region at the N-terminus.

The pol-NS-238-GFP-374 and pol-HA-334-GFP-464 constructs were created by Dr. Yuying Liang at Emory University. Construction of pol-NS-238-GFP-374 is detailed in our published report. The pol-HA-334-GFP-464 construct was created using the *Bgl* II and *Eco*RI sites within the HA coding sequence to insert an inframe PCR amplified GFP gene. The fusion reporter encodes 111 N-terminal residues of the HA gene, fused to the N-terminus of GFP.

The pol-NA-363-GFP-286 reporter was constructed by PCR amplifying the GFP gene from the pEGFP-N1 vector (Clontech) using primers that contained neuraminidase sequence. The primers used for this purpose included: NA-Kas 5'-TGACTCAAGGCGCCATGGTGAGCAAGGGCGAGGAGCTGTTC-3' and NA-BamHI 5'-ATCCATTAGGATCCCTATTACTTGTACAGCTCGTCCATGCCGAGA-3'. The resulting PCR product was cut with *Kas*I and *Bam*HI, and cloned into the pol-NA vector that had been digested with the same restriction enzymes. The resulting plasmid generates a neuraminidase-GFP fusion protein, in which the 121 N-terminal residues of neuraminidase are fused in-frame with GFP, which is followed by a stop codon.

Similarly, the pol-M-309-GFP-259 vector was created by PCR amplifying the GFP gene from pEGFPN-1 with the following primers that contain matrix sequence: M-Hind 5'-TGTATAGGAAGCTTATGGTGAGCAAGGGCGAGGAGCTGTTC-3' and M-Stu 5'-TTTCTGATAGGCCTCTATTACTTGTACAGCTCGTCCATGCCGAGA-3'. The PCR product was cut with *Hin*d III and *Stu* I and cloned into the pol-M vector that was similarly cut. The resulting vector encodes a fusion protein that includes the 103 N-terminal residues of the matrix protein fused to the N-terminus of GFP.

293T cells were transfected with both WT and J10 PA using the 5-plasmid system that included either a GFP fusion reporter or a native matrix reporter. Cells were harvested for protein and RNA. The results suggest that although transcription appears to




**UnPub. Fig.5 J10 Transcription and Translation**

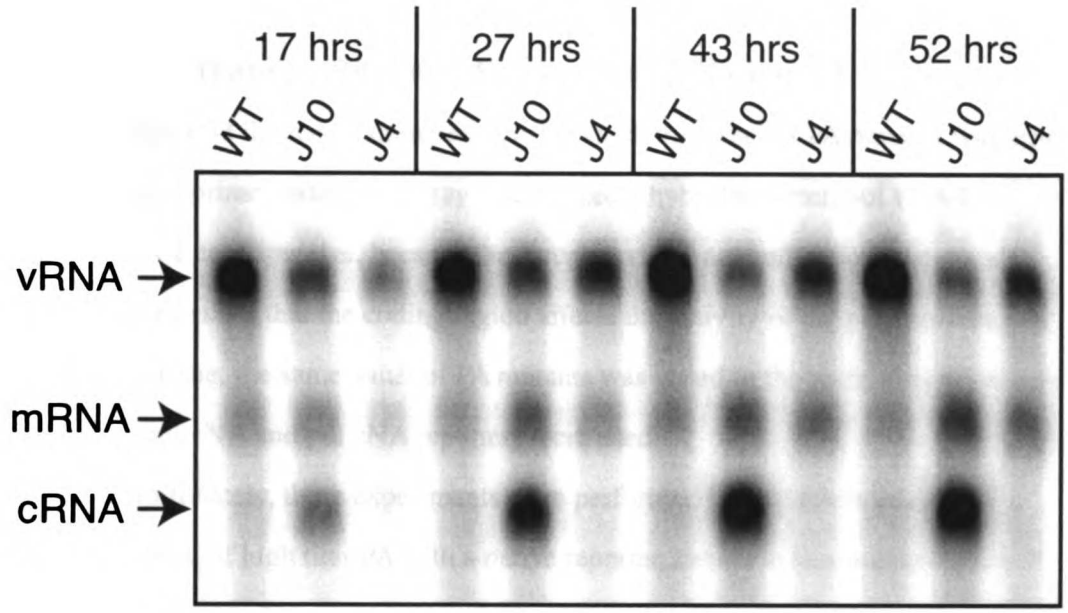
293T cells were transfected with the 5-plasmid system and at 49 hours post-trasnsfection, cells were harvested with Accutase to preserve the extracellular NA-GFP. Cells were divided in two for protein and RNA isolation, using a Triton-X100 lysis buffer and Trizol, respectively. Primer extension was performed using the primers mentioned in Unpub. Fig. 3. and the Western blot was performed with anti-GFP, as described in our published report.

be equal between WT and J10 polymerases, there may be a slight segment-specific defect in replication (Unpub. Fig. 5). Both HA-GFP and M-GFP reporters were translated to a lower level in J10 expressing cells when compared to WT. This discrepancy was also observed by microscopy and FACS analysis (data not shown). Additional experiments are required to confirm this trend. Despite these data that suggest there may be a segment-specific defect in expressing the fusion constructs, translation of native matrix was equal between both WT and J10 polymerases. It is important to note that the lack of the central and C-terminal portions of the fusion products may affect the manner in which the polymerase regulates transcription and/or translation.

## F. 5-Plasmid Neuraminidase Time Course Expression

The 5-plasmid transfection system does not accurately mimic a natural infection. Considering that many of our experiments rely on this assay, it was important to determine 1) how the accumulation of RNA species changes over time and 2) the time at which it is optimal to harvest RNA from the transfected 293T cells. To answer these questions, we performed a time-course experiment that included WT, J10, or J4 forms of PA. RNA was harvested at 17, 27, 43, and 52 hours post-transfection, and analyzed by primer extension.

At 17 hours, a significant amount of RNA accumulates in WT and J10 transfected cells, and as expected less RNA was expressed by the low-titer mutant J4 (Unpublished FIG. 6). The pattern of RNA expression that was seen at 17 hours remained the same, but increased in intensity as the time-course progressed out to 52 hours. The level of increase for WT between 17 and 52 hours was 2-3 fold, whereas for J10, it was approximately 10 fold, which suggests that J10 synthesizes RNA at a slower rate than WT. As previously observed, pattern of RNA accumulation for J10 and WT were at polar opposites; WT accumulated more vRNA and J10 accumulated more cRNA. These results suggest that RNA can be harvested as early as 17 hours, and the pattern or RNA expression does not




**UnPub. Fig.6 Time Course of 5-Plasmid Transfection**

293T cells were transfected with the 5-plasmid system that included 1 $\mu$g each of protein expression vectors for xPB2/PR8, xPB1/PR8, xPA/WSN, and xNP/WSN, and a native reporter (pPol-vNA/WSN). RNA was harvested with Trizol at 17, 27, 43, and 52 hours post-transfection, and reverse transcription was performed as previously described, using the NA-specific primers (Unpubl. Fig. 3). The cDNA products were separated on a 6% PAGE/Urea gel.

change appreciably over time under the transfection conditions used.

Of note, the pattern observed in this time-course experiment may change under different concentrations of PA used in the transfection (See Unpub. Fig. 1).

## G. Transcription and Replication of Native NA

Chapter 43 describes an experiment in which the panel of PA mutants was tested in a 5-plasmid primer extension assay that utilized a hybrid reporter, pol vNA-Luc and pol cNA-Luc. However, because these reporters are not authentic flu segments, the possibility exists that the coding region affects the activity of the polymerases. To address this issue, the same panel of PA mutants was tested in the same manner, except that native pol vNA and pol cNA reporters were used.

Unfortunately, these experiments were performed before determining that over-expression of high titer PA with a native reporter, results in less accumulation of mRNA (See Unpub. Fig. 1). As a result, these data are difficult to interpret since high titer viruses (WT, J5, J9, and J16) produce relatively little mRNA from a native vNA reporter in comparison to low titer viruses (J1, J4, J8, and J12)(Unpub. Fig. 7). A similar pattern is observed when the mutants are given a cNA reporter, however, the trend is not completely consistent. The lack of consistency may be due to the fact that the polymerase needs to synthesize a vRNA template prior to making mRNA, therefore a specific defect in vRNA synthesis would affect the level of mRNA synthesis.

It is interesting that over-expression of high titer PA results in less RNA synthesis from a native reporter, but not from a hybrid reporter. This finding suggests that the lack of RNA when using a native reporter is not due to toxicity directly related to PA, but potentially due to toxicity related to the products PA synthesizes. Possibly the native vRNA or the corresponding mRNA or protein (NA), is toxic to the cells, and causes a host-antiviral response that is either apoptotic or dependent on host endonucleases to degrade synthesized transcripts. Alternatively, the polymerase is capable of recognizing

native sequence and limiting viral transcription. The last option is the least likely, since lower levels of PA were shown to be very active in viral transcription, see Unpub. Fig. 1.

