Biological characterization of RNA polymerase proteins from human 2009 pandemic H1N1 and low pathogenic avian H5N2 and H9N2 influenza viruses

Myaing, Myint Zu

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NANYANG
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**BIOLOGICAL CHARACTERIZATION OF RNA POLYMERASE PROTEINS
FROM HUMAN 2009 PANDEMIC H1N1 AND
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ABBREVIATIONS

aa	amino acid
AIV	Avian Influenza Virus
ATCC	American Type Culture Collection
AVA	Agri-Food and Veterinary Association
BSA	Bovine Serum Albumin
BSL	Biosafety Laboratory
CDC	Centre for Disease Control and Prevention
cDNA	complementary Deoxyribonucleic Acid
cRNA	complementary Ribonucleic Acid
CT149	A neutralizing human monoclonal antibody
dpi	days post-infection
dsRNA	double stranded Ribonucleic Acid
dsDNA	double stranded Deoxyribonucleic Acid
EF	Elongation Factor
EM	Electron Microscopy
HA	Hemagglutinin
HPAI	High Pathogenic Avian Influenza
hpi	hours post-infection
hpt	hours post-transfection
M1	Matrix protein 1
M2	Matrix protein 2
moi	Multiplicity of infection
mRNA	messenger Ribonucleic Acid
NA	Neuraminidase
NLS	Nuclear Localization Signal
NP	Nucleoprotein
NS1	Non Structural protein 1
NS2	Non Structural protein 2
nt	nucleotide
PA	Polymerase Acidic protein
PB1	Polymerase Basic protein 1
PB2	Polymerase Basic protein 2
PCR	Polymerase Chain Reaction
pfu	plaque forming unit
qPCR	quantitative Polymerase Chain Reaction
RISC	RNA-induced silencing complex
RLU	Relative light unit
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid interference
RNP	Ribonucleoprotein
RT	Reverse Transcription
shRNA	short hairpin Ribonucleic Acid
siRNA	small interfering Ribonucleic Acid
ssRNA	single stranded Ribonucleic Acid
UPL	Universal Probe Library
vRNA	viral Ribonucleic Acid
vRNP	viral Ribonucleoprotein
WHO	World Health Organization

ABSTRACT

The influenza A genome replication and gene transcription are mediated by polymerase complex composed of three polymerase subunits PA, PB1 and PB2. This polymerase complex is also essential for host adaptation and pathogenesis. To characterize the polymerase complex of 2009 pandemic H1N1 (pH1N1) and low pathogenic avian (LPAI) H5N2 influenza viruses, monoclonal antibodies (mAbs) against these complexes are essential. Therefore, we have challenged mice with purified soluble PA protein (pH1N1/471 and H5N2/F118 strains), insoluble PB2 and PB1 proteins (pH1N1/471 strain). Then, mAbs directed against PA subunit of both strains and PB2 subunit of 2009 pH1N1 strain were successfully generated, while mAb against PB1 subunit was not successful at fusion stage for two attempts. Interestingly, two mAbs-PA(9F5 and 2E2) against pH1N1/471 strain recognized PA protein in virus-infected cells with different staining patterns, which may be explained by their different epitope recognition. In addition, smaller protein products (PA*) were identified by 9F5 in recombinant protein expression, virus-infected cells and mature virus particles. We proved the PA* are PA related and demonstrated the first evidence of their association with the RNP complex. Similar to PA*, PB2* was also revealed by mAb-PB2(4G3), but in the virions, suggesting that PB2* was not virus-associated. However, significance of PB2* remained unclear and it needs further investigation.

For the functional analysis of viral polymerase complex, NP and three polymerase subunits of four influenza virus strains have been successfully cloned using a mammalian expression system. H1N1/WSN polymerase complex was found to be the most active than polymerase complexes of other strains tested. In our gene reassortment study, a single reassortant bearing a human-origin PA or PB2 subunit against an avian polymerase background could overcome the host range barrier of avian polymerase in

human 293T cells, indicating the PA or PB2 as a major determinant of species tropism and pathogenicity. Again in our RNAi study, potent *in vitro* inhibition of virus replication was achieved with eight siRNAs against NP and polymerase genes of LPAI H5N2 virus as observed by reduction in mRNA levels, protein expressions and virus titers.

CHAPTER 1. INTRODUCTION

1.1. Influenza A virus

1.1.1. Virus structure, genome and functions of viral proteins

Influenza A virus belongs to the family *Orthomyxoviridae*. It is an enveloped virus consisting of a genome with eight gene segments of negative sense single-stranded RNA (Fodor and Brownlee, 2002). The eight influenza viral RNA segments encode 15 known proteins to date. Currently, there are five genera in this family, which are influenza A virus, influenza B virus, influenza C virus, Thogotovirus and Isavirus. All of the influenza viruses have different characteristics. Influenza A and B virus encode eight segments of RNA and eleven proteins, while influenza C encodes seven segments and nine proteins only (Palese, 1980). Among them, Influenza A virus is the most common and can infect a wide range of species including birds, humans, and other mammals such as swine and horses.

The structure of an influenza virion is illustrated in Figure 1.1A. The viral envelope of influenza A consists of a lipid bilayer containing two surface glycoprotein, hemagglutinin (HA) and neuraminidase (NA), which are essential for the import and export of virus through their interactions with cell surface receptors. They are embedded in the viral envelope together with the M2 ion-channel protein derived from the host plasma membrane. In virions, each viral negative-strand RNA (vRNA) segment is associated with multiple nucleoprotein (NP) copies and three viral polymerase subunits (PA, PB1 and PB2) forming a viral ribonucleoprotein (vRNP) complex (Lamb and Krug, 2001; Fodor and Brownlee, 2002) as shown in Figure 1.1B. The matrix protein (M1) interacts with both the viral envelope and the RNP, while the non-structural protein 2 (NS2), also recognized as nuclear export protein (NEP) is present in the purified viral preparations (Richardson and Akkina, 1991).

Figure 1.1. (A) Schematic representation of the structure of influenza A virus particle (Adapted from (Horimoto and Kawaoka, 2005)). (B) Enlarged schematic of a helical vRNP structure (Adapted from (Portela and Digard, 2002)).

The morphology of influenza A virus particles is characterized by protruding spikes at the surface. A representative Electron Micrograph (EM) image of influenza A virus is shown in Figure 1.2. These spikes are nearly 10 to 14 nm long, and contain HA and NA proteins with the ratio of approximately 400-500 HA to 100 NA. High quality images of influenza A virus morphology have also been successfully obtained by electron microscopy. For example, Fujiyoshi *et al.* have managed to visualize the influenza A virus by cryo-electron microscopy (Fujiyoshi *et al.*, 1994). The influenza A particles were grouped into the diameter of approximately less than 150 nm spherical particles with well-organized interiors, with spikes protruding from the surface. Furthermore, the internal components of the influenza A virus have also been imaged successfully as demonstrated by Murti *et al.* using immuno-gold labelling/electron microscopy (Murti *et al.*, 1980; Murti *et al.*, 1988). The EM image of influenza A virus and its internal part components are shown in Figure 1.2. Noda *et al.* also reported

spherical morphology of influenza virus in grown eggs and the diameter of each spherical virus particle is a nearly 100 nm (Noda *et al.*, 2006). In contrast, filamentous forms of the virus can elongate up to 300 nm in length, which derived from tissue culture cells (Chu *et al.*, 1949).

Figure 1.2. Electron microscope images of (A) spherical influenza virus buds attaching to infected cells (40,000x magnification) (Adapted from Itoch *et al.*, 2009), (B) a negatively stained influenza virus particle, (C) influenza A virions showing a specific arrangement of eight rod-like structures, and (D) the magnification of one of the virions (Adapted from Noda *et al.*, 2006).

The evidence of the segmented nature of influenza A RNA was first shown by using polyacrylamide gel electrophoresis analysis, where 8 bands of RNA were visualized, which later were assigned as segments (Palese and Schulman, 1976). Additionally, each viral segment also contains 5' and 3' non-coding regions. The non-coding regions are conserved among all segments, and this is followed by a segment-specific coding region (Desselberger *et al.*, 1980; Palese *et al.*, 1980). The schematic representation of the genomic organization, the segment numbering referring to the

respective gene and protein of influenza A virus are shown in Figure 1.3. Generally, each gene segment in the influenza A virus encodes one protein, but there are exceptions. Segment 2, 3, 7 and 8 encode more than one protein as summarized in Table 1.1. Two additional products of PB1 protein, PB1-F2 and PB1-N40, are encoded by the segment 2 via alternative translation initiation mechanism (Chen *et al.*, 2001; Wise *et al.*, 2009). Besides, PA-X and two additional products truncated from N-terminus (PA-N155 and PA-N182) are originated from the segment 3 due to +1 ribosomal frameshifting in an new open reading frame and alternative AUG start codon sites respectively (Jagger *et al.*, 2012; Muramoto *et al.*, 2013). Moreover, the segment 7 and 8 also encode more than one protein. M1 and M2 proteins are products of the segment 7, while NS1 and NEP/NS2 are translated from the segment 8. As shown in Figure 1.3, M2 and NS2 proteins arises from alternative splicing mechanism by cellular spliceosome (Fodor and Brownlee, 2002). Table 1.1 summarizes the eight viral genome RNA segments of influenza A virus including the lengths of individual segments, their encoded polypeptides with predicted molecular weights, and their brief functions.

Figure 1.3. The genome organization of influenza A virus H1N1/PR/8/34 (Adapted from Lamb and Krug, 2001).

RNA segment	Length (nt)	Encoded polypeptide	Predicted M.W (kDa)	Functions
1	2341	PB2, polymerase basic protein 2	85.70	Polymerase subunit that recognizes and binds to the 5' mRNA cap of host cell
2	2341	PB1, polymerase basic protein 1	86.50	Polymerase subunit that initiates the replication cycle through catalyzing the RNA elongation by nucleotide addition
		PB1-F2	10.54	Pro-apoptotic protein with membrane-disrupting properties; contributes to viral pathogenicity; possible virulence factor
		PB1-N40	82.31	Interacts with PB2 and the polymerase complex during infection
3	2233	PA, polymerase acidic protein	82.65	Polymerase subunit; involved in the RNA synthesis and possesses protease activity
		PA-X	29.00	Modulates cellular gene expression; host cell shutoff and limitation of viral pathogenesis
		PA-N155	62.00	Required for efficient virus replication by a process that appears to be independent of virus gene transcription
		PA-N182	60.00	
4	1778	HA, hemagglutinin	61.46	Surface glycoprotein that binds to host receptor to fuse and enter the host cell; antigenic determinant
5	1565	NP, nucleoprotein	56.10	Possesses RNA binding activity and is required for replication; facilitates nuclear import of RNA
6	1413	NA, neuraminidase	50.08	Surface glycoprotein; neuraminidase activity which allows the virus release and spread; antigenic determinant
7	1027	M1, matrix protein 1	27.80	Matrix protein which forms a shell surrounding the nucleocapsid; interacts with vRNPs and glycoproteins; aids in viral budding
		M2, matrix protein 2	11.01	Integral membrane protein with ion channel activity to control pH for uncoating of virus inside the cell
8	890	NS1, non-structural protein 1	26.81	Non-structural protein; regulates host genome expression and antagonize interferon activity of host cell
		NS2, non-structural protein 2	14.21	Interacts with M1 and involved in nuclear export of RNPs

Table 1.1. List of eight viral genome RNA segments of influenza A virus and their encoded polypeptides.

1.1.2. Virus replication cycle

1.1.2.1. Virus entry, uncoating and nuclear import of viral genome

Figure 1.4. Replication cycle of influenza A virus (Adapted from Itzstein, 2007). The process can be divided into 3 main parts: (1) binding and entry of the virus into host cell, (2) replication and transcription of the viral genes occurs inside the nucleus, and (3) assembly and release of the virus to infect a new host cell.

A schematic diagram of a single cycle infection of an influenza virus is shown in Figure 1.4 (Itzstein, 2007). Influenza A virus binds to glycoproteins and glycolipids on the target cell surface through the interaction of terminal sialic acid residues and the HA protein. A shallow depression at the top of the HA molecule is where the binding takes place, and it contains conserved residues (Tyr-98, Trp-153, His-183, Glu-190, Leu-194) in all influenza strains (Wilson *et al.*, 1981; Skehel and Wiley, 2000). Two major linkages are formed between the penultimate galactose residues of carbohydrate side chains and the sialic acid, namely NeuAc(α 2,3)Gal and NeuAc(α 2,6)Gal. Different HA proteins recognize these linkages specifically, as determined by the HA receptor binding pocket (Weis *et al.*, 1988). The recognition of HA molecules to particular sialic acid receptors expressed by human trachea (NeuAc(α 2,6)Gal), avian intestine

(NeuAc(α 2,3)Gal) and pig trachea (NeuAc(α 2,3)Gal and (α 2,6)Gal) enables the virus to infect the host effectively (Ito *et al.*, 1998; Vines *et al.*, 1998; Matrosovich *et al.*, 2000; Skehel and Wiley, 2000). This HA specificity is an important factor for species barrier in influenza virus transmission.

Upon binding of the HA to host cell surface receptor, the virus is internalized into endosomes. The internalization of influenza virus occurs with or without clathrin-mediated endocytosis (Matlin *et al.*, 1981; Sieczkarski and Whittaker, 2002). At the low pH of the endosomes, fusion of the viral and endosomal membranes mediated by HA is activated (Maeda and Ohnishi, 1980; Huang *et al.*, 1981; White *et al.*, 1981). Moreover, the cleavage products of the HA0 protein (HA1 and HA2) are required for membrane fusion and infectivity (Skehel and Waterfield, 1975). This cleavage by cellular proteases such as furin occurs on released viruses or on the host cell surface, or intracellularly in the case of high pathogenic H5 and H7 influenza subtypes (Stieneke-Gröber *et al.*, 1992). Numerous experiments support a model of fusion mechanism in which the fusion peptide (the exposed N-terminal of HA2) inserts into the endosomal membrane, bringing the endosomal membrane and viral membrane together via N- and C-terminal ends of HA respectively (Skehel and Wiley, 2000). A schematic diagram of HA-mediated membrane binding and fusion is shown in Figure 1.5.

The interior of the virus particle is further acidified by the ion channel activity of the M2 protein, and this is essential for the uncoating process of the virus (Bukrinskaya *et al.*, 1982). The acidification process causes the vRNPs to dissociate from the viral matrix protein M1 into the cytoplasm (Martin and Helenius, 1991). The free vRNPs are then imported into the host cell nucleus for virus replication.

Figure 1.5. Membrane binding and fusion mediated by HA between the endosomal and viral membranes (Adapted from (Goodsell, 2006)). (A) HA₁ (blue) binds to a sialic acid group on the host cell membrane (light green); (B) HA₂ undergoes a conformational change after a decrease in endosomal pH, driving the fusion peptides (red) into the cell membrane; (C) The outermost leaflets of the opposing membranes are brought together by an additional conformational change; (D) Formation of a stalk.

1.1.2.2. Transcription of viral mRNA and replication of vRNA

Influenza virus is one of the RNA viruses in which transcription and replication take place in the host nucleus. The vRNA serves as a template for both the viral messenger RNA (mRNA) and complementary RNA (cRNA) production. The process of mRNA synthesis is catalyzed by the viral RNA-dependent RNA polymerase (composed of three subunits PB2, PB1 and PA), and this process requires the host nuclear machinery. This was demonstrated by the addition of α -amanitin, which inhibited not only the host cell DNA-dependent RNA polymerase II, but also viral mRNA transcription (Mahy *et al.*, 1972; Lamb and Choppin, 1977). In transcription, the viral RNA-dependent RNA polymerase will not initiate viral mRNA synthesis until a host-capped primer is supplied. This host-capped primer, m⁷GpppXm (containing a methylated 5' guanosine residue), is obtained from the cellular pre-mRNA transcripts synthesized by the host cell DNA-dependent RNA polymerase II. The PB2 subunit binds the 5' cap of cellular pre-mRNAs which are subsequently cleaved after 10–13 nucleotides by the PA endonuclease activity in a process known as “capsnatching” (Decroly *et al.*, 2012).

Figure 1.6 showed that the activation of viral mRNA synthesis occurs in three steps:

(1) Initiation step, whereby the 5'-vRNA binding to the PB1 protein activates the host-capped primer binding to the PB2 protein, together with the binding activity of the PB1 to the 3'-vRNA (Kawakami *et al.*, 1985).

(2) Elongation step, in which the host-capped mRNA is cleaved by the PA (Nakagawa *et al.*, 1995; Dias *et al.*, 2009) and serves as a primer for the initiation and transcription by the PB1 protein, until the polyadenylation is reached (Beaton and Krug, 1981; Shaw and Lamb, 1984).

(3) Termination step, where the PB1 protein binds both the 5'- and 3'- ends of the viral mRNA, the polyadenylation and termination of the mRNAs occurs before the 5' end is reached. The polyadenylation is formed due to the steric hindrance of the polymerase itself, resulting in the stuttering production of five to seven adenine residues (Emtage *et al.*, 1979; Robertson *et al.*, 1981; Luo *et al.*, 1991; Li and Palese, 1994; Poon *et al.*, 1999; Zheng *et al.*, 1999)

Finally, viral mRNAs are then exported to the cytoplasm for translation using cytoplasmic ribosomes.

Figure 1.6. Schematic representation of the viral mRNA transcription (Adapted from Lamb and Krug, 2001). Three polymerase proteins (PB1, PB2 and PA) are involved. C in the PB2 denotes the cap-snatching site. R5 and R3 in PB1 denotes the vRNA 5' and 3' binding regions, respectively. (UUUUU) denotes poly-uracil sites on the vRNA for mRNA polyadenylation (AAAAAAA) synthesis. The arrow in mRNA denotes direction of the transcription. The arrowhead in vRNA denotes 3' end.

The replication of vRNA occurs in two steps: (1) the synthesis of template RNAs in full length anti-genomic or cRNA, and (2) the copying of the cRNAs into vRNAs. The switch from viral mRNA transcription to viral replication requires the change from capped RNA-primed initiation to unprimed initiation. This different initiation strategy will prevent termination and polyadenylation at the poly(A) site, which is used during viral mRNA synthesis (Deng *et al.*, 2006b). The copying of cRNA to vRNA requires the addition of NP protein to the elongating vRNA molecules (Shapiro and Krug, 1988), and no vRNAs are made in the absence of the NP protein. The newly synthesized vRNAs in complex with NP and three polymerase proteins (known as vRNPs) are ready for exporting from the nucleus and packaging into the virus progeny (Shapiro and Krug, 1988).

1.1.2.3. Virus assembly and budding

Since only the mature viral proteins are found inside the cells, the production of new virions from the infected cells required the important processes of virus assembly, budding and release of virus particles at the plasma membrane. In the final stage of infection, the nuclear vRNPs move to the plasma membrane, where they incorporate with the other viral proteins required to assemble the complete virus particle (Figure 1.7).

Figure 1.7. Schematic representation of influenza virus budding (Adapted from (Prescott *et al.*, 1996).

The viral surface glycoproteins, HA and NA, are directed to lipid rafts which cause the enlargement of the raft domains and a deformation of the membrane and the initiation of the virus budding event (Rossman and Lamb, 2011). The M1 and M2 proteins also assist viral assembly and budding especially in filamentous particle budding (Roberts *et al.*, 1998). The matrix protein M1 polymerizes at the cytoplasmic tails of HA and NA upon binding to form the interior structure of the newly-synthesized virion. Moreover, it serves as a docking site for the recruitment of not only the viral RNPs, but also the M2 to the virus budding site. After the recruitment, M2 protein first stabilizes budding site, possibly enabling the M1 protein polymerization and the filamentous formation of virus particles. Consequently, M2 protein is able to change membrane curvature at the neck of the budding virus which causes membrane cleavage (Rossman and Lamb, 2011). Finally, progeny virus is released from plasma membrane of the host cell by the sialidase activity of the NA protein.

1.1.3. Classification of subtypes

Classification of the influenza A virus is based on the antigenic properties of the surface proteins, the hemagglutinin (HA) and the neuraminidase (NA) (Palese and Shaw, 2007). HA serves as a major determinant of host range due to its role in host cell recognition and attachment. Although all HA subtypes have been detected in avian species, some particular HA subtypes are more commonly found in mammalian species such as H1 and H3 in humans and swine, and H7 in equines (Chan *et al.*, 2006). Of the 18 known HA and 11 known NA subtypes circulating in animals, only three subtypes of H1N1, H2N2, and H3N2 subtypes are associated with pandemics in humans (Nakazono *et al.*, 2012). Moreover, the role of bats became a concern in influenza virus ecology after identification of two influenza-like viruses (H17N10 and H18N11) in bats (Tong *et al.*, 2012; Tong *et al.*, 2013).

1.1.4. Clinical features of influenza disease

Influenza A virus infection is characterized by abrupt onset of symptoms inclusive of high fever, severe headache, myalgia, pharyngitis, cough and coryza. The virus is able to function effectively as it has developed an intricate mechanism to enter host cells through host cell receptors and is able to evade host cell immunity to carry out transcription and replication using host cell machinery. Pathological findings are mostly confined to the respiratory tract, and death is secondary to pneumonia and respiratory failure. Despite no evidence of systemic viral infection, most patients die of secondary bacterial pneumonia. In some patients, fulminant acute pulmonary hemorrhage or pulmonary edema is a cause of death, depending on the virus virulence (Palese and Shaw, 2007).

1.1.5. Host range determinants

Influenza A viruses can infect a wide range of mammalian species, including birds, sea mammals, pigs, horses and humans. A phylogenetic analysis suggests that the main reservoir of all influenza viruses are wild aquatic birds. These viruses are able to perform interspecies transmission through the frequent mutation nature of the influenza virus (Gorman *et al.*, 1991). The molecular basis for host-range restriction and pathogenicity includes the viral glycoproteins, polymerase proteins, matrix protein and non-structural protein, which allow interspecies transmission to other hosts, either indirect transmission (through an intermediate host such as chicken or pig) or direct transmission (for example the Asian H5N1 virus) (Neumann and Kawaoka, 2006).