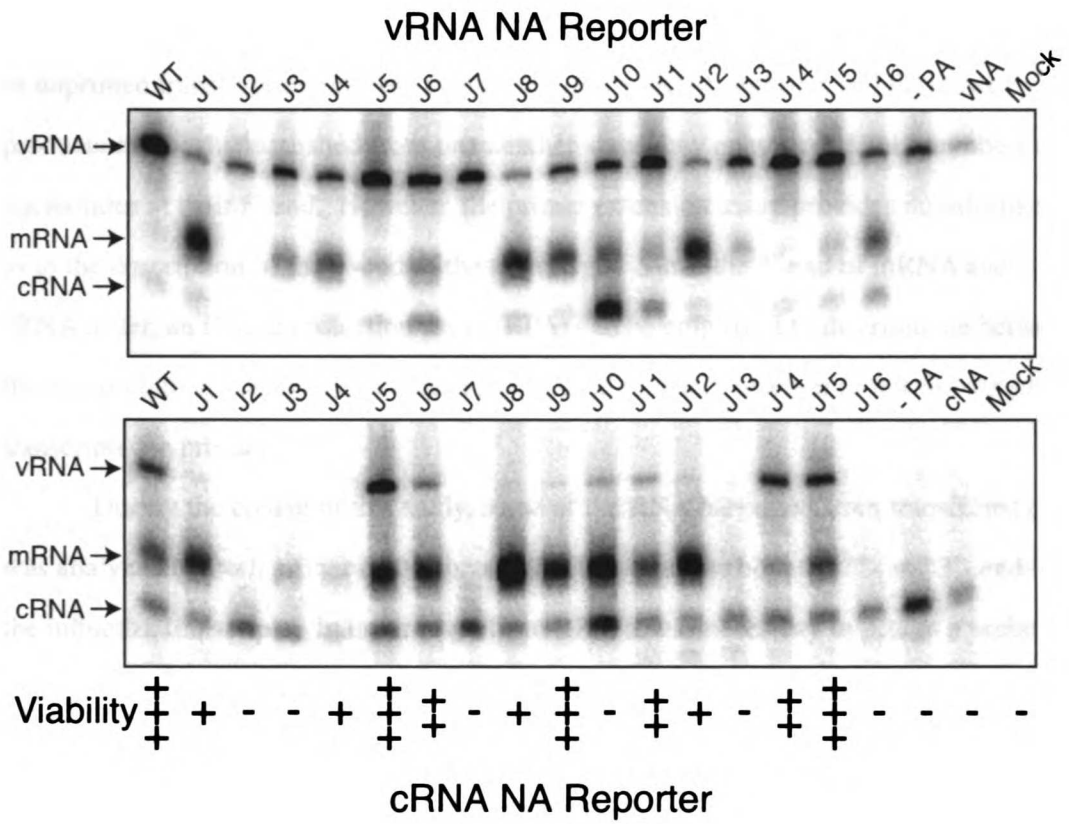
It would be interesting to test for signs of either activated programmed cell death or an activated anti-viral immune response. Further work could be done to determine if it is the vNA, cNA, mRNA, or NA protein that is initiating this interesting response. It may also be necessary to do a time-course to determine if viral RNA products were synthesized but later degraded. It is important to note that the hybrid reporters, pol-vNA-Luc and pol-cNA-Luc do not contain any NA coding region that has been shown by Fujii *et al.* to be necessary for packaging [303]. This packaging signal may initiate an anti-viral response.

The ability of low titer viruses to accumulate more mRNA than high titer viruses suggests that these mutants or their products are less toxic to the cells and do not activate apoptosis or host endonucleases as readily as high titer PA. It is unclear how their products would be less toxic, since they should be the same, the only difference may be the rate at which they are synthesized. A higher rate of synthesis may more easily create an anti-viral response.

Of note, all high and medium titer viruses (except J9) are capable of synthesizing and accumulating vRNA, when given a cRNA reporter. The low titer and dead mutants fail to either synthesize or stabilize generated vRNA. Potentially, some of the low and dead titer mutants are capable of RNA synthesis, but are not capable of stabilizing the vRNA template for protection against host endonucleases.

## H. Examination of Termination

There are several different assays that can be employed to monitor the level of influenza RNA generated by an infected or transfected cell. The three most popular assays include the primer extension assay, the RNase protection assay, and the Northern blot. The primer extension assay is useful for studying how the polymerase initiates




**UnPub. Fig.7 Transcription and Replication of Native NA Gene**

Native reporter RNA expression by mutant influenza polymerase in cells. 293T cells underwent 5-plasmid transfections that included either the wild-type (WT) PA protein vector or the indicated mutants, along with a native neuraminidase reporter that generates either a vRNA-NA (top panel) or cRNA-NA (bottom panel) transcript. Total RNA was harvested at 44 hours post-transfection and probed for vRNA, cRNA, and mRNA by primer-extension assay, using the NA-specific primers described in Unpub. Fig. 3. Mock = sham-transfected cells. vNA = vRNA reporter plasmid only. cNA = cRNA reporter plasmid only. –PA = PA vector omitted. Relative infectivities of the PA mutants (from Pub. Table 1) are indicated at bottom of each panel.

transcription since it focuses on the 5'-end of influenza generated transcripts. For example, the polymerase can initiate either primed transcription (mRNA synthesis) or unprimed transcription (cRNA- synthesis) from a vRNA reporter; and these two products can be distinguished from one another since they possess different numbers of nucleotides at their 5'-end. However, the primer extension assay provides no information as to the description of the 3'-end of the transcripts. Since the 3'-end of mRNA and cRNA differ, an RNase protection assay (RPA) can be employed to discriminate between the two products. Lastly, the Northern blot is used to detect whether or not full length transcripts are present.

During the course of this study, some of the RNA harvested from transfected cells was analyzed by both primer extension and RPA to examine both the 5'- and 3'- ends of the influenza transcripts. In order to perform RPA, it was necessary to design a probe that would distinguish between cRNA and mRNA products and can be internally labeled with $^{32}$-P. To do this, a probe was designed that would bind to the 300 nucleotides at the 3' end of mRNA and cRNA species. This probe binds fully to cRNA, but does not bind fully to mRNA due to the mismatches that are created with the polyadenylated tail. These base-paired products are treated with RNases that target single stranded RNA, to yield protected fragments that represent either mRNA or cRNA. The mRNA fragment of the neuraminidase gene is approximately 16 nucleotides shorter than the cRNA fragment, since mRNA transcripts have a poly (A) tail that begins at the polyuridine stretch, which is 16-21 nucleotides from the 5'-terminus of the vRNA. Due to the 16 nucleotide size difference between mRNA and cRNA, these species can be separated on a PAGE/Urea gel.

DNA plasmids were generated that contained the sequences that can be linearized and transcribed with T7 polymerase to generate the probe, as well as transcripts that can be used as controls for the expected length of mRNA and cRNA products. The clone used to generate the probe is called pBS-NA300(-). It was generated by PCR

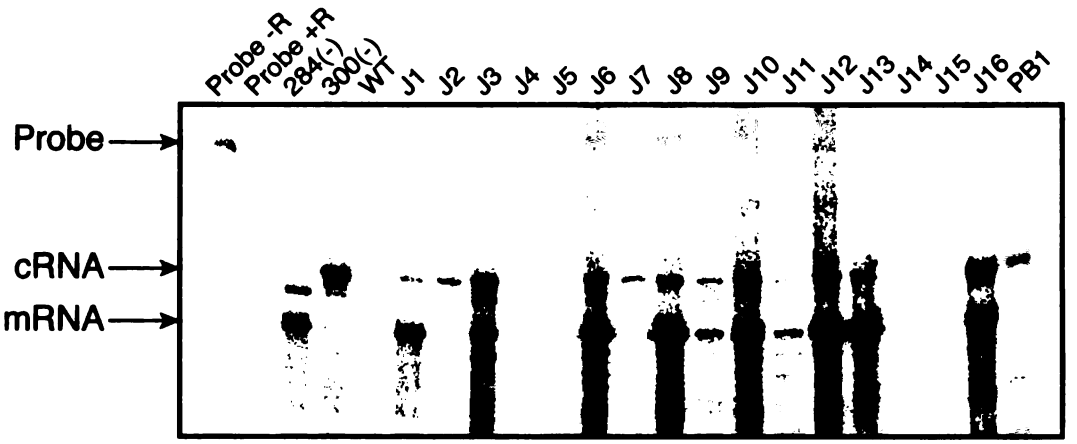
amplification of the NA gene using the following primers:
5'-GCCGCCCGCCTCTAGAAGTAGAAACAAGGAG-3' and
5'-GCCGCCCGCCAAGCTTGGACAGAGACTGATA-3'.

As positive controls for size, clones were constructed that would allow the *in vitro* transcription of mRNA and cRNA-like transcripts. To synthesize mRNA-like transcripts, the following primer pair was used to create the pBS-NA284(+) clone:
5'-GCCGCCCGCCTCTAGAGGACAGAGACTGATA-3' and
5'-GCCGCCCGCCAAGCTTTTTTGAACAAACTACTT-3'. To synthesize the cRNA-like transcripts, the following primer pair was used to create the pBS-NA300(+) clone:
5'-GCCGCCCGCCTCTAGAGGACAGAGACTGATA-3' and
5'-GCCGCCCGCCAAGCTTAGTAGAAACAAGGAG-3'.