1.2. Role of polymerase proteins, nucleoprotein and their host adaptation

Influenza A virus polymerase proteins are encoded by the largest gene segments in the genome with over 2.2 – 2.3 kbps in size. The polymerase complex in influenza A virus is composed of the three subunits: PB2, PB1 and PA proteins. According to gene nomenclature, they are encoded by segment 1, 2 and 3 for PB2, PB1 and PA proteins, respectively. PA protein possesses acidic amino acids prevalence while PB1 and PB2 proteins were named for their basic amino acids prevalence. As shown in Figure 1.8A, both PB1 and PB2 segments are approximately 2,341 nucleotides in length which correspond to 759 amino acids and 757 amino acids for PB2 and PB1 proteins respectively, while the PA segment is approximately 2,233 nucleotides in length and 716 amino acids (Lamb and Choppin, 1976; Horisberger, 1980). In SDS-PAGE protein, PB1, PB2 and PA have molecular weights of approximately 86.5 kDa, 85 kDa and 83 kDa, respectively (Lamb and Choppin, 1976; Muramoto *et al.*, 2013). Moreover, the viral nucleoprotein (NP) is a structural RNA-binding protein (Figure 1.8B), and is found to be the most abundant viral protein in the influenza A virus. The length of RNA segment 5 which encodes NP protein is 1,565 nucleotides in total with 498 amino acids corresponding to a molecular weight of ~56 kDa (Winter and Fields, 1981). The three polymerase proteins, together with NP, form a complex with the viral RNA, to form the viral ribonucleoprotein (RNP) complex.

Unlike many, but not all other RNA viruses, the nucleus of infected host cells is the unique site of both transcription and replication of influenza viral genome (Boulo *et al.*, 2007). Hence, nuclear translocations of the components and assembly of the polymerase complex with NP in the nucleus play important roles in viral growth. Expression of the NP or polymerase protein alone shows that each component has the ability to migrate into the nucleus independently due to the presence of putative nuclear

localization signals (Akkina *et al.*, 1987; Biswas *et al.*, 1998; Boulo *et al.*, 2007; Loucaides *et al.*, 2009; Huet *et al.*, 2010). Besides, it has been reported that PB1 co-migrates with PA in nuclear import (Fodor and Smith, 2004; He *et al.*, 2008) and replication and transcription take place together with PA-PB1 complex (MacDonald *et al.*, 2012). However, PB2 was found to translocate to the nucleus independently of the other polymerase proteins by the interaction between the NLS located at PB2 C-terminus and α/β -importin pathway (Gabriel *et al.*, 2008; Gabriel *et al.*, 2011).

In addition, it was reported that the viral polymerase of influenza virus confers a major role in host adaptation and pathogenesis (Gabriel *et al.*, 2005; Gabriel *et al.*, 2007; Gabriel *et al.*, 2008) with nuclear and cytoplasmic host proteins serving as cofactors of the viral polymerase (Engelhardt *et al.*, 2005; Deng *et al.*, 2006a). PA, PB1 and PB2 proteins are found to co-fold together, and the 3D structure of the complete polymerase complex has been elucidated (Pflug *et al.*, 2014; Reich *et al.*, 2014; Chang *et al.*, 2015). Structural models showing the interaction of the polymerase complex with the viral RNA promoter have also been described (Figure 1.9). This has provided an insight into the structural assembly of the RNP and the mechanistic mode of action of the polymerase complex. The fully functional polymerase complex requires all the activities that are associated with the individual polymerase proteins, and recent structural analysis of the entire polymerase complex has provided new insights into how these different activities are adapted.

The functionally important domains, interactions and host adaption of three polymerase proteins including NP are elaborated individually in the following paragraphs.

Figure 1.8. (A) Features of the polymerase subunits (Adapted from Boivin *et al.*, 2010). Linear representations of the three polymerase subunits (PA, PB1 and PB2) are annotated. The structurally characterized domains are shown as large boxes, while residues involved in host adaptation are presented in black. Independently foldable crystal structures of interaction regions of PA-PB1 and PB1-PB2 are shown with helix colors. (B) Linear representation of the NP and its regions responsible for binding to PB2-purple, RNA-red, and NP itself-orange are presented (Adapted from Li *et al.*, 2009).

Figure 1.9. Structure of influenza polymerase complex with the vRNA promoter (Adapted from Pflug *et al.*, 2014).

1.2.1. Polymerase acidic (PA)

Based on the proteolysis studies from Hara *et al.*, PA subunit has two major domains, a smaller N-terminal domain (~25 kDa), and a larger C-terminal domain (~55 kDa) (Hara *et al.*, 2006). In addition, they investigated that amino acid residues within the N-terminal domain of PA are mainly associated with critical functions of polymerase and play important role in endonuclease activity, protein stability, cap binding, and viral RNA promoter binding. The N-terminal endonuclease domain (residues 1-196) which exhibits RNA endonuclease activity generates methylated capped primers from the host cell mRNA during viral RNA transcription (Dias *et al.*, 2009). Besides, the PA N-terminus associated with binding to the viral genome promoters was also found to regulate vRNA/cRNA synthesis (Maier *et al.*, 2008). No enzymatic activity has been identified in the C-terminal domain (residues 257-716), but it contains PB1 protein binding region (He *et al.*, 2008; Obayashi *et al.*, 2008). The interaction with the PB1 protein also allows nuclear transport of the PA protein as a PA-PB1 complex (Deng *et al.*, 2006a; Hutchinson *et al.*, 2011) although PA protein has its own bipartite NLSs located at two regions (residues 124-139 and 186-247) of N-terminus (Nieto *et al.*, 1994; Yuan *et al.*, 2009).

Moreover, the structures of the N- and C-terminal domains have been showing that they are separated by a long linker region (Guu *et al.*, 2008) which is found between amino acid residues 197/198-256/257 (Yuan *et al.*, 2009; Pflug *et al.*, 2014) as shown in Figure 1.10. Based on the secondary structure predictions, the PA-linker region should have more random coil than 60% and this was proved by Guu's group. They also highlighted that this linker region plays an essential role in the PA-PB1 interface, and their study confirmed that none of the two PA domains (either the N- or the C-terminal domain) could establish a stable interaction with PB1 unless there is the linker (Guu *et*

al., 2008). Moreover, the study of protease digestion on the PA-PB1 complex also revealed the importance of PA linker in the subunit interface. From this proteolysis analysis, PA-PB1 bound form was found to be notably more stable than PA free form, implying that PB1 protects the linker from protease digestion. In addition to the previous structural data, Pflug *et al.* presented new interactions between the PA-linker and the external surface of PB1 domains mediated by the highly conserved hydrophilic and hydrophobic residues of PA (Pflug *et al.*, 2014).

Figure 1.10. Structure of PA domains extracted from full length PA subunit indicating N- and C-terminal domains, and the linker region between two domains (Adapted from Pflug *et al.*, 2014).

Hara *et al.* examined three amino acid positions (102K, 108D, and 134K) in the PA N-terminal region of influenza A/WSN/33 in details to ensure PA protein activity from a complex with PB1 and PB2 proteins (Hara *et al.*, 2006). They introduced systematic point mutations to study the polymerase activity, and found that mutation K102A inhibited both viral RNA promoter and cap binding activities of PB1 protein, resulting in a severe reduction of both replication and transcription *in vivo*. However, mutations D108A and K134A specifically inhibited transcription by a complete inhibition on endonuclease activity *in vitro*, indicating their particular defect in transcription. Dias *et al.* also discovered that the folding and active-site arrangement of PA endonuclease domain shows similar to members of the PD-(D/E)XK family of

nucleases at amino acid position 107 and it has been confirmed by Yuan *et al.* in their X-ray crystallographic study (Dias *et al.*, 2009; Yuan *et al.*, 2009). In addition, recombinant viruses with T157A and T157E mutations revealed a significant drop in both protease activity and RNA replication (Huarte *et al.*, 2003). This result indicated the importance of amino acid residue at 157T in the N-terminus of PA and its role in the modulation process of vRNA/cRNA synthesis in infectious viruses. In the PA protein, host signatures appear to cluster in the endonuclease of N-terminus, but other mammalian adaptive mutations have also been identified in the C-terminus. Mutations at various residues comprising 336M, 552S, and 615N from C-terminus of avian-origin PA contributed to increased avian influenza polymerase activity in mammalian cells as well as pathogenicity in mice (Gabriel *et al.*, 2005; Song *et al.*, 2009; Kim *et al.*, 2010a; Bussey *et al.*, 2011; Mänz *et al.*, 2012; Mehle *et al.*, 2012).

Previously, how host factors associate with PA in virus infection was poorly understood. However, Wang *et al.* have recently identified host factors such as the 26S proteasome, minichromosome maintenance complex (MCM) and the coat protein I (COPI) complex which show the interaction with PA protein in chicken embryo fibroblast cells infected with H5N1 virus (Wang *et al.*, 2016b). These functional protein complexes demonstrated the key roles in the process of RNA replication during the course of infection.

Figure 1.11. Influenza A virus PA segment showing for ORF (in frame 0) and X-ORF (in frame 1), with the frameshift motif are indicated. The X-ORF region and structural domains of PA are shown by yellow and cyan shadings respectively. Either 61 or 41 amino acids encoded by the X-ORF is also shown. The X-ORF product which lies mainly within a linker region between the N- and C-terminal domains of PA is noted (Adapted from Shi *et al.*, 2012).

Furthermore, the PA gene has been reported to produce a 29 kDa fusion protein, known as PA-X protein, encoded from a new X open reading frame (X-ORF) by +1 ribosomal frameshifting as shown in Figure 1.11 (Jagger *et al.*, 2012). In most of the influenza A virus isolates, this fusion protein was found to consist of 191 amino acid PA N-terminus endonuclease domain and 61 amino acid PA C-terminus from the X-ORF. However, it becomes a shorter 41 amino acid X domain in 2009 pandemic H1N1 isolate from a truncation of 20 amino acid residues of PA-X due to the presence of an internal stop codon in the swine-originated influenza A strains. Previous studies have revealed that it is involved in regulation of host gene expression and provides limited host response to virus infection, resulting in a host-cell shutoff (Jagger *et al.*, 2012; Yewdell and Ince, 2012). This host cellular repression was due to selective degradation of host mRNAs by PA-X using the N-terminal endonuclease domain of PA (Yewdell and Ince, 2012; Khapersky *et al.*, 2016). In addition to host immune response modulated by PA-X product, many studies have also investigated the contribution of PA-X to the viral pathogenicity in different animal models using the generated PA-X-lacking recombinant influenza A viruses (Jagger *et al.*, 2012; Gao *et al.*, 2015; Hu *et al.*, 2015). Their results revealed that PA-X deficient high pathogenic avian H5N1, human pandemic 1918 and 2009 H1N1 viruses exhibit higher level of infection in comparison to the wild type viruses, indicating that absence of PA-X expression in these viruses enhanced virus growth and viral replication. Moreover, it is also important to note the species-specific effect from truncated forms of PA-X which may contribute to the evolutionary conservation of PA-X (Shi *et al.*, 2012).