The above primer pairs include *Xba* I and *Hind* III sites that were used for cloning into Stratagene's pBluescript II KS (+) vector. To generate transcripts, the clones were linearized with *Hind* III, phenol-chloroform cleaned, and transcribed with Ambion's MAXIscript T7 Kit. The length and sense of the transcribed RNA product is indicated by the clone name. Only the probe was internally labeled with an alpha $^{32}$-P nucleotide, the controls for mRNA and cRNA length were unlabeled.

As mentioned earlier, it is unfortunate that this experiment was performed prior to realizing that over-expression of PA with a native NA reporter causes reduced amounts of mRNA accumulation. Therefore, the RPA results, like the results from the primer extension analysis using a native neuraminidase reporter, are of limited use. As expected, the polymerase of medium and high titer viruses (WT, J5, J9, J11, J14, J15) produce mRNA and cRNA at very low levels (Unpublished FIG. 8). In contrast, the polymerase of dead or low titer viruses (J1, J3, J8, J10, J13, J16) yielded high levels of mRNA and detectable levels of cRNA.

According to the primer extension assay, PA is required for initiation of mRNA and cRNA synthesis. However, according to the RNase protection assay, PA is not




**UnPub. Fig.8 Examination of Termination**

The 3' end of the (+)-sense RNA products produced by the mutant polymerases given a pol vNA reporter were analyzed by RPA. 293T cells were transfected with protein expression vectors for xPB2/PR8, xPB1/PR8, xPA/WSN, and NP/WSN, and reporter vRNA expression vector pPol-NA/WSN. At 48 hours, RNA was harvested with Trizol. 10 ug of total cellular RNA was incubated with 80,000 cpm of internally labeled $^{32}$P-NA300(-) probe. In addition, probe was incubated with *in vitro* transcribed cold NA300(+) and NA284(+), which were included to provide length controls for the expected size of cRNA and mRNA, respectively. RNase protection was performed according to manufacturer recommendations (Ambion's RPA III), with the exception that RNase T1 was used rather than RNase A/T. Protected fragments were run on a 6% PAGE gel with 7.6 M urea, dried and exposed on a phosphor-imager screen for densitometry analysis using a Typhoon Phosphor Imager. Probe -R = Probe without RNase T1; Probe +R = Probe with RNase T1; 284(-) = control for mRNA length; 300(-) = control for cRNA length; PB1 = 5-plasmid transfection without PA, but twice the amount of PB1 (2μg).

necessary to get terminal cRNA synthesis. These data suggests that the PB1-PB2 can internally initiate RNA synthesis. In addition, because the synthesis does not get polyadenylated and terminates as a cRNA product, it suggests that PA is necessary for polyadenylation but not for RNA synthesis. Potentially, without PA, PB1-PB2 can enter the NA vRNA template internally without binding the 5'-end, and as a result, no polyadenylation occurs. However, in the presence of PA, the polymerase binds the 5' vRNA and helps hold on to it during transcription, which allows for stuttering and creation of a poly (A) tail.

## I. Fidelity

It is quite possible that some of the mutations created in PA affect the fidelity of the polymerase. The literature states that the mutation frequency for influenza A virus HA and NA genes for any single nucleotide appears to be about $10^{-5.5}$ per replication [669].

To examine whether the J10 polymerase has reduced fidelity, viral RNA generated from a pol vNA-Luc reporter was extracted at 36 hours from transfected cells and reverse transcription was performed to selectively enrich for particular RNA species. To selectively clone luciferase mRNA, cRNA, and vRNA species, the following primers were used for first strand synthesis by Superscript™ II (Invitrogen): 5'-CCCCCCCCCTC TAGATTTTTTTTTTTTTTTTT-3', 5'-CCCCCCCCCTCTAGAAGTAGAAACAAGG AGTTTTTTGAACAA-3', and 5'-CCAGCCAGCAAGCTTATGGAAGACGCCAAAA ACATAAAG-3', respectively. The first two primers have *Xba* I restriction enzyme sites and the last primer has a *Hin*d III restriction enzyme site. After first-strand synthesis, the cDNA was incubated with RNase H and RNase A/T prior to PCR amplification. The primers for second strand synthesis included: 5'-CCAGCCAGCAAGCTTATGGAAGAC GCCAAAAACATAAAG-3', 5'-CCAGCCAGCAAGCTTATGGAAGACGCCAAAAA CATAAAG-3', and 5'-CCCCCCCCCTCTAGAAGTAGAAACAAGGAGTTTTTTGAA

CAA-3', for mRNA, cRNA, and vRNA, respectively. The first two primers contain *Hind* III sites and the last primer contains a *Xba* I site. After PCR amplification using high fidelity Tgo polymerase (Roche), products were cut with *Hind* III and *Xba* I for ligation into pBSIIKS+. The PCR amplified luciferase coding region was approximately 1690 nucleotides long. A caveat of this cloning strategy is that incomplete transcripts were selectively excluded.

A total of 30,400 nucleotides of WT transcribed RNA was sequenced and compared to a total of 26,200 nucleotides of J10 transcribed RNA (Unpub. Table 1). Of note, vRNA species produced by the transfection system are the product of both human RNA polymerase I and influenza polymerase synthesis. Due to this artifact of the transfection system, more cRNA clones were sequenced to obtain a more accurate and representative look at the genome mutation rate. Each of the four sequenced mRNA clones of J10 and WT had poly(A) tails of different length, suggesting non-redundant clones were selected for sequencing. Similarly, none of the seven clones that contained non-wild-type sequence were identical, again suggesting non-redundant clones were selected for sequencing. Any ambiguous sequence was confirmed by sequencing the opposite strand.

The mutations found in WT-generated RNAs consisted only of skipped adenosine nucleotides. A total of two skipped nucleotides were found in cRNA, which corresponds to a replication mutation rate of one per 8,400 nucleotides ($1.1 \times 10^{-4}$). In contrast, six mismatch mutations were found in RNA from the J10 polymerase, which corresponds to a four-fold increase in replication mutation rate, equating to approximately one mutation per 2,200 nucleotides ($4.5 \times 10^{-4}$). The six mutations found in J10 cRNA were spread throughout the genome, revealing no mutation hot-spots exist.

## J. Dominant Negative - Silencing

To determine whether any of the PA mutants act in a dominant negative manner

| | WT | | J10 | |
|---|---|---|---|---|
| mRNA | 7,800 | Skipped 1 'A' | 6,500 | No Mutations |
| cRNA | 16,600 | Skipped 2 'A' | 13,200 | 6 Mutations* |
| vRNA | 6,000 | No Mutations | 6,500 | No Mutatoins |
| | | | * 2G→U, 2A→U, 1G→C, 1C→U | |

**Unpub. Table.1 Fidelity of J10**

RNA generated from WT and J10 transfected cells were selectively cloned and sequenced to examine the fidelity of the polymerases.

against WT PA, 293T cells were transfected with both WT and mutant PA, and examined for defects in luciferase production (Unpublished FIG. 9).

Interestingly, when pEGFP-N1 was used in the presence of WT PA, the expression of ß-galactosidase was selectively depressed or silenced. As a result, the control transfection that included wild-type PA with only pBSIIKS(+) was used for comparison to the mutants. The mutants reduced luciferase activity to the following: J2 = 91%, J3 = 11%, J7 = 70%, J9 = 63%, and J16 = 23%, when compared to WT PA that was set to equal 100%. Similar results were observed in another experiment, but due to the silencing of ß-galactosidase activity by wild-type PA and pEGFP-N1, averages were not calculated.

In the transient transfection system, J3 acts as a dominant negative. To test whether this effect would be stronger if J3 was expressed prior to WT expression, stable transfectant cell populations were created. Protein expression vectors for WT, J3, and pEGFP-N1 were transfected individually into different lots of MDCK cells with Fugene-6. To select for transfectants, 400 $\mu$g G418/mL of media was added two days after the transfection. Selection was maintained for growth and passage, and after sixteen days, most cells transfected with pEGFP-N1 showed GFP expression by fluorescence microscopy. Subconfluent monolayers of stably transfected MDCK cells (WT, J3 and pEGFP-N1), were infected with wild-type virus at an M.O.I. of 0.0002, $2x10^{-5}$, and $2x10^{-6}$. After 48 hours, plaques were examined by crystal violet staining, and no significant difference between wild-type and J3 was observed. To test whether this negative result was due poor stable expression of J3, MDCK cell lysates were tested by Western blot against PA. Western blot analysis failed to detect J3 or WT (data not shown), suggesting that a different methodology should be employed to achieve stable expression of PA in MDCK cells.

To examine the possibility that mutants may be dominant negative for other functions of PA not associated with transcription and translation of a non-influenza

reporter; the mutants were included in a competition experiment with wild-type for reconstitution of VLPs. The following mutants were tested: J1, J2, J3, J7, J9, J10, J13, and J16. In the transfection, mutant PA protein expression vector was included at ten fold excess over wild-type (1.0:0.1 μg/35 mm dish), and no mutant PA vRNA expression vector was included. Because WT PA vRNA expression was included in the experiment, any successfully assembled and packaged VLP would form a wild-type plaque. The transfection was performed as previously described, with the exception that only the 12 essential plasmids were included, excluding protein expression vectors for HA, NA, M1, M2, and NS2. Supernatants were harvested at 48 hours and titered.

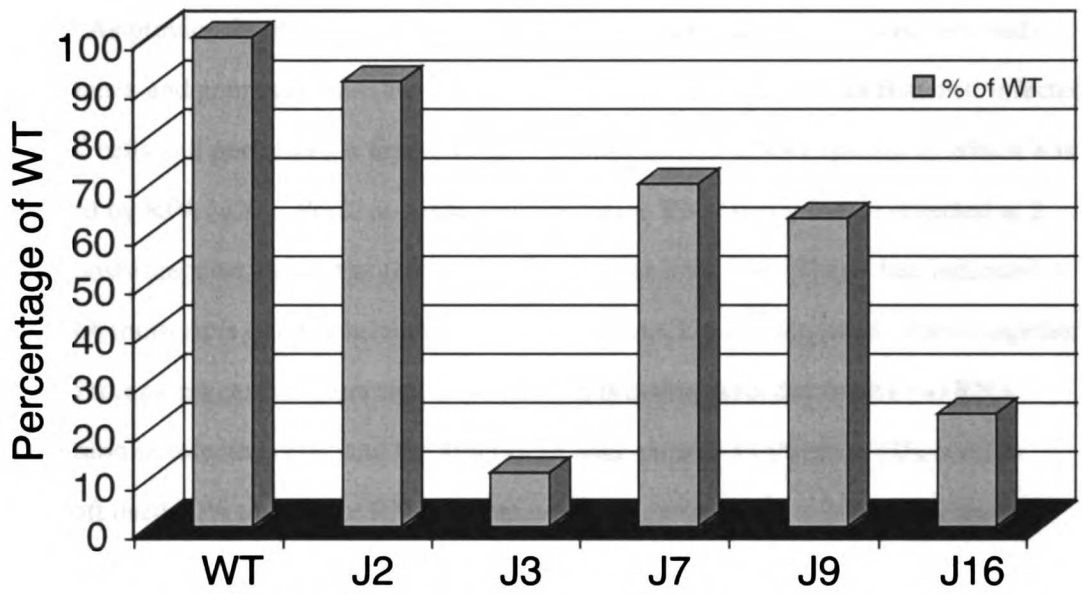
No significant difference was observed by including any of the mutant protein expression vectors (data not shown). In retrospect, this transfection should have been only permitted to continue for 24 hours to reduce to possibility that re-infection of the 293T cells has obscured the results.

## K. (+) RNA Matrix Expression in Infected MDCK Cells

To determine the optimal time at which to harvest RNA for the detection of matrix mRNA and cRNA in infected MDCK cells, a time-course experiment was performed (Unpublished FIG. 10).

At three hours post-infection, both matrix mRNA and cRNA are detectable, and the levels rise to maximum expression at five to six hours post-infection. After six hours, both species exhibit reduced expression levels. By nine hours post-infection, mRNA levels dropped to levels seen at three hours, and cRNA is practically undetectable. Of interest, a slower migrating species that ran at approximately 140 nucleotides, became detectable at five hours post-infection, and increased in intensity through nine hours (data not shown). It is possible that the expression of this product is induced by the viral infection and it has cross-reactivity with the matrix primer.

These data suggest that mRNA expression is concomitant with cRNA expression.




**UnPub. Fig.9 Dominant Negative Experiment**

293T cells were transfected with a mutant PA protein expression vector plus a master mix consisting of protein expression vectors xPB2, xPB1, xPA (WT), and xNP, reporter pPol-vNA-Luc, and a ß-galactosidase expression vector, pCH110, to control for transfection efficiency. Each PA mutant protein expression vector was added to the master mix in five-fold excess to wild-type PA protein expression vector. Two controls were included. The first control had no mutant PA, but included pEGFP-N1 and pBSIIKS(+), to equalize each transfection to DNA content and the number of CMV promoters. The second control also lacked mutant PA, but did included pBSIIKS(+), but no pEGFP-N1. Each control contained the 5-plasmid transfection plus pCH110. At 22 hours, cells were lysed with 1X RLB, and lysates were normalized to ß-galactosidase activity. Normalized lysates were diluted with 1 mg/mL BSA/PBS, and luciferase activity was measured with Turner Luminometer.

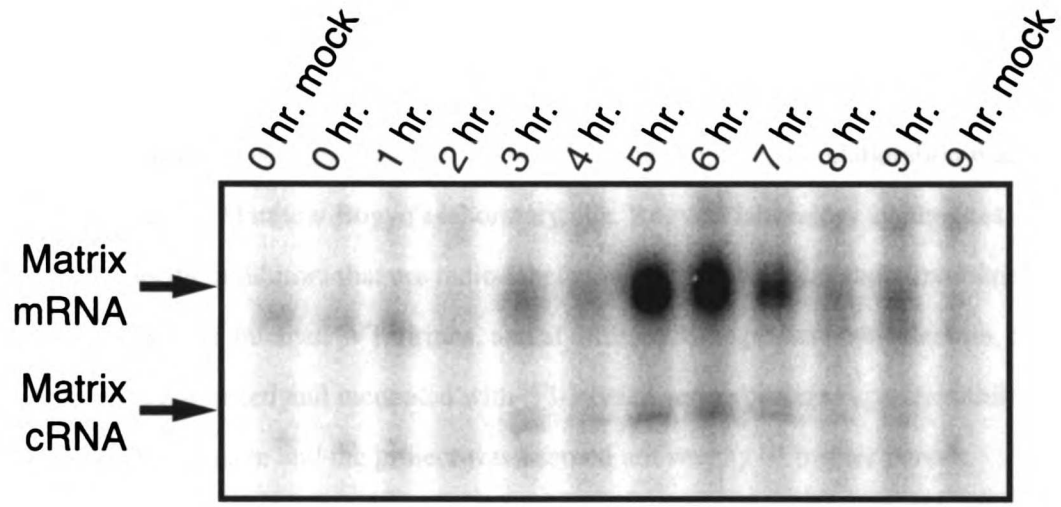
This finding is not in agreement with the dogma that primary transcription proceeds replication, yet other laboratories have published similar results [480, 515].

As previously mentioned, influenza has temporal regulation of transcripts and both matrix and neuraminidase are late genes. Interestingly, Dr. Christa Bancroft infected MDCK cells and performed a time-course of neuraminidase RNA expression, which was detected by RPA [670]. Positive-sense neuraminidase RNA species were detected at 2 hours post-infection, but not at zero or four hours post infection. These data indicated viral NA transcripts quickly accumulate, but just as quickly are degraded. Taken together, these findings suggest neuraminidase (+) RNA expression precedes matrix (+) RNA expression in infected cells, and the kinetics of degradation is different. Of note, Dr. Bancroft used RPA to analyze RNA, whereas primer extension was used in the present report.

## L. Serine Protease / RRL Work

An abstract presented at the Options for the Control of Influenza IV Conference, at Hersonissus, Crete, Greece in September 2000, suggested that PA may be a serine protease [652, 671]. This finding supported earlier work by another group that indicated that PA induced generalized proteolysis [646, 647]. In light of these published results, we decided to investigate whether PA would cleave itself or other viral proteins when expressed in a rabbit reticulocyte lysate. To examine potential cleavage, influenza proteins were transcribed and translated from the pGEM vectors in the presence of $^{35}$S-methionine. Radio-labeled proteins were incubated with unlabelled PA and the reaction mixture was examined by SDS-PAGE to see whether any of the radio-labeled products would be cleaved.

Protein expression from the pGEM vectors was found to vary depending on the lot of rabbit reticulocyte lysate used. PB2, PB1, PA, NP, and M1 expressed well, whereas HA, NA, M2, NS1, NS2 were expressed poorly or not at all. The subunits of




**UnPub. Fig.10 Timecourse of mRNA Expression**

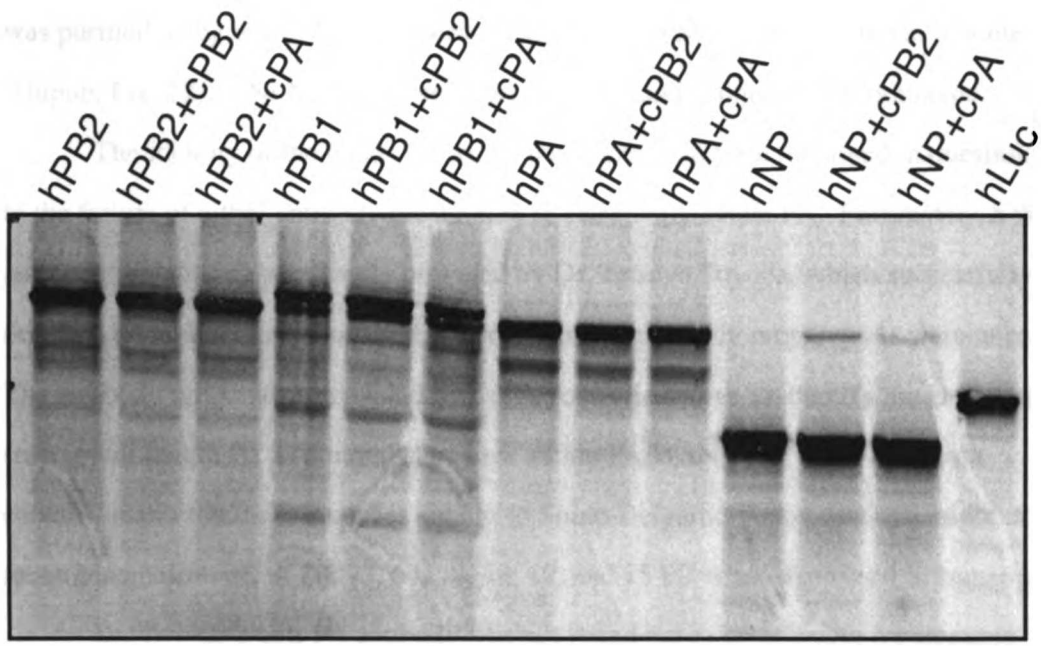
MDCK cells were infected at an M.O.I. of 3 with wild-type virions. RNA was harvested by Trizol at one hour increments for nine hours, including mock infection time-points that were harvested at zero and nine hours post-infection. The RNA was analyzed by primer extension to assess the abundance of matrix mRNA and matrix cRNA. The primer M90(-), 5'-GCTTTGAGGGGGCCTGAC-3', was 5' - end labeled with $^{32}P$, and 150,000 cpm was added to 5 $\mu$g of total RNA. Primer extension by Superscript II was carried out as previously described. Products specific to the matrix gene, represented mRNA and cRNA species of 99-102 and 90 nucleotides in length, respectively, were analyzed on a 6% PAGE, 7.6 M urea gel.