Additionally, other truncated forms of the PA protein from N-terminal domain have been identified as 62 kDa and 60 kDa products during influenza virus infection (Akkina *et al.*, 1991) and they are found to be translated from two initiation codons

(11th AUG and 13th AUG) at amino acid residue 155 and 182 on the mRNA of PA (Muramoto *et al.*, 2013). These two PA protein variants (termed PA-N155 and PA-N182) could be identified from virus-infected cells using several influenza A isolates from various species (Akkina *et al.*, 1991). They were found to be required for efficient virus replication by a process independent of virus gene transcription (Muramoto *et al.*, 2013). Due to the absence of the regions essential for endonuclease activity in N-truncated PAs, only limited effect on polymerase activity was shown when they were expressed together with PB2 and PB1 using mini-replicon assay (Muramoto *et al.*, 2013). Moreover, N-truncated PAs deficient mutant viruses showed significantly lower replication in *in-vitro* and pathogenicity in mice when compared to wild-type virus, indicating the important roles of these PA-truncated proteins in both virus replication cycle and pathogenicity (Muramoto *et al.*, 2013).

1.2.2. Polymerase basic 1 (PB1)

The PB1 subunit is known to be associated with the PB2 and PA subunits to create the viral polymerase required for the transcription and replication of the viral genome. It plays a central role in viral RNA binding during transcription due to the presence of RNA binding domains on PB1 protein. These domains are found in the first 83 amino acids from N-terminus and last 263 amino acids from C terminus (González and Ortín, 1999). PB1 binds to the terminal ends of both cRNA and vRNA for initiation of replication and transcription. The interaction with the 3' end of the vRNA activates the endonuclease activity of PB1 which generates the capped primer required for mRNA synthesis. During RNA chain elongation, the process of sequential nucleotide addition is catalyzed by PB1 protein. Moreover, PB1 takes part in the structural formation as well as catalytic activities of the RNA polymerase (Chu *et al.*, 2012). PB1 is imported into the nucleus using two NLS regions within residues 180 to 252 located at its N-

terminus when it is expressed alone (Nath and Nayak, 1990). However, PB1 necessitates the coexpression of PA for its efficient nuclear localization (Fodor and Smith, 2004). During infection, PB1 and PA subunits become a dimer in the cytoplasm, and the dimer is transported into the nucleus, facilitated by NLS-dependent nuclear import factor Ran-binding protein 5 (RanBP5) (Deng *et al.*, 2006a; Hutchinson *et al.*, 2011). The nuclear localization signal (NLS) motifs, NLS-1 (residues 187-190) and NLS-2 (residues 207-211), identified from the previously discovered bipartite PB1-NLS were also found to be important for binding to RanBP5 (Hutchinson *et al.*, 2011). In addition, recent structural analysis has revealed residues 670-679 from the PB1 C-terminal region as functionally important promoter 3' end binding sites for initiation of RNA synthesis (Reich *et al.*, 2014).

Figure 1.12. (A) Functional regions of PB1 showing conserved polymerase motifs: motif pre-A/F (residues 229-257, yellow), motif A (residues 296-314, lime), motif B (residues 401-422, cyan), motif C (residues 436-449, magenta), motif D (residues 474-486, green), and motif E (residues 487-497, orange), and key functional residues present in these motifs. (B) PB1 domain related to vRNA promoter (Adapted from Pflug *et al.*, 2014).

Furthermore, it has been reported that highly conserved polymerase motifs in PB1 (motifs pre-A/F, A, B, C, D and E, ranging in size from 11-29 amino acids) shown in Figure 1.12 appear to be closely associated with other RNA-dependent RNA

polymerases (Poch *et al.*, 1989; Pflug *et al.*, 2014). Moreover, Biswas and Nayak (1994) observed that mutations in these motifs cause disruption in the transcriptional activity (Biswas and Nayak, 1994). Majority of the mutations cause a significant reduction in polymerase activity, but exceptionally mutation K480R found in multiple early 2009 pandemic H1N1 virus isolates showed slightly increased polymerase activity and virus replication in comparison to wild type PB1. (Biswas and Nayak, 1994; Chu *et al.*, 2012). Besides, mutations at amino acid positions of 473V and 598P in avian-origin PB1 contributes to high activity of the H5N1 virus polymerase in mammalian cells, and that of 473V appears to be associated with efficient viral replication of the pH1N1 strain (Xu *et al.*, 2012). In addition to the above mutations, Ghanem *et al.* described that 25 residues from N-terminus of PB1 which bind to the C-terminus of PA interfere with the viral replication by inhibiting the activity of polymerase complex (Ghanem *et al.*, 2007). Together with this information, two structural studies suggested the essential PB1 binding region on PA protein as a potential target for designing new anti-influenza drugs which block the polymerase assembly (He *et al.*, 2008; Obayashi *et al.*, 2008).

There is also a minor PB1 gene product known as PB1-F2, acting as a pro-apoptotic virulence factor. It is a short polypeptide and its lengths of certain variants have been reported as 57, 87, or 90 amino acids (Zell *et al.*, 2006; Zell *et al.*, 2007). Using an alternative +1 open reading frame of PB1 gene accessed via leaky ribosomal scanning, PB1-F2 is translated from a start codon AUG located downstream of the PB1 start site (Chen *et al.*, 2001; Chen *et al.*, 2004; Wise *et al.*, 2009). During influenza virus infection, PB1-F2 binds to the mitochondria where it induces apoptosis. This effect has been found in both *in vitro* (Chen *et al.*, 2001) and *in vivo* (Zamarin *et al.*, 2006). In addition, Yamada *et al.* revealed the mitochondrial targeting sequence of PB1-F2 located from residues 63 to 75 as an essential domain for apoptotic function (Yamada

et al., 2004). This domain appears to induce apoptosis by interacting with two mitochondrial apoptotic mediators (ANT3 and VDAC1) (Zamarin *et al.*, 2005). Moreover, PB1-F2 also appears to be associated with high pathogenic avian H5N1 and human pandemic 1918 strains, and it augments the pathogenicity of primary viral and secondary bacterial infection in mice (Zamarin *et al.*, 2006; McAuley *et al.*, 2007; Kamal *et al.*, 2015). However, it is also found that the effects of PB1-F2 protein on viral polymerase activity are strain specific (McAuley *et al.*, 2010b; Buehler *et al.*, 2013). In the case of 2009 pandemic influenza H1N1, it does not encode the full length PB1-F2 protein due to mutations after 11 amino acids which create three stop codons within the coding region (Trifonov *et al.*, 2009). This could be a possible determinant for the low virulence of 2009 pandemic H1N1 isolates. In addition, PB1-F2 production through genetic mutations and its expression by 2009 pandemic H1N1 virus also revealed little impact on virulence in mice models (Rong *et al.*, 2010). Moreover, the amino acid position 66 of PB1-F2 is shown to be important for increased virulence in mice. It has been observed in 1918 H1N1 pandemic and high pathogenic H5N1 avian influenza viruses which possess a serine (S) at position 66 instead of an asparagine (N) typically seen in other viruses (Conenello *et al.*, 2007). The variant of N66S amino acid substitution reduced interferon (IFN) production and enhanced inflammatory response of the lung, resulting in increased pathology (McAuley *et al.*, 2010a; Varga *et al.*, 2011).

Besides PB1-F2, ribosomal scanning produces another PB1 polypeptide product using a different AUG start codon. It is an N-terminal product directly translated from PB1 mRNA codon 40 (termed as PB1-N40) which does not possess transcriptase function and cannot interact with PA due to lack of an N-terminal PA binding region (Wise *et al.*, 2009). However, PB1-N40 has been shown to interact with PB2 and the polymerase complex during infection (Wise *et al.*, 2009) and its overexpression

contributes to the virus replication *in vitro* and *in vivo* (Tauber *et al.*, 2012). Moreover, recent structural analysis revealed the secondary pseudoknot structure which contains the start codons for PB1-F2 and N40 (Priore *et al.*, 2015). This pseudoknot may affect the translational activity of PB1-F2 and N40 with ribosomal interaction and offer a possible target for drug design.

1.2.3. Polymerase basic 2 (PB2)

The PB2 subunit in influenza A virus plays an essential role in the initiation of viral transcription. It is responsible for binding the 5' cap structure of host pre-mRNA molecules (10–13 nucleotides) and uses the cap structure as a primer for viral mRNA synthesis (Guilligay *et al.*, 2008). In addition, PB2 is the most essential polymerase for efficient viral replication as it mediates the binding of two other polymerases (Obayashi *et al.*, 2008).

Recent studies on PB2 revealed the crystalline structure as shown in Figure 1.13. Based on the NMR and crystal structure analysis, PB2 protein contains a short N-terminal domain (residues 1-247) and a long C-terminal domain (residues 248-759). Each domain is formed by several folded subdomains. In the N-terminal domain, there are 2 major subdomains: N1 (residues 55-103) and N2 (residues 110-247) (Pflug *et al.*, 2014). N1 facilitates interaction between PB2 and PB1, while the N2 region facilitates binding to PA. The PB2 N-terminus containing a chain of linked modules directs towards the PB1 by interacting with the PB1 C-terminal extension and the polymerase thumb domain.

Moreover, the C-terminal of the PB2 protein mainly consists of two important domains: the cap (m⁷GTP) binding domain located at one end (residues 318-483) (Guilligay *et al.*, 2008), and nuclear localization signal (NLS) domain located at the other end of the C terminus (residues 687-759) (Tarendeau *et al.*, 2007). The structural

analysis of Guilligay *et al.* revealed that the PB2 cap-binding domain was different from other host cap binding proteins. Based on their functional studies with point mutations, viral cap-dependent transcription could be inhibited by interfering the PB2 cap binding site (F325) in addition to F363 and F404 reported by another group (Fechter *et al.*, 2003). These ligands could be usable targets to develop new anti-influenza drugs. Besides, C-terminal NLS domain has been found to mediate the importin α -dependent nuclear import of the PB2 subunit (Tarendeau *et al.*, 2007). Using NMR structure analysis, a linear bipartite nature of NLS sequence ($^{736}\text{KRKR}^{739}$ and $^{752}\text{KRIR}^{757}$) which has a critical role in the nuclear translocation of PB2 has also been identified in the NLS region by co-crystallization with the nuclear import receptor (importin $\alpha 5$) (Mukaigawa and Nayak, 1991; Tarendeau *et al.*, 2007; Pumroy and Cingolani, 2015). In addition, host chaperone proteins such as heat shock proteins (Hsp90, Hsp70) and chaperonin containing TCP-1 (CCT) have been found to interact with PB2 protein and suggested to take part in the nuclear transport and assembly of trimeric RNA polymerase complex (Deng *et al.*, 2005; Naito *et al.*, 2007; Fislová *et al.*, 2010; Manzoor *et al.*, 2014).

Figure 1.13. Structure of PB2 domains extracted from full length PB2 subunit including N-terminal domain (residues 1-247), C-terminal domain (residues 248-759) and the PB2 cap-627 linker (Adapted from Pflug *et al.*, 2014).

In addition to the above two domains, another key structure of the PB2 C-terminus next to the NLS domain, known as 627-domain (residues 539-675), has also been resolved by X-ray crystallography (Tarendeau *et al.*, 2008). From this structural study, host-specific PB2 residue 627 could be identified on the surface of the 627-domain and found to interact with either components of the polymerase complex or host factors. Moreover, it has been reported that the 627-domain is involved in functional association of NP by the interaction of NP arginine-150 to either lysine-627 or arginine-630 of PB2 (Ng *et al.*, 2012). This association directly correlated an increased RNP activity with the stronger NP and polymerase interaction. Hence, these 627 and NLS domains could be important in the packaging process with incoming NP to form newly made vRNPs or cRNPs.