the polymerase and NP were incubated with unlabelled PA or PB2 for a control for 0, 6, or 24 hours (Unpub. Fig. 11). No apparent processing is evident in rabbit reticulocyte expressed proteins, suggesting that if processing or induction of proteolysis occurs, it does not occur in the rabbit reticulocyte lysate system.

To further investigate whether PA was a serine protease, a collaboration was initiated with Dr. Matthew Bogyo's laboratory. Dr. Bogyo's laboratory synthesizes protease specific inhibitors that are radio-labeled with $^{125}I$. To utilize these inhibitors, MDCK cells were infected WT virions, and at different time points post-infection, cell lysates were harvested and incubated with $^{125}I$-labeled serine protease specific inhibitors. Results were negative and the project was deemed not worthy of further pursuit.

## M. Anti-PA Antibodies / Bacterial Expressed PA

Initially, the PA project was in jeopardy due to the inability to detect PA by Western blot. First, we had anti-serum generated in rabbits against two PA decamer peptides that resided in the region of M2 and M9 and had been KLH-conjugated. Despite a strong titer against these peptides, we were unable to detect PA protein in virions or in transfected cells. Similarly, we received anti-serum as a kind gift from Dr. Mark Krystal and again we were unable to detect PA. Because PA had been shown to be a target of the proteolysis that it induces, we were fearful at the time that we were not expressing the protein to a high enough level for detection, or that even if it was being expressed, it was being quickly degraded. Due to our inability to detect PA, we decided to express PA in bacteria to make a positive control to test the two antibody preparations. To this end, two constructs were made the encoded full-length PA and a truncated form of PA that contained 204 N-terminal residues, respectively. Both constructs contained a C-terminal six-histidine tag and were cloned into bacterial expression vectors pTrcHis 2A (Invitrogen) or pET 21d (Novagen). The pTrc vectors were expressed in XL-1 Blue cells




**UnPub. Fig.11 Rabbit Reticulocyte Expression**

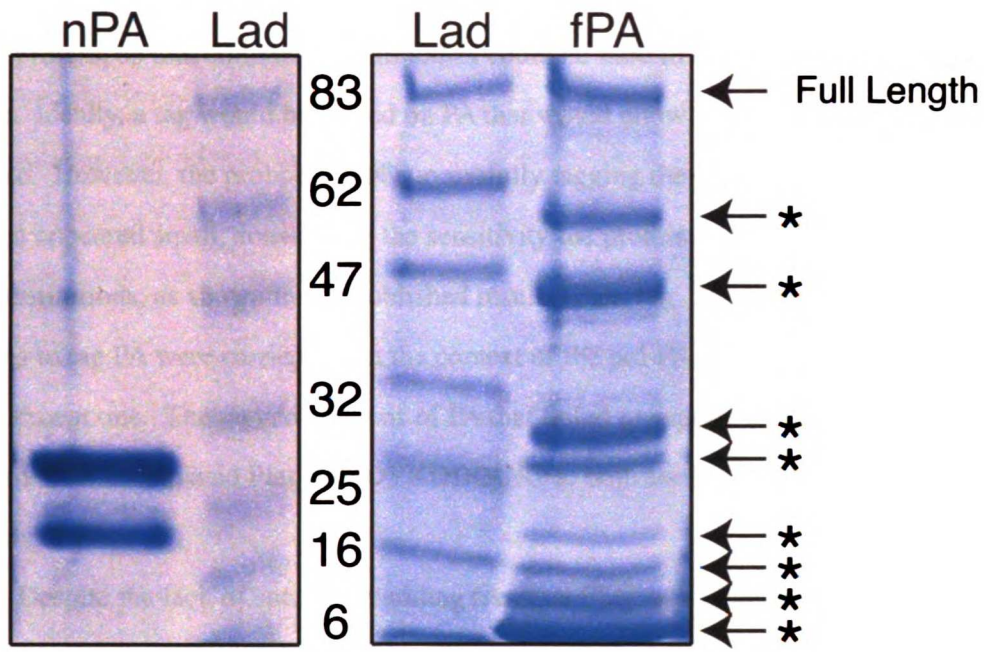
$^{35}$S-radiolabelled influenza proteins that were generated in rabbit reticulocyte lysate were incubated with unlabelled PA to determine whether proteolytic processing occurs. Labelled and unlabelled protein preparations were incubated at 30°C for 0, 6, and 24 hours. The zero hour time point is shown here, but the results did not differ with longer incubation periods. The 'h' and 'c' refer to $^{35}$S-radiolabelled and unlabelled protein, respectively. PB2 is used as a negative control.

and DH5α cells and the pET vectors were expressed in BL21 (DE3) pLys E cells.

IPTG induction resulted in recombinant expression in both systems within one hour, which increased slightly over four hours (data not shown). Recombinant protein was purified using either Talon Superflow Metal ($Co^{2+}$) Metal Affinity Resin (Clontech) (Unpub. Fig. 12) or Ni-NTA Spin columns (Qiagen) and stained with Coomassie.

The identity of the bands shown by the Coomassie stain remained in question due to the failure of either antibody preparation to selectively detect PA. Fortunately, a third antibody preparation was kindly provided by Dr. Tetsuya Toyoda, which successfully detected PA and demonstrated that the previous two antibody preparations were unusable. The antibody preparation provided by Dr. Toyoda allowed us to identify the laddering seen in full length PA as internal start sites within PA/WSN. The PA/WSN strain contains many internal sequences similar to Shine-Delgarno boxes, and one might expect recombinant proteins of 84, 77, 64, 58, 39, 19, and 15 kD when expressed in bacteria.

Dr. Toyoda's anti-PA antibody was not found not to be effective for immuno-fluorescent staining. To address this issue, the N-terminal PA that was describe above, was expressed in bacteria and purified using Talon Resin. The N-terminal PA fragment was purified by gel electrophoresis, and the un-stained band was electro-eluted from the gel slices. The protein was dialyzed against TE, but during the dialysis it became insoluble. The precipitate, in dialysis tubing, was solubilized with urea and refolding was carried out at room temperature by diluting the dialysis solution that contained urea with equal volume of TE. Step-refolding was carried out until essentially no urea remained in the sample. The sample was then soluble and could be frozen and thawed without forming precipitate. The protein was quantified using a Lowry assay and sent to Covance for antibody production in rabbits. The resulting antibody was found to be effective for both Western blot and immuno-fluorescent staining. The quality of this antibody was




**UnPub. Fig.12 Bacterial Expression of PA**

Expression and purification of the N-terminal 204 amino acids and full length of PA/WSN that have C-terminal six-histidine tags. Expression was induced by IPTG in XL-1 Blue cells and purification was performed utilizing Talon $Co^{2+}$ resin.

superior to the others we had available and was used for all subsequent experiments.

## N. Viable Virus with a Tagged PA

The initial struggles with bad PA antibody preparations forced us to try to tag the protein, so that commercial antibodies could be used to detect and purify the protein. Ideally, a tag would be placed on PA that would allow the virus to successfully replicate. However, the probability of successfully tagging the protein without affecting function appeared small, considering the sensitivity the protein exhibits to di-amino acid substitutions, as shown in our published results (Pub. Fig. 2). Nevertheless, several attempts to tag PA were carried out in the context of the pol-PA vector, and all of them failed, except one. The tagged versions of PA that failed to support viral replication included the 8 amino acid Flag tag (DYKDDDDK) at both the N-terminus and C-terminus of PA.

Despite the lack of success in adding terminal Flag tags to the PA protein, we attempted to insert an internal tag. Under my supervision, Jeff Stone, a rotation student in the laboratory, attempted to place an internal tag in the Pol-PA vector. We decided to try both internal Flag and His tags. We searched for a region of PA that was not highly conserved, was hydrophilic, and ideally was flanked by proline residues, which create kinks in the secondary structure. We decided to insert the tags between residue 268 and 269. This site was attractive since type B viruses have 7 residues that fall between these residues in type A viruses, and residues 267 and 271 are both prolines.