Using the crystalline structures of bat influenza A and human influenza B polymerase proteins, the detailed mechanism of cap-snatching process initiated by PB2 in the RNA synthesis has been described (Reich *et al.*, 2014). With in situ rotation of the PB2 cap-binding domain, it firstly leads the capped primer towards the endonuclease site, and then into the polymerase active site where the polymerase likely faces conformational changes to alter the pre-initiation state into the active initiation and elongation states.

Furthermore, previous studies have shown that PB2 is important in the adaptation of an avian virus once it infects humans. The amino acid at position 627 in PB2 is shown to be essential for this adaptation process (Subbarao *et al.*, 1993). Influenza A viruses which infect birds typically have a glutamic acid (E) at 627 position, while those wholly adapted to humans usually possess a lysine (K) at the same position. Avian viruses with an E627K mutation has been proved to possess the increased viral polymerase activity in human cells and virulence in mice (Hatta *et al.*, 2001; Mase *et*

al., 2006; Van Hoeven *et al.*, 2009; Zhang *et al.*, 2014; Sang *et al.*, 2015). There is another mammalian adaptive mutation, a substitution of an aspartate (D) to asparagine (N) at residue 701, in PB2 protein of H5N1. This mutation also appeared to enhance virulence and widen the host range of avian H5N1 virus to mammalian hosts (Li *et al.*, 2005c; de Jong *et al.*, 2006; Steel *et al.*, 2009). This D701N mutation improved the binding of PB2 to the importin α 1 in mammalian cells, facilitating the nuclear translocation of PB2 (Gabriel *et al.*, 2008). In addition to these two mammalian adaptation substitutions, the PB2 mutation T271A has been found to improve the activity of polymerase in human cells (Bussey *et al.*, 2010). Another data revealed that PB2 residue 158 is an important pathogenic determinant of H5 and pandemic H1N1 influenza viruses in mice (Zhou *et al.*, 2011).

Recently, Zhu *et al.* discovered that combined mutations, D701N and E627K, in the PB2 protein of H7N9 influenza virus enhanced viral replication in mammalian cells and improved its virulence in mice (Zhu *et al.*, 2015). Furthermore, the most recent data highlighted the PB2-E712D mutation which affects the viral growth and increases pathogenicity to mice (Katsura *et al.*, 2016). Although the 627K and 701N have been identified as essential mammalian adaption markers in PB2, it has been found that pH1N1 does not possess these two residues which are generally present in other human influenza viruses. Interestingly, major impact on virulence was not also observed in the study with the substitution of E627K or D701N mutation in pH1N1 (Herfst *et al.*, 2010). However, a substitution of serine (S) to arginine (R) at residues 590 and 591 in pH1N1 PB2 has been found to compensate for the two adaption markers and enhance virus replication in mammals by affecting the protein interaction between viral and cellular factors (Mehle and Doudna, 2009).

1.2.4. Nucleoprotein (NP)

The NP protein is one of the major components found in the viral ribonucleoprotein (vRNP) complexes. NP is known as a multifunctional protein and its major function is encapsidation of viral RNA genome to form functional RNPs with the polymerase proteins for the purposes of RNA replication, transcription and packaging into new virions (Biswas *et al.*, 1998; Portela and Digard, 2002; Zheng and Tao, 2013; Sanchez *et al.*, 2014).

Figure 1.14. Structure of NP subunit showing (A) RNA binding groove, and (B) Head, body and tail loop domains (Adapted from Ye *et al.*, 2006). (C) Schematic diagram of NP structure including head, body and tail loop domains with contributing residues, along with the viral RNA binding groove (Adapted from (Cianci *et al.*, 2012).

The NP protein is highly conserved among influenza A viruses and its sequence is rich in arginine residues which are important for binding to RNA genome (Gorman *et al.*, 1991; Scholtissek *et al.*, 1993; Elton *et al.*, 1999b; Tarus *et al.*, 2012). Ye *et al.* has revealed the crystal structure of NP with head, body and tail loop domains, along with the RNA binding groove where arginine rich residues are present on its surface

(Ye *et al.*, 2006) (Figure 1.14). Similar to the three polymerase proteins, NP protein consists of amino acid sequences which act as an unconventional NLS (NLS1, residues 3-13) and a bipartite NLS (NLS2, residues 198-216) (Wang *et al.*, 1997; Weber *et al.*, 1998). In addition to NLSs, it also involves a cytoplasmic accumulation signal (CAS, residues 327-345) (Davey *et al.*, 1985) and a tail loop (residues 402-428) (Ye *et al.*, 2006; Ng *et al.*, 2008). Both NLS1 and NLS2 of NP facilitate the nuclear import of NP protein (Neumann *et al.*, 1997; Ozawa *et al.*, 2007; Wu *et al.*, 2007), while CAS of NP binds to F-actin which causes cytoplasmic accumulation of NP protein during late infection (Avalos *et al.*, 1997; Digard *et al.*, 1999). Moreover, the NP tail loop mediates the process of NP oligomerization and RNP activities which are required for vRNA transcription (Elton *et al.*, 1999a; Chan *et al.*, 2010; Ng *et al.*, 2012).

Furthermore, NP associates with importin α family directly via its NLS1 motif, and these interactions allow the nuclear import of vRNPs, by which the viral replication and transcription are initiated (Melen *et al.*, 2003; Cros *et al.*, 2005; Gabriel *et al.*, 2008; Nakada *et al.*, 2015). In addition to NLS mediating nuclear transport of NP, Batra *et al.* recently found that cellular heat shock protein 40 (Hsp40) appears to interact with NP and facilitates the transport of newly synthesized vRNPs to the nucleus. This protein was also found to be necessary for efficient association of NP with importin α (Batra *et al.*, 2016). NP protein also contains an N-terminal RNA-binding region (residues 1-181) where arginine rich residues are located, and two NP-NP auto-interaction domains (residues 189-358 and 371-465) required for the assembly of vRNPs (Kobayashi *et al.*, 1994; Albo *et al.*, 1995; Elton *et al.*, 1999b; Li *et al.*, 2009). Moreover, the interactions of NP with the viral polymerase proteins, PB2 and PB1, have been reported to indicate the important role of NP in the modulation of polymerase activity (Biswas *et al.*, 1998; Poole *et al.*, 2004; Ng *et al.*, 2012).

Several studies have shown the association between NP and M1 or NEP/NS2 proteins in the nuclear export of vRNPs (Martin and Heleniust, 1991; Yasuda *et al.*, 1993; Whittaker *et al.*, 1996; Avalos *et al.*, 1997; O'Neill *et al.*, 1998; Ye *et al.*, 1999; Noton *et al.*, 2007). Besides the M1 or NS2 protein binding, NP also plays a major role in vRNP export through its nuclear export signal (NES3) recognized by the cellular export receptor CRM1/exportin-1 (Elton *et al.*, 2001; Yu *et al.*, 2012; Chutiwitoonchai *et al.*, 2014; Li *et al.*, 2015).

Moreover, NP is also known as a phosphoprotein and a number of highly conserved phosphorylation sites on NP have been found with unknown functions (Petri and Dimmock, 1981; Kistner *et al.*, 1989; Arrese and Portela, 1996; Bui *et al.*, 2002; Hutchinson *et al.*, 2012). Recently, Zheng *et al.* extensively characterized three phosphorylation sites on NP (S9, Y10, and Y296) and discovered their essential functions in the nucleocytoplasmic shuttling of NP protein during virus replication (Zheng *et al.*, 2015). In this study, phosphorylation and dephosphorylation of S9 and Y10 regulated the binding affinity of NP for different importin- α isoforms for the nuclear transport of NP, while the phosphorylation of Y296 decreased the interaction between NP and CRM1, causing the nuclear retention of NP. In addition to the above phosphorylation sites, phosphorylation of S165 residue also appears to inhibit the oligomerization of NP, and subsequently reduce the RNP activity and viral growth during infection (Hutchinson *et al.*, 2012; Chenavas *et al.*, 2013; Turrell *et al.*, 2015).

NP protein also plays an essential role in interspecies transmission of influenza virus. Unlike PB2 and PA, only a few mammalian adaptive mutations have been described in NP till now (Gabriel *et al.*, 2005; Gabriel *et al.*, 2007). Among these mutations, N319K in NP has been shown to increase the binding affinity of NP to importin $\alpha 1$ and facilitate the nuclear import of NP, which in turns enhances viral

replication in mammalian hosts (Gabriel *et al.*, 2008; Gabriel *et al.*, 2011). Furthermore, there is also a functional association between NP and PB2 in host adaptation through host specific residues such as NP-R150, PB2-K627 and PB2-R630 (Ng *et al.*, 2012). The combined effect of adaptive mutations in NP and PB2 of avian influenza viruses were shown to enhance the interaction between NP and polymerase protein which directly increases the RNP activity in mammalian cells. (Mänz *et al.*, 2013; Danzy *et al.*, 2014).

1.3. Avian influenza virus (AIV)

Influenza A viruses which are found mainly in birds are referred to avian influenza viruses (AIV), but these viruses can also infect humans. In general, the risk from avian influenza is minimal to most people. However, it has been confirmed that there were multiple cases of human infection from many different avian influenza subtypes since 1997. H3 and H6 subtypes of influenza A viruses are observed most particularly in ducks whereas H7 subtype is found in chickens. Moreover, H4, H9, H11, and H13 subtypes are found more frequently in shorebirds and seagulls. Interestingly, H5 subtypes have been found in wide range of both domesticated and wild birds, especially in Southeast Asia region (Webster and Kawaoka, 1988). The N2, N6, and N8 subtypes of the NA gene predominate in ducks, while N6 and N9 are more common in shorebirds and gulls. Outbreaks of avian influenza virus infections not only occur in wild and domestic birds, but also spread in other species including humans. Most human cases with avian influenza infection are found to be transmitted from direct contact with infected domestic poultry (e.g. turkeys, ducks and chicken) or contaminated surfaces with the excretions/secretion from these infected birds.

Among several subtypes of AIVs, H5 and H7 subtypes have been particularly highlighted in most of the AIV surveillance programs due to their capability to undergo

mutations from low pathogenic AIV (LPAI) to high pathogenic AIV (HPAI). For instance, outbreak of HPAI of subtype H7 has been reported in Europe region. Countries such as Italy (Capua *et al.*, 2002), Canada (Pasick *et al.*, 2005), Chile (Suarez *et al.*, 2004), Netherlands (Fouchier *et al.*, 2004) and Britain (Hirst *et al.*, 2004) were affected by the H7 subtype pandemic. Later, human infections with the most recent novel avian influenza virus (H7N9), resulting from human exposure to infected poultry, were first reported in China in 2013, and the virus isolates from humans were extensively characterized (Chen *et al.*, 2013; Lam *et al.*, 2013; Watanabe *et al.*, 2013). Although genetic analysis has pointed out all six internal genes of H7N9 originated from H9N2 viruses (Gao *et al.*, 2013; Liu *et al.*, 2013), the H7N9 was found to be more highly pathogenic in humans than H9N2 due to continual reassortment and mutations in the internal genes of H7N9 (Lu *et al.*, 2014; Bi *et al.*, 2015).

1.3.1. Outbreaks of High Pathogenic Avian Influenza (HPAI) H5N1

Among outbreaks of HPAI, the most well-known outbreak was HPAI H5N1. The H5N1 virus was first detected in geese of Guangdong, China in 1996 (Wan, 1998), while the first outbreak of H5N1 HPAI in humans was originated from Hong Kong in 1997 (Shortridge *et al.*, 1998). Later, this virus was classified as HPAI because of its high mortality rate (70% - 100%) in chickens (Suarez *et al.*, 1998; Subbarao, 1998). It was also reported >60% mortality rate in human population although no efficient human to human transmission was observed (Gambotto *et al.*, 2008). The major characteristic of the HPAI H5N1 viruses is its multibasic sequence (R-X-K/R-R) at the HA cleavage site, which is a characteristic of highly pathogenic viruses (Shortridge *et al.*, 1998) (Figure 1.15). This cleavage site that is recognized by intracellular, subtilisin- and furin-like proteases secreted by most of the host cells, enables the virus to infect multiple organs and causing systemic infection. Many of the H5N1 isolates possess a shortened

NA stalk and this characteristic of viruses is found in an adapted influenza virus in land-based poultry (Matrosovich *et al.*, 1997). Since late 2003, HPAI H5N1 viruses have spread widely in Southeast Asia countries (CDC, 2009a) and numerous cases of direct avian-to-human transmission and death in humans have been reported. Based on the surveillance data from 2009 to 2014, avian influenza viruses were found to be continuously evolving genetically and antigenically. Recently, increasing H5N1 infections in both poultry and human cases were observed in Egypt (Ghazi *et al.*, 2016). This continuous circulation of panzootic H5N1 viruses in poultry and the increase in human infections have raised both animal and public health concerns.

Figure 1.15. HA cleavage site in LPAI and HPAI (Adapted from Horimoto and Kawaoka, 2005)

1.3.2. Outbreaks of Low Pathogenic Avian Influenza (LPAI) H5N2 and H9N2

Not only H5N1, but also H5N2 subtype of avian influenza virus was also found to infect poultry. Although no cases have been reported for the emergence of HPAI H5N2 in South East Asia, there have been reported cases of LPAI H5N2 in poultry of several Asian countries. In addition, emergence from LPAI H5N2 subtype to HPAI in domestic poultry was first reported in North America, and next in Mexico and Europe. In addition, the first report of H5N2 avian influenza in humans was from Japan during

2005 (Okamatsu *et al.*, 2007), and the seropositive cases were detected among workers working in the chicken farms where a huge H5N2 bird flu outbreak occurred (Yamazaki *et al.*, 2009).

Generally, LPAI causes minimal or no clinical signs in birds whereas HPAI can manifest severe clinical signs with high mortality in birds. The mechanism for the emergence from LPAI to HPAI remained unclear, but it was believed that the LPAI H5 virus evolved into the HPAI isolates in poultry (Okamatsu *et al.*, 2007). Previous reports pointed out that gradual mutations in LPAI allowed the virus to emerge as HPAI. In the case of LPAI virus outbreak in Pennsylvania in 1983, the progression from LPAI to HPAI was due to the loss of N-linked carbohydrate side chain located near the cleavage site of HA (Kawaoka *et al.*, 1984). In recent years, HPAI H5N2 bird flu has re-emerged and spread across the United States, causing outbreaks in chicken, turkeys and other domestic birds (CDC, 2015). Although this recently identified HPAI H5 subtype is not known yet to infect humans, its appearance in North American birds may increase the chance of human infection. Besides the U.S, this HPAI H5N2 has also been observed sporadically among chicken in Taiwan via reassortment between two different viruses (HA and NA genes derived from a Mexican-like H5N2 virus and internal genes derived from a local enzootic H6N1 virus) (Lee *et al.*, 2014).

Hence, most HPAI strains are commonly derived from LPAI strains that acquire gene reassortment or mutations in the HA gene. In addition, other viral internal genes such as polymerase or NS1 genes can also potentially be responsible for its pathogenicity, virulence and mode of transmission among avian and human populations (Shortridge *et al.*, 1998). It is hypothesized that the genome constellations resulting from genetic reassortment in influenza viruses determines their ability to cross species barrier, spread in new hosts, and potentially cause pandemics (Dugan *et al.*, 2008).

Another subtype of LPAI H9N2 viruses has also been circulating worldwide. The first detection of the H9N2 in 1966 was from turkeys in Wisconsin (Homme and Easterday, 1970). Many of the H9N2 viruses in North America were detected in wild ducks and gulls (Kawaoka *et al.*, 1988; Shaw *et al.*, 2002), but there was no H9N2 virus infection report in chickens (Perez *et al.*, 2003). Before 1990, ducks were the only source of H9N2 viruses in Asia. Since then, infections caused by H9 subtype have been identified in many Asian countries, especially in chickens (Naeem *et al.*, 1999; Guo *et al.*, 2000). Later in Hong Kong and Mainland China, the H9N2 viruses could be isolated from humans with influenza-like illness (Guo *et al.*, 1999; Peiris *et al.*, 1999)). In general, LPAI viruses cause mild to moderate symptoms only in humans. However, they may cause high morbidity if there is co-infection with other respiratory pathogens such as *pneumococcus* (Nili and Asasi, 2002; Joseph *et al.*, 2013). Based on the studies of H9N2 viruses isolated from the last 20 years, they have evolved rapidly and reassorted with other avian influenza viruses to give rise to new genotypes of virus (Li *et al.*, 2003; Xu *et al.*, 2004; Li *et al.*, 2005b; Xu *et al.*, 2007). Iqbal *et al.* discovered that the H9N2 viruses in the Indian sub-continent have extensive genetic reassortment, causing a novel genotype of H9N2 virus with NS gene similar to HPAI viruses, H7N3 and H5N1 (Iqbal *et al.*, 2009). In addition, H9N2 viruses donated all six of reassortant's internal genes to novel H7N9 viruses, and facilitated the H7N9 virus formation by their genotypic evolution (Pu *et al.*, 2015; Ge *et al.*, 2016). The most recent study by Jia Wang and his group also highlighted the infectivity and interspecies transmission of enzootic H9N2 influenza viruses from birds to pigs (Wang *et al.*, 2016a). This could be an early warning of the emergence of novel reassortants with potential to infect humans.

1.4. Human influenza virus

According to Centre for Disease Control (CDC) terminology, human influenza A viruses refer to those subtypes that generally infect humans and can be found widespread throughout the world (CDC, 2007). The most known subtypes of influenza A viruses with common pattern of circulation among humans are H1N1, H1N2 and H3N2, which termed “currently” circulating influenza virus (CDC, 2007). CDC also sets a rule, where any case of human infection with a novel influenza A virus that is different from currently circulating human influenza H1 and H3, such as H5 strain classified as a “recently” circulating virus with the confirmation process from CDC laboratory (CDC, 2010). Due to antigenic drift (accumulation of mutations within the genes that code for antibody-binding sites) and antigenic shift (reassortment or viral shift that confers a phenotypic change), the current circulating human influenza virus will be antigenically different from those circulating in the past year. This type of influenza is commonly referred as seasonal influenza, or “seasonal flu”, “common flu” or “annual flu” (CDC, 2007). It generally causes annual influenza epidemics that are mostly not lethal to humans except to the elderly (>65 years old) or young (<5 years old) (CDC, 2007). The seasonal influenza is a recurring time period characterized by the prevalence of outbreaks of influenza in a specific region. This occurs during the winter period in both northern and southern hemispheres (CDC, 2007). With genetic analysis, seasonal influenza activity can sometimes be predicted according to geographic regions (NIAID, 2009). The beginning of the seasonal influenza emergence may vary by location. The disease will take approximately 3 weeks to reach the peak, followed by another 3 weeks to diminish (NIAID, 2009).

Currently, there are two circulating HA subtypes (H1 and H3) and two NA subtypes (N1 and N2) that can transmit efficiently among humans (CDC, 2009b). This

will increase the possibility for genetic reassortment between the two viruses. The human seasonal influenza prevalence of different subtypes may vary between different seasons. For example, the H3N2 strain has been the worldwide main influenza A strain for the last two decades, except from 1988–1989 and 2000–2001 seasons when H1N1 infections were mostly predominant (Lin *et al.*, 2004). A new reassorted human strain from two dominant human influenza virus H1N1 and H3N2 to form H1N2, had emerged in Europe continent and became dominant in the September–November 2001 in the Northern hemisphere (Paget *et al.*, 2002; Ellis *et al.*, 2003).

1.4.1. Outbreaks of Human Pandemic Influenza H1N1

Human Influenza A H1N1 virus is one of the influenza A virus subtypes that is currently endemic in the human population. The typical seasonal influenza virus infects approximately 5% of total population with 100,000 death annually (CDC, 2007). The first H1N1 subtype was first successfully isolated by Wilson and Smith in 1933, which is named A/WS/33 (H1N1) (Wilson and Smith, 1933). There was a major influenza pandemic caused by H1N1 influenza virus strain in 1918, commonly known as “Spanish Flu” (Cox and Subbarao, 2000). After the 1918 “Spanish Flu” pandemic, the H1N1 subtype of virus diminished rapidly for almost 50 years, and the pandemic occurred with H2N2 and H3N2 in 1957 and 1968, called “Asian Flu” and “Hong Kong Flu” respectively (Cox and Subbarao, 2000). The first re-emergence of the H1N1 was in 1977, known as “Russian Flu” (Webster *et al.*, 1992). Since then, both H3N2 and H1N1 recirculate in human population.

Based on the knowledge that waterfowl is the natural reservoir of the influenza virus, the genetics analysis have been carried out on the H1 strain of the influenza circulating in 1930s by Hinshaw *et al.* in order to determine the origins and relationships of the H1N1 virus with the older H1N1 strain from other species. They found that the

human H1 variants circulating in the early 1930s were closely related to swine influenza viruses and these variants were later found to be similar to viruses from wild ducks antigenically.

The most recent pandemic involving H1N1 influenza virus emerged in 2009, known as “pandemic 2009 H1N1 flu” or “swine-origin H1N1 flu”. It was first detected in Mexico and United States in April 2009 and this novel virus appeared to be a new strain of H1N1 of swine origin, which had not infected human previously (CDC, 2009b). It was resulted from a reassortant of triple reassortment as shown in Figure 1.16: i) classical swine RNA-segments from the North American lineage (HA, NP, NS), ii) avian influenza RNA-segments from the North American lineage (PB2 and PA), iii) human seasonal H3N2 lineage (PB1), and iv) Eurasian avian-like swine H1N1 lineage (NA and M) (Neumann *et al.*, 2009).

Figure 1.16. The origin of 2009 swine influenza virus (Adapted from Neumann *et al.*, 2009).

On June 2009, the WHO declared this novel virus as a global pandemic H1N1 influenza A and raised an alert level from phase 3 to phase 5 (human-to-human spread in at least two countries, and signs of an imminent pandemic). The new H1N1 virus has continue spreading and the infected cases have expanded to more than 440,000 with more than 5,700 deaths worldwide as of November 2009 documented by WHO. Many studies have been performed to try to elucidate the mechanism of action of the novel 2009 H1N1 influenza virus. This includes growth characteristics *in vitro* and *in vivo* (Itoh *et al.*, 2009), transmission study in ferrets and mice (Maines *et al.*, 2009), cytokine profiling of swine influenza virus (Woo *et al.*, 2010), and some unanticipated host and viral protein interactions (Shapira *et al.*, 2009). Moreover, Furuse *et al.* also performed internal genes analysis from 673 strains of influenza A virus derived from human, avian and swine host (Furuse *et al.*, 2010). Base on their finding, the adaptation of 2009 swine influenza virus in human host was due to mutations from continual genetic reassortment.