The internal Flag tagged PA did not permit viral propagation, but the internal His-tagged PA allowed for the propagation of virus to a titer comparable to wild-type. Based on the published literature, this is the first tagged protein in the polymerase complex that allows for successful viral reconstitution. This construct may prove valuable for purifying the 3P complex for biochemical experimentation and analysis, although the HIS epitope is not ideal for this purpose. A Western blot of the recombinant WSN virus which

contained an internal His-tagged PA was performed, and the blot suggested an anti-His antibody can be used to detect His-tagged PA (data not shown).

The clone was created in the following manner. The reverse primer used to create this clone was 5'-GGGAGGCCCATCCGGAAGTCTATGATGATGATGATGATGGAG CGGTCGTGGTGTTGATTTC-3', which contains a *Bsp*E I site for cloning and a *Bsr*B I site for diagnostics. This primer was used for PCR in conjunction with a sequence specific primer upstream of the *Sfi* I site in PA/WSN. The PCR product was cloned into pBSIIKS+PA/WSN using the mentioned restriction enzymes and then subcloned into Pol-PA with *Bbs* I and *Afl* II. Sequencing confirmed the presence of the tag.

## O. Fluorescent Microscopy of PA

To determine whether J10 experienced a localization defect, attempts were made visualize J10 and WT PA in transfected cells using confocal fluorescent microscopy. 293T cells were transfected with protein expression vectors for PA and indirectly stained. Stained cells were Dapi counterstained, and Z-sections were taken using confocal microscope, with the assistance of Kelly Dubois and Marla Abodeely. Generally, by microscopy 75% of the cells transfected showed WT PA localized to the nucleus (Unpubl. Fig. 13A). In a time-course experiment, cells were processed at 18, 24, 30, and 48 hours, and the localization pattern of 75% nuclear to 25% cytoplasmic remained constant. Strangely, this result was not supported by cellular fractionation of cells transfected with PA that were harvested at 51 hours, which demonstrated that greater than 80% of WT PA localized to the cytoplasm (Western blot - not shown). The reason for WT appearing to be localized in the nucleus by immuno-fluorescent microscopy, but fractionating to the cytoplasmic compartment is unclear. In contrast, J10 PA localized to the cytoplasm in the majority of transfected cells by both immuno-fluorescent microscopy and cellular fractionation. Although no time-course was performed with J10, cytoplasmic localization was observed at both 48 and 52 hours post-transfection. Of note, our published data

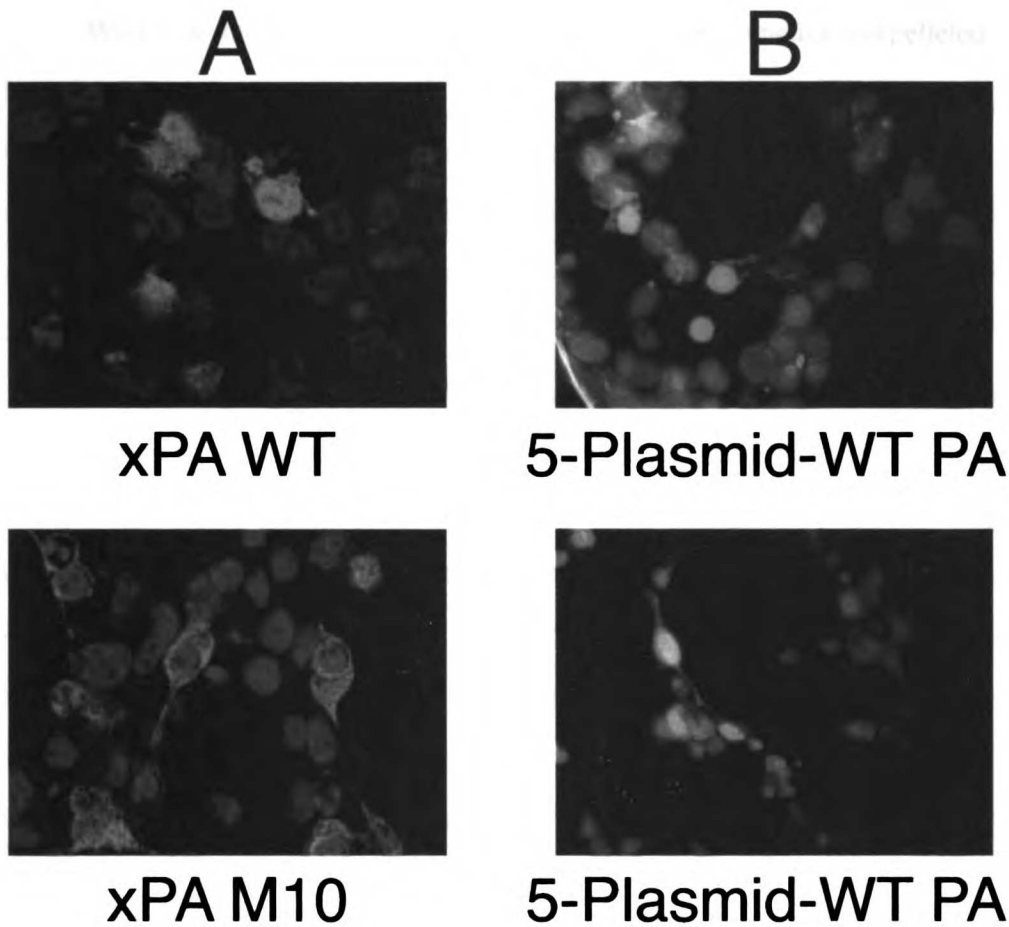
demonstrate that after cellular fractionation of cells expressing each of the 5-plasmids (Moflo sorted), WT and J10 PA are equally distributed between the nucleus and cytosol, and cells without the full complement of 5-plasmids have approximately 75% of the PA in the cytoplasm (Pub. Fig. 6).

Work from other laboratories has suggested that the localization of PA is largely dependent on the expression of the other polymerase subunits. To this end, cells were transfected with the 5-plasmid system using a pol-NA-363-GFP-286 reporter (previously described). Only cells expressing all of the influenza polymerase subunits, the protein expression vector for NP, and this fusion reporter will turn green.

Microscopy on 5-plasmid transfected cells was performed using WT PA. Overlay of the green, red, and blue channels indicated that GFP+ cells, which require PA expression, did not stain with the PA antibody (Unpub. Fig. 13B). However, some red cells were observed, which presumably express PA, but do not express the other components of the 5-plasmid system. These data suggest that the antigenic epitopes of PA that are recognized by the polyclonal sera are masked when PA is expressed in the 5-plasmid system. Masking could be due to a conformation change in PA that occurs when it binds to PB1, or other viral or cellular protein/RNA may sterically prevent antibody binding. This finding is particularly interesting, since the anti-sera was raised against the N-terminal 204 amino acids of PA, which are not thought to be involved in binding PB1.

## P. EM Images

Virion particles were purified through a glycerol gradient as described in our published data. Virus containing fractions were dialyzed to remove the glycerol, and fixed with 2% formaldehyde. Fixed particles were placed on Holey Carbon grids (Tedpella, Inc.), negative stained with phosphotungstic acid, and visualized by transmission electron microscopy with the assistance of Dr. Chris Regan at the University




**UnPub. Fig.13 Localization of PA by Fluorescent Microscopy**

293T cells were transfected and fixed with 2% formaldehyde at 52 hours post-transfection. Cells were permeabilized with 0.2% Triton X-100 in 5% skim milk/PBS and stained for PA with polyclonal sera followed by rhodamine red conjugated donkey anti-rabbit sera (Jackson ImmunoResearch Laboratories). Stained cells were incubated with ProLong Gold Antifade Reagent (Molecular Probes) that contained Dapi stain, and visualized using a fluorescent microscope. Cells were transfected with either xPA alone (WT and J10) and visualized by confocal microscopy-Z-sections (A) or the 5-plasmid system including a pol-NA-363-GFP-286 reporter (WT PA only) and visualized with conventional fluorescent microscopy (B).

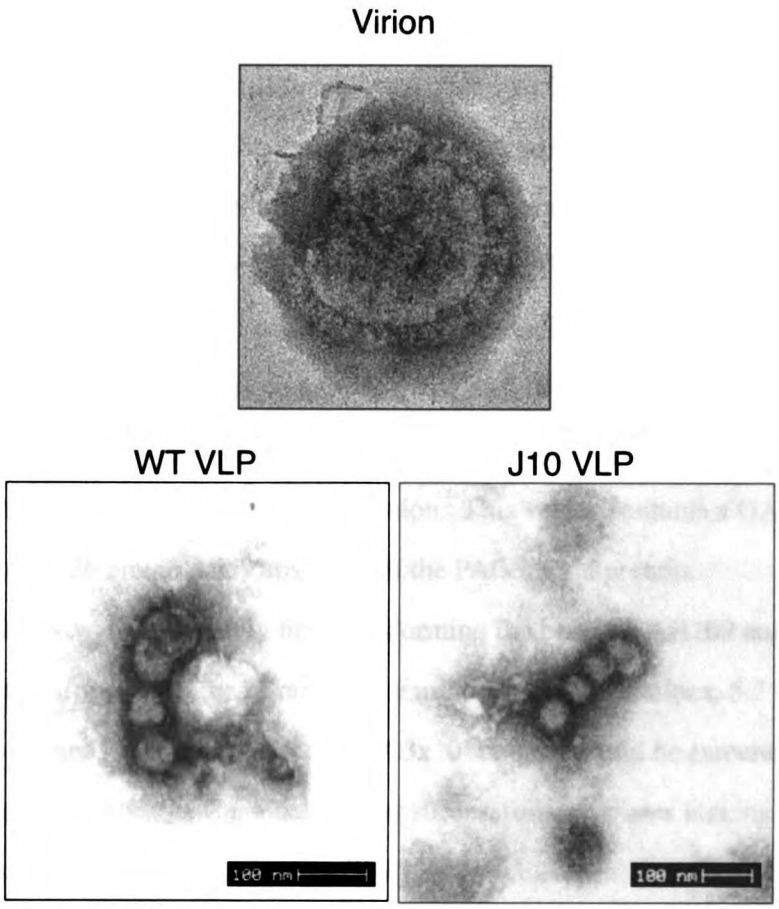
of California, Berkeley (Unpub. Fig. 14).