From 2010 onward, this novel virus continued to circulate in several northern and western states of the country. It caused more severe illness in England in the year 2010/11 than in the pandemic year due to absence of vaccination for children, public education and awareness (Mytton *et al.*, 2012). Along with the previously circulating influenza A H3N2 and influenza B viruses with seasonal activity, the 2009 H1N1 strain re-emerged in several Indian states in 2012-2013 during winter (Broor *et al.*, 2012; Dangi *et al.*, 2014). The Mullick group detected a D222G mutation in HA gene of circulating Indian isolates which showed binding ability to both α 2-3/ α 2-6- sialic acid linkage, leading to a possible cause of concern (Mullick *et al.*, 2011). Moreover, multiple influenza viruses including 2009 H1N1 co-circulated in Southern China during the winter of 2013-14, and a fatal case of co-infection with human 2009 H1N1 and avian H7N9 subtypes was also reported (Wanju *et al.*, 2015). The ongoing resurgence of 2009

H1N1 virus could be still a disease burden, and annual flu vaccination as recommended by CDC could possibly have controlled the severity of this resurgent wave.

1.5. Isolation and characterization of LPAI H5N2, H9N2 and human pandemic 2009 H1N1 isolates used in our study

The Agri-food & Veterinary Authority of Singapore (AVA) performs testing on samples from live avian species imported into Singapore as part of routine surveillance for AIVs. Between 1993 and 2007, three LPAI H5N2 viruses were isolated from live broiler ducks imported into Singapore by AVA named as A/duck/Malaysia/F59/2004 (H5N2/F59), A/duck/Malaysia/F118/2004 (H5N2/F118), and A/duck/Malaysia/F189/2004 (H5N2/F189). With the help of colleagues from Defence Medical and Environmental Research Institutes (DMERI) at DSO national laboratories, our lab performed rapid subtyping and complete molecular characterization of these isolates such as sequence and phylogenetic analyses of all eight full-length genes, studying of their replication and growth characteristics in different tissue culture cell lines, and profiling of virus-host gene expression with microarray technology (Yeo *et al.*, 2009; Sutejo *et al.*, 2012). In addition, three H9N2 influenza viruses were also identified from poultry imported into Singapore between 1990 and 2011 (NCBI Influenza Databanks). Our lab also carried out full characterization on one of the isolates namely A/duck/Malaysia/01/2001 (H9N2) (Accession: CY073797.1...804.1) obtained from AVA.

Furthermore, influenza A 2009 pandemic H1N1 viruses were isolated from nasal swabs of positively infected patients in Singapore during 2009 pandemic. Four of the highest positive isolates namely A/Singapore/276/2009 (pH1N1/276), A/Singapore/471/2009 (pH1N1/471), A/Singapore/478/2009 (pH1N1/478), and A/Singapore/527/2009 (pH1N1/527) were cultured in DSO lab and tissue culture

supernatants were grown in embryonated eggs. Additionally, in-depth molecular characterization including replication characteristics and antiviral signaling responses were also carried out with these isolates in our lab (Yeo *et al.*, 2009; Sutejo *et al.*, 2012).

1.6. Introduction to specific monoclonal antibody in the study of molecular interactions

Monoclonal antibodies are antibodies that are specific to a particular antigen and produced by plasma cells from a particular clone of unique parent cells. So they have only monovalent affinity (i.e., they bind to only one same epitope). Given that specificity and ability to produce monoclonal antibodies against almost any substance, this has become an important tool in biochemistry, molecular biology and medicine.

This modern era of monoclonal antibodies originated with the invention of hybridoma technology to produce mouse monoclonal antibodies in 1975. Before that technology, there were many problems to generate monoclonal antibodies. For examples, the response of the immune system to any antigen is polyclonal i.e., a pool of different antibodies which vary in epitope recognition and classes, and even if a single antibody-secreting cell could be isolated and cultured, it would die out after a few generations due to limited growth capacity of normal somatic cells.

Köhler and Milstein overcame these shortfalls by inventing hybridoma technology (Figure 1.17). It combined the unlimited growth capacity of myeloma (cancerous B cells) and the antibody specificity of immune spleen cells which was immunized against a particular antigen. In this method, a fusion agent was used to facilitate the fusion of plasma membranes of these cells, and a special selective media, hypoxanthine-aminopterin-thymidine (HAT) medium, to select and grow fused cells. The idea of using selective media was based on a blockage of the *de novo* pathway for DNA synthesis by aminopterin. Unfused myeloma cells, which are unable to utilize the

salvage pathway for nucleotide synthesis, die due to lack of hypoxanthine-guanine phosphoribosyl transferase (HGPRT). Therefore, only fused B cells-myeloma hybrids possessing HGPRT from the B cells, can survive. These fused cells can grow unlimited (a property of myeloma cells) and secrete antibodies (a property of B cells).

Figure 1.17. An overview of the hybridoma method used to generate monoclonal antibodies (Adapted from (Jones, 2010)).

This mixture of cells is then diluted and grown in culture media on microwell plates. The supernatant is tested for specific antibodies, and the culture cells are subsequently subcloned (till a single clone is achieved), hence the antibodies are monoclonal. The hybridoma can be grown unlimitedly and in a large scale as well. They can be grown in a suitable culture medium or injected into peritoneal cavity of mice. Later, the antibodies can be purified from culture medium or ascetic fluid of mice by various methods such as protein A/G affinity chromatography, ion exchange chromatography. Because of its specificity and ability to conjugate with different molecules such as fluorescent molecule or radioactive atom, the application potential of

monoclonal antibodies is unlimited. They are widely used in research, diagnosis and human medicine.

To use mouse antibodies as therapeutic agents, there are some major limitations such as lack of effector functions, immunogenicity and short serum half-life. These limitations could be overcome by antibody chimerization and humanization technologies. Many modern antibody therapies are chimeric or humanized versions of mouse antibodies. However, some monoclonal antibodies are now fully human which are likely to be safer than earlier monoclonal antibodies. An even newer approach uses fragments of antibodies instead of whole ones which may make them more effective. Antibodies have rapidly become established as a clinically important therapeutic choice. More than 25 antibodies have been approved for human treatment and hundreds of antibodies are currently in clinical development worldwide for a wide range of diseases, including cancer, organ transplantation, ophthalmological diseases, cardiovascular diseases, infections, inflammations and autoimmunity (Chan and Carter, 2010). Wang *et al.* have characterized many fully human monoclonal antibodies against a consensus-sequence of influenza A virus M2e peptide from M2 protein using transchromosomal engineered-mice (Wang *et al.*, 2008). One of these antibodies strongly bound to the M2e variants from most of the viral isolates, including HPAI strains. When this antibody was used as a passive immunotherapeutic agent, it demonstrated a significant decrease in virus replication in the mice lung and protected the animals from lethal infection. Human monoclonal antibodies have been developed not only against influenza A virus, but they could also be broadly used as universal prophylactic agents or potential therapeutics against influenza B virus (Mayo *et al.*, 2013).

In the field of virology, monoclonal antibodies have contributed important advances in knowledge of molecular epidemiology of viruses and structures of viruses.

There are extensive studies about antigen drift and shift of influenza A virus using monoclonal antibodies. In addition, studies of antigenic variants of different viruses such as influenza and rabies, using monoclonal antibodies, revealed interesting findings and potential application. For example, Webster *et al.* have reported several monoclonal variants of A/Hong Kong/68 (H3N2) virus which showed single amino acid changes in or close to peptide HA1 (Webster *et al.*, 1980). That particular region of the HA molecule is important for change in antigenicity of the HA. Such detailed knowledge will be essential for future genetic cloning or a synthetic vaccine against antigenic portion of HA. Recently, a neutralizing human monoclonal antibody, CT149, isolated from recovered patients infected with 2009 pandemic H1N1 was shown to recognize the fusion domain in HA2 and inhibit the HA-mediated membrane fusion (Wu *et al.*, 2015). This antibody also revealed the binding characteristic to the HA protein, providing more information to design high potency flu vaccines.

In addition, a panel of mouse monoclonal antibodies against HPAI H5N1 virus was developed as a rapid diagnostic tool by Sonja Linke group (Linke *et al.*, 2011). Most of their antibodies were found to react strongly with the HA protein of H5 subtypes, whereas one antibody was specifically reacted to H5N1 in the immunofluorescence assay and two antibodies were shown to neutralize H5 subtypes.

The potential use of monoclonal antibodies in virology will not be exhausted and it will be expanding with time. Therefore, our group also plan to carry out the biological characterizations on polymerase subunits of influenza A virus by using monoclonal antibodies, with the main purposes of specific viral polymerase protein detection in influenza virus-infected cells, studying virus-host interactions and identifying important host factors.

1.7. RNA interference (RNAi) and its potential as therapeutics

1.7.1. Mechanism of RNAi

RNA interference (RNAi) is a simple and natural process of sequence-specific gene silencing firstly described as posttranscriptional gene silencing in virus-infected plants (Vaucheret *et al.*, 2001). The phenomena of RNAi is also conserved in a broad range of eukaryotes and mediated by double-stranded RNA (dsRNA) using a two-step mechanism (Fire *et al.*, 1998; Sontheimer, 2005; Bumcrot *et al.*, 2006) (Figure 1.18). The first step involves cleavage of long dsRNA substrates into small interfering RNAs (siRNAs) which are about 21-25 nucleotide in length, by an RNase Dicer protein. In the second step, the siRNAs join to a protein complex, RISC (RNA-induced silencing complex), where the antisense strand separated from the sense strand of siRNA looks for and binds to the complementary mRNA. These mRNA substrates are then cleaved within the RISC by an Argonaute2 enzyme, resulting in mRNA degradation and subsequently mRNA down-modulation.

Figure 1.18. RNAi mechanism in mammalian cells (Adapted from Bumcrot *et al.*, 2006).

1.7.2. RNAi as an antiviral in *in vitro* and *in vivo*

One of the advanced potential applications of the RNAi pathway is to use it in therapeutic approaches, especially in the development of an antiviral therapy (Elbashir *et al.*, 2001; Dorsett and Tuschl, 2004; Bumcrot *et al.*, 2006; van Rij and Andino, 2006). This can be achieved by introducing the alternative strategy of naturally occurring siRNAs such as delivery of synthetic siRNAs or plasmids expressing short hairpin RNAs (shRNAs) in *in vitro* tissue culture cells. By pre-treating the cells with virus-specific siRNAs or shRNA, it will program the RNAi machinery to target virus for degrading the viral mRNA and inhibiting the virus replication. These RNAi-based antiviral studies have been observed in cultured cell lines with various human viral pathogens including respiratory viruses (Table 1.2). Later on, these *in vitro* observations were successfully extended to *in vivo* studies by challenging the animal models with virus infections. Generally, the *in vivo* studies demonstrated effective silencing of disease genes in animals by systemic or local application of siRNAs or shRNAs. In addition, the most effective antiviral effect in these studies was observed with animals treated with siRNAs prior to virus infections, in either dose or concentration-dependent fashion. Overall, these studies proved the principle of novel RNAi-based therapeutic and prophylactic approaches in controlling acute and chronic viral infections.