Wild-type and M10 VLPs were generated by reverse genetics and pelleted through a 20% sucrose cushion, at 27,000 rpm for 3 hours in Sw41 Ti Beckman Rotor, with slow acceleration and deceleration. Pellets were resuspended in Karnovsky's morphology fixative and negative stained with phosphotungstic acid. Samples were placed on carbon coated grids and visualized with transmission electron microscopy with the aid of Ivy Hsieh.

These EM images WT and J10 VLPs are of little value for the several reasons. First, these preparations were not sufficiently purified and there was a significant amount of cell debris, which made visualization of the VLPs difficult. Secondly, not enough VLPs were observed to make any statements regarding structural differences between WT and J10. And lastly, it is unclear if these particles contain influenza antigens since immuno-staining was not performed. Presumably, the virus-like particles in the J10 sample do not contain viral RNA, as suggested in our published results (Pub. Fig. 7). In addition, we would argue that in comparison to WT, there are few J10 generated particles, and they do not have the same buoyant density as normal viral particles, which contain matrix and nucleoprotein (Pub. Fig. 6). It is possible that these virus-like particles are simply spherical forms of cellular lipid bilayers that have no influenza associated proteins or RNA.

## Q. Yeast Two-Hybrid and Hsp40 Work

To examine whether PA interacts with any cellular factors to facilitate the viral replication cycle, a yeast two-hybrid assay was performed with the aid of a Matchmaker cDNA library that was purchased from Clontech. The library had been created from the lungs of two female Caucasians, ages 24 and 32. The library was constructed by priming reverse transcription with *Xho* I-(dT)15, and directionally cloning the products into pACT 2. The library contained $3.3 \times 10^6$ independent clones/mL (2 mL total). The number of




**UnPub. Fig.14 Transmission Electron Microscopy**

Virion and VLP samples were fixed, negative stained, and examined by transmission electron microscopy.

clones in the library was titered at approximately $1x10^8$ clones/mL. In order to fully screen the library, the manufacturer recommends screening three times the number of independent clones. Therefore, $1x10^7$ independent clones were amplified by diluting 100 $\mu$L of the library into 125 mL of LB, which was plated on 250 150 mm ampicillin plates (0.5 mL/plate). The plates were harvested after an overnight incubation at 37°C and maxipreped by CsCl to yield 1.89 mg of DNA. To construct the bait, PA A/Victoria/3/75 from the pGEM-3Z vector was removed with *Eco* RI and *Pst* I. The fragment was cloned into pGBKT7-BD, cut with *Nco* I and *Pst* I. Cloning was facilitated by the addition of a synthesized oligonucleotide linker containing *Nco* I and *Eco* RI sites. The cloned vector, pGEM-PA/Victoria was sequenced and found to be the same as previously published [630], with the exception of a G485A substitution. This vector contains a GAL4 BD, and a c-myc tag that is 26 amino acids upstream of the PA/Victoria protein.

The library was screened by first transforming the bait into AH109 and subsequently transforming in the library. After multiple transformations, $5.7 \times 10^6$ clones were screened. Ideally, an additional $4.3x10^6$ clones should be screened in order to examine the entire library. Candidates grew successfully on plates lacking Ade/Leu/ Trp/His and turned blue in the presence of x-alpha galactosidase. In total, 30 candidates were selected, some of which grew poorly in liquid culture and no DNA was extracted. The candidates were identified as RNA helicase SkiW2, human hnRNP H, mammalian translation initiation factor INT-6, clathrin heavy chain, arylsulfatase, IG Mu chain C region, hspC024, hsp40, and hspC152. The clathrin heavy chain was represented twice as two separate clones that contained overlapping regions. Other candidates include unidentified proteins that share homology with the following: translation initiation factor eIF3, Class I histocompatibility antigen, ubiquitin, and pinch protein.

Many of the candidates could conceivably be involved in influenza biology. Hsp40 was followed up, since it has been shown that influenza infection results in the release of hsp40 from the $p58^{IPK}$, the inhibitor of PKR. This action allows influenza

to inhibit PKR from shutting down the host translation machinery by phosphorylating eIF2α. To examine the possibility that PA may be involved in this process, a CMV driven hsp40 construct, created by Steve Massa, was obtained from Dr. Bill Welch's laboratory. To test whether PA binds and sequesters hsp40 upon influenza infection, the CMV-hsp40 was transfected concomitant with the 17-plasmid system to overload the amount of hsp40 in the system. The hypothesis was that PA would bind to the exogenous hsp40, leaving the endogenous hsp40 bound to $p58^{IPK}$. Because hsp40 would never be released from $p58^{IPK}$, PKR would become active and shut down host protein synthesis and the viral infection would be compromised. However, the addition of hsp40 had no effect on viral titer. A better experiment would have been to transfect 293T cells with CMV-hsp40 24 hours prior to low M.O.I. infection (rather than a transfection), and titer the subsequent supernatant. In order for this experiment to work, transfection efficiency would need to be high. This project is worthy of pursuit, but unfortunately, at the time, it was not a high enough priority to warrant additional work.

# Chapter 45. Discussion

My discovery that the J10 mutant fails to produce RNA-containing VLPs suggests that the PA subunit plays a role in viral assembly. Many questions regarding influenza viral assembly remain unanswered, including how the vRNPs are transported from the nuclear pore to the site of budding, how assembly of viral proteins and packaging of the genome occur at the plasma membrane, and what the driving force is behind viral budding. The PA subunit may be involved in one or more of these steps.

The sequence of PA that was altered by the J10 mutation, 502-GFIIKGRS-509, resembles a nucleotide binding motif [630], also called a P-loop whose consensus sequence is (A/G)xxxxGK(S/T) [666]. It is questionable whether the conservative lysine to arginine mismatch would maintain the functionality of this motif. However, the

consensus sequence of P-loops is not absolute, especially for the residues flanking the glycine-rich loop, as EGFR and BRAF, both of which have functional P-loops, contain the sequence GxxxxGTV [667]. In PA, the two glycines and the serine residue are 100% conserved in the 52 type A and type B influenza isolates used in the alignment for my study. Previous studies have mutated residues near or within this putative motif of PA. Mutation S509A was found not to affect the polymerase function, whereas H510A was shown to selectively inhibit the activation of cap endonuclease activity [632]. Insertion of a single serine residue between positions 509 and 510 yielded a dominant negative mutant that did not support polymerase activity [590]. There are some data that argue against a functional P-loop. These data include mutation G502A, which eliminates polymerase function [632]. This mutation replaces a critical glycine residue with an alanine, which according to the consensus sequence should maintain functionality of the P-loop. Collectively, these results cast doubt on whether the P-loop is functional, however, they do not preclude the possibility.

Initially, I was hopeful that the P-loop binds ATP and directs the placement of the first nucleotide during primer-independent replication, since the 5'-end of both vRNA and cRNA usually begin with ATP. However, my primer extension data suggest that the 5'-ends of cRNAs and vRNAs generated from WT and J10 polymerases are the same, which makes this possibility unlikely.

If the P-loop turns out the be functional, I have several hypotheses regarding its enzymatic function. First, J10 may fail to assemble free-floating particles that contain vRNA due to a defect in the ability of the polymerase to transport newly synthesized vRNP from the nuclear membrane to the plasma membrane for budding. Potentially, PA hijacks kinesin, which is a motor that transports cargo to the plus-end of microtubules, and transports vRNPs along the microtubule network up to the plasma membrane where packaging occurs. My second hypothesis is that PA is a kinase, whose activity is required

for late-stage assembly.

Alternatively, if the P-loop is not functional, this region of the protein may facilitate the assembly of the virus by way of its structural characteristics.

Aside from the speculation surrounding the J10 mutation, PA is likely to have other functions that are independent of this motif. In our published data, we describe several mutants that appear to have a specific bias toward accumulating certain RNA species. Aside from J10, which has been shown preferentially to accumulate cRNA over vRNA, J14 accumulates vRNA without much mRNA, and J8 appears to favor mRNA accumulation. My primer extension data suggest that the 3P polymerase fluctuates between two conformational states: one that promotes transcription and the other promotes replication. These data suggest that PA may indeed modulate transcription and replication, and that such modulation may be dependent on the concentration of PA in the cell, as suggested by Unpub. Fig. 1.