1.7.3 RNAi-based antiviral approaches in influenza A virus infection and current progress

Several RNAi-based therapeutic approaches with respiratory virus infections have been extensively studied. The first proof of a synthetic siRNA as an antiviral can be used in 2001 where the replication of respiratory syncytial virus (RSV) was inhibited by directly targeting viral mRNAs which code for the critical viral RNA transcription factor (Bitko and Barik, 2001). Later, many studies have been carried out for antiviral

drug development against influenza virus infection in the human population. The successful antiviral approach of siRNA was first reported with influenza virus-infected mammalian cells (Ge *et al.*, 2003). In the report, potent viral inhibition was observed using the sequence specific siRNAs against conserved regions of the influenza NP and PA genes.

To see the anti-influenza effect of RNAi in animal models, lentivirus vector-expressing NP shRNA and PB1 shRNA were injected intravenously into mice and the result showed pronounced inhibition of influenza virus production (Ge *et al.*, 2004). In addition to systemic mode of delivery, the profound inhibition of viral replication with intranasal application of siRNA in mouse models has been observed within a few days after RSV or influenza virus infection (Ge *et al.*, 2004; Tompkins *et al.*, 2004; Bitko *et al.*, 2005; Zhang *et al.*, 2005; Bitko and Barik, 2007; Zhou *et al.*, 2007; Barik, 2010). This type of local administration was less invasive and provided a prompt delivery of siRNA to the lungs, causing a significant decrease in lung virus titers of infected mice and protection of animals from lethal challenge with highly pathogenic avian influenza H5 and H7 subtypes (Tompkins *et al.*, 2004).

Virus Family	Genome	Virus	<i>In-vitro</i> study	<i>In-vivo</i> study	Outcome from <i>in vivo</i>	Reference
<i>Orthomyxoviridae</i>	Segmented, (-) ssRNA	Influenza A virus	siRNA, shRNA, SD powders (Peptide/siRNA complexes)	Intravenous injection of siRNA or shRNA, Intranasal administration of siRNA	Decreased lung titers, Potent inhibition of viral replication	Refer to Table 3
<i>Paramyxoviridae</i>	(-) ssRNA	(i) Respiratory syncytial virus (ii) Human parainfluenza virus-3	(i) siRNA (ii) siRNA	(i) Intranasal administration of siRNA or shRNA (ii) Intranasal administration of siRNA	(i) Decreased lung titers and pulmonary pathology (ii) Decreased lung titers	(Bitko and Barik, 2001; Bitko <i>et al.</i> , 2005; Zhang <i>et al.</i> , 2005; Alvarez <i>et al.</i> , 2009) (Barik, 2005; Bitko <i>et al.</i> , 2005)
<i>Coronaviridae</i>	(+) ssRNA	SARS-associated coronavirus	siRNA	Intratracheal administration of siRNA	Decreased viral RNA and lung pathology	(Wang <i>et al.</i> , 2004; Li <i>et al.</i> , 2005a; Pyrc <i>et al.</i> , 2006)
<i>Flaviviridae</i>	(+) ssRNA	(i) West Nile virus (ii) Japanese encephalitis virus	(i) shRNA (ii) siRNA, shRNA	(i) Hydrodynamic injection of siRNA (ii) Intracranial injection of siRNA or shRNA	(i) Decreased viral load, partial protection from lethal infection (ii) Protection from lethal encephalitis	(Bai <i>et al.</i> , 2005; Geiss <i>et al.</i> , 2005) (Kumar <i>et al.</i> , 2006)
<i>Retroviridae</i>	ssRNA	Human immunodeficiency virus-1	siRNA, shRNA	Intravenous injection	Decrease viral load and disease-associated CD4 T-cell loss	(Lau <i>et al.</i> , 2007; Kim <i>et al.</i> , 2010b; Bobbin <i>et al.</i> , 2015)
<i>Hepadnaviridae</i>	Circular, partially dsDNA	Hepatitis B virus	siRNA, shRNA	Hydrodynamic injection and intravenous injection of siRNA and shRNA	Decreased virus replication and viral antigen	(Giladi <i>et al.</i> , 2003; McCaffrey <i>et al.</i> , 2003; Morrissey <i>et al.</i> , 2005; Uprichard <i>et al.</i> , 2005; Hean <i>et al.</i> , 2010)
<i>Herpesviridae</i>	dsDNA	Herpes simplex virus-2	siRNA	Intravaginal application of siRNAs complex with lipid	Protection from lethal viral challenge	(Palliser <i>et al.</i> , 2006; Wu <i>et al.</i> , 2009)

Table 1.2. RNAi-based antiviral approaches in *in vitro* and *in vivo* with various human viral pathogens including respiratory viruses.

Target gene	Strain of influenza virus	Test animal/ cell lines	Reference
PB2	H1N1, H5N1	<i>in vitro</i>	(Ge <i>et al.</i> , 2003; Behera <i>et al.</i> , 2015)
PB1	H1N1, H3N2, H5N1	<i>in vitro and in vivo</i>	(Ge <i>et al.</i> , 2003; Ge <i>et al.</i> , 2004; Jain <i>et al.</i> , 2014; Mahmoud <i>et al.</i> , 2014)
PA	H1N1, H5N1, H7N7, H9N2	<i>in vitro and in vivo</i>	(Ge <i>et al.</i> , 2003; Ge <i>et al.</i> , 2004; Tompkins <i>et al.</i> , 2004; Mahmoud <i>et al.</i> , 2014)
NP	H1N1, H1N2, H3N2, H5N1, H7N1, H7N7, H9N2	<i>in vitro and in vivo</i>	(Ge <i>et al.</i> , 2003; Ge <i>et al.</i> , 2004; Tompkins <i>et al.</i> , 2004; Zhou <i>et al.</i> , 2007; Behera <i>et al.</i> , 2015; Stoppani <i>et al.</i> , 2015)
M	H1N1, H5N1	<i>in vitro</i>	(Ge <i>et al.</i> , 2003; Hui <i>et al.</i> , 2004; Zhou <i>et al.</i> , 2007; Sui <i>et al.</i> , 2009)
NS	H1N1, H5N1	<i>in vitro and in vivo</i>	(Ge <i>et al.</i> , 2003; Jiao <i>et al.</i> , 2012; Rajput <i>et al.</i> , 2012)

Table 1.3. Different gene segments from various influenza A virus strains targeted to silence the expression, via siRNA.

A number of influenza viral gene-specific siRNAs have now been designed and their degrees of inhibition have been tested *in vitro* and *in vivo* (Table 1.3) (Khanna *et al.*, 2015). These results indicate that RNAi-based antiviral approaches are promising for both treatment and prophylaxis of influenza virus infection. However, there are certain limitations such as undesirable cell toxicity and systemic adverse effects from the modes of siRNA administration. Therefore, efficient local siRNA delivery to pulmonary cells is necessary to reduce the therapeutic dose and minimize the limitations. In recent years, pulmonary delivery of inhalable spray-dried (SD) powder formulation of siRNA has been considered as a therapeutic potential due to its highly effective gene silencing, good stability and low toxicity (Lam *et al.*, 2012; Abbate *et al.*, 2013; Liang *et al.*, 2014). The SD powder formulated against H1N1 influenza virus was characterized by Liang *et al.* and it was shown to be suitable for inhalation with the antiviral activities (Liang *et al.*, 2015). The formulation can therefore be a promising therapeutic agent for the treatment of influenza and other respiratory viral infections.

The siRNA-based therapeutic compounds are already entered into clinical trial pipeline for the treatment of respiratory diseases (Ballarín-González *et al.*, 2012). To translate the successful animal results into human trials, the antiviral activity of ALN-RSV01 for RSV infection has been conducted in a Phase II human clinical trials by Alnylam Pharmaceuticals, and the results claimed to be promising with its safety and tolerability (Alnylam, 2007; Bitko and Barik, 2007). Similarly to ALN-RSV01, Alnylam Pharmaceuticals also collaborated with Novartis and Arrowhead Pharmaceuticals to develop an RNAi drug against all strains of influenza (Alnylam, 2007; Alnylam/Arrowhead, 2016). These groups have demonstrated the activities of ALN-FLU01 (Patent: US7579451) for both pandemic flu and H5N1 isolate with advanced progress.

1.8. Research Objectives

1.8.1. Generation and characterization of antibodies against polymerase proteins from human 2009 pandemic H1N1 (pH1N1) and low pathogenic avian (LPAI) H5N2 subtypes of influenza virus

Different strains of influenza virus have been known to behave differently and interact with the host in several ways. Interestingly, some strains of influenza virus can infect and replicate well in a particular host, but not in the others. This indicates that there are specific interactions between the virus and the host which can control the virus growth. Moreover, the influenza viral RNA polymerase complex composed of PA, PB1 and PB2 subunits plays a key role in transcription and replication of viral genome, and its pathogenicity. To have a better understanding of the association between host cells and viral polymerase proteins, it is important to identify host factors and study the possible virus-host interactions which influence species adaptation and transmission. In addition, one of the major interests in influenza virus research involves the biological characterization of viral transcription and replication complexes at the molecular level. It contains the study of functionally relevant domains in each protein component of the complex and those involved in the interaction between the different subunits.

To address these issues, obviously there is a need to generate specific antibodies against the individual polymerase subunits which are not readily available and use them as detection reagents in the study of virus-host interactions and functional analyses. For that reason, one major scope of my project is to generate and characterize monoclonal antibodies (mAbs) against human and avian influenza A viral RNA polymerase proteins (PA, PB1 and PB2). In this study, the polymerase proteins of two well-characterized virus isolates (pH1N1/471) and (H5N2/F118) will be used in the generation of mAbs directly against these proteins. Subsequently, the generated antibodies will be

characterized by using recombinant expressed proteins in mammalian cells for testing the specificity of antibodies, and virus-infected cells to study the replication kinetics/expression profiles of these proteins in the course of virus infection. In addition, purified influenza virus particles will also be involved to show the antibody ability to detect the viral polymerase proteins of mature infectious virus. The molecular biology techniques such as IFA and Western blotting will be conducted in the process of characterizations.

1.8.2. Functional analysis of influenza viral polymerase complex via polymerase reporter assay system

Reassortants from human and avian influenza viruses were shown to cause influenza pandemics. To gain a better understanding on these reassortant viruses, one way to address is the functional study of their polymerase genes after reassortment. Therefore, reassortment between human and avian strains will be performed to study the compatibility among NP and polymerase subunits. In our reassortment study, laboratory-adapted influenza strain (H1N1/WSN), pandemic H1N1 2009 (pH1N1/471), and low pathogenic avian influenza (H5N2/F118 and H9N2) strains will be the main focuses. This study will involve mammalian cells expressing the reporter gene and the viral genes (NP, PA, PB1 and PB2) either from individual strain or reassortment. Then, the polymerase activities between parental complex and reassortants will be analyzed using a reporter assay system. The findings from this study may provide the possibility of recombinant influenza vaccine for the control of future pandemics.

1.8.3. Silencing of influenza polymerase genes via RNAi

The recent emergence of new avian influenza viruses, their ability to cross species barriers and their high virulence have led to a public health concern. In recent years, RNA interference (RNAi) therapy has rapidly evolved as a new therapeutic

approach. Therefore, our aim is to assess of RNAi