In addition, the RPA data suggest that in absence of PA, PB1 and PB2 can bind internally to at least the neuraminidase gene, and allow for "transcription" without poly (A) addition. The addition of PA to complete the 3P polymerase enhances the specificity of the polymerase for initiating transcription at the 3' end of the vRNA. These results suggest that PA is necessary for proper recognition of the promoter, and such binding is required for poly (A) addition.

The finding that J1, J4, J6, J8, J9, J10, and J12 fail to accumulate vRNA (Pub. Fig. 2) despite high levels of mRNA accumulation suggests that PA may be involved in stabilizing vRNA complexes. This hypothesis is supported by Vreede *et al.*, who showed that newly synthesized polymerase was sufficient to bind to, and stabilize (protect) cRNA from host nucleases [480]. One would presume that vRNA also needs to be stabilized

and transported to the assembly site prior to packaging into the viral particle.

# Chapter 46. Future Directions

## A. Specific Aim #1

**Determine the localization of matrix protein in the transfected cells.** Although the localization of PA within the nucleus and cytoplasm of cells transfected with the 5-plasmid system appears to be normal, there is the possibility that a sub-compartment localization defect will be visible by electron microscopy. Initially, this issue will be examined indirectly by examining the localization of the matrix protein, to which we have a good monoclonal antibody. Electron microscopy will be performed on cells transfected with all 17 plasmids excluding the xM1, xM2, pol NA, and xNA plasmids. The exclusion of NA should trap the VLPs on the surface of the cells, and any cells expressing matrix protein will minimally have the polymerase, xNP, and pol M vectors. At 36 hours, cells will be trypsinized and washed prior to pelleting. Pellets will be fixed, dehydrated, embedded, and sectioned. Cell pellet slices will be immuno-stained using an anti-matrix antibody and an anti-mouse gold conjugated antibody. Additional work may be carried out using anti-PA antibody with a rabbit secondary antibody that is conjugated to a different sized gold bead. These experiments should shed light on whether matrix shells are forming within the cytoplasm and whether PA is near these shells.

## B. Specific Aim #2

**Determine if PA contains a functional nucleotide-binding motif.** The J10 mutation falls in a region of PA that is a putative nucleotide-binding motif [630]. I will examine whether PA binds nucleotides by purifying the J10 and WT polymerase

complexes. This will be achieved by running lysates that contain the polymerase over a streptavidin column that has already bound biotin conjugated to RNA that contains the sequence of the influenza promoter. Bound polymerase will be washed before adding radiolabelled nucleotides. If differential binding is observed between J10 and WT that is nucleotide specific, it would suggest that the J10 region of PA facilitates the binding of nucleotides and that PA may have enzymatic activity.

If the nucleotide-binding motif is functional, PA can be included in the nucleotide triphosphate hydrolase superfamily of proteins. This superfamily is extremely diverse and has 21 functions in terms of enzyme commission number [672]. Structure analysis on this superfamily has divided the members into four functional clusters, including various functional proteins, signal transduction proteins, hydrolases, and transferases. The tertiary structure of PA will be modeled and placed into the multidimensional scaling plot for this superfamily to determine the functional cluster to which PA most likely belongs.

Review of the literature suggests that PA may be an ATP-dependent kinase, which is a type of transferase. Many influenza proteins are phosphorylated, although the exact function of the phosphorylation status of these proteins is largely unknown. Potentially, phosphorylation may facilitate the assembly of the virus for budding. The influenza proteins that have been shown to be phosphorylated, include: M1 [340], M2 [357], NP [435], and PA [650]. In addition, the influenza NS1 protein has been shown to be phosphorylated by the viral RNP, which includes the polymerase [435].

**1a) Determine if PA is a kinase.** To determine if PA is a kinase it is necessary to identify its target(s). To achieve this, recombinant viruses will be generated that lack neuraminidase and are unable to bud out of the producer cells. Lysates will be made from these cells and the flu-specific proteins will be immuno-precipitated with anti-influenza antibodies. These proteins will be examined by gel electrophoresis using phospho-specific antisera. Positive targets will be extracted, trypsinized, and identified by mass

spectrometry. Particular attention will be paid to NS1. If NS1 is phosphorylated in cells with wild type PA, but not in cells with J10 PA, it would suggest that PA is an active kinase or indirectly promotes phosphorylation.

In order to confirm a role of PA in phosphorylation, attempts will be made at generating an *in vitro* system to examine the transfer of a radiolabelled isotope. In addition, radiolabelled ATP analogs can be cross-linked to the binding site, which can be localized after treatment with trypsin and identification by mass spectroscopy. Work with ATP analogs has already shown that ATP binding and hydrolysis is required for viral budding, which is consistent with the hypothesis that PA may have kinase activity that is required for viral assembly [268].

Furthermore, if PA is found to have kinase activity, specific kinase inhibitors will be used to determine if they can prevent wild type viral assembly. The inhibition of viral assembly will be indirectly assessed using reverse genetics with a flu-like luciferase reporter that is packaged into viral particles. These viral particles will be used to infect new cells and the level of luciferase activity in the infected cells should represent the level of viral assembly in the producer cells.

Lastly, if PA is found to phosphorylate viral proteins, the phosphorylation sites of the recipient proteins will be mutated and these mutants tested in the reverse genetics system to determine if these sites are required for viral assembly.

If PA is found to bind ATP, but does not have kinase activity, alternative hypotheses will be examined. One such hypothesis is that PA is involved in the transport of the vRNPs from the nuclear pore to the plasma membrane via the microtubule network.

**1b) Determine if PA is involved in the transport of vRNPs to plasma membrane for packaging.** Published data have shown that treatment of infected cells with microtubule depolymerizing agents promotes basolateral rather than apical budding and causes an accumulation of viral particles in the intercellular space [326]. However,

it has not been determined whether the viral particles that bud from treated cells were infectious or contained RNA.

I propose to use immuno-electron microscopy to localize influenza nucleoprotein and matrix in the cytoplasm of the J10 producer cells to determine whether the vRNPs make it to the plasma membrane and the extent of matrix shell formation. In addition, wild type infected cells will be treated with microtubule depolymerizing agents and the viability of the budded particles will be assessed by plaque assay. Budded particles will be examined for RNA content by treating them with micrococcal nuclease followed by quantitative PCR. If cells that lack a functional microtubule network fail to assemble infectious particles, like the J10 mutant, and have a similar phenotype according to immuno-electron microscopy, this would suggest that the microtubule network is involved in directing the packaging of the genome. To confirm that PA facilitates the transport of vRNPs along the microtubule network, immuno-precipitation studies will be carried out on the transport machinery to see if viral RNA is associated.

## C. Specific Aim #3

**Examine the role of PA in host/viral transcription and translation.**

During influenza infection, host protein synthesis is shut down and viral proteins are preferentially translated. The shutdown of host protein synthesis has largely been attributed to the actions of the NS1 protein [399, 407] and the viral polymerase's role in cellular cap snatching [499, 572]. However, PA may also play a role in these processes by mediating the association of the viral polymerase with the host RNA polymerase II [673], and by interacting with the host ribosomes.

I will examine whether PA mediates the association of the influenza polymerase with the host RNA polymerase II by screening the PA mutants that I have generated for defects in binding to polymerase II by immuno-precipitation experiments. Such an interaction would suggest that PA is involved in modulating host and viral transcription,

and additional experiments would be carried out to characterize this interaction.

In addition, I will determine whether PA affects cellular and viral translation in the cytoplasm. The sequence analysis I have performed suggests that regions of PA share homology with ribosome subunits and translation initiation factors. Furthermore, my yeast two-hybrid screen has identified several polysome-associated proteins of interest. To examine this hypothesis, the panel of PA mutants will be screened to determine if any mutants have a discordant relationship between the amount of mRNA synthesized and the level of protein translated. This will be achieved by analyzing the level of mRNA synthesis by primer extension and protein synthesis by Western blot for both a selected host and viral gene. Interesting mutants will be compared to wild type in a yeast two-hybrid assay to determine if the mutants have diminished binding ability to the ribosome-associated candidates that have already been identified. To support any findings, mutant polymerases will be purified from the cytoplasm and examined for the presence of bound cellular polypeptides by mass spectroscopy.

## D. Specific Aim #4

**Identify drugs that inhibit the ability of the influenza polymerase to replicate a reporter.** I have developed a functional assay that can be used to screen drug libraries in a high throughput manner. The assay involves transfecting cells, adding drug treatment, and then measuring the level of both firefly and Renilla luciferase activity. The Renilla luciferase is included as a control for transfection efficiency and this level should remain equal between samples, unless the drug is toxic to the cells. However, the level of firefly luciferase activity is directly dependent on the activity level of the influenza polymerase. If a drug specifically inhibits the influenza polymerase, the level of firefly luciferase will be low, whereas the level of the Renilla luciferase will be relatively high.

Drugs that inhibit the polymerase will be titered and tested in animal models of influenza.

The above proposal begins to analyze the mystery that currently surrounds the influenza PA subunit. **By better understanding of the functions of PA in the influenza polymerase, we may be able to develop new therapeutics that will better prepare us for a future pandemic.**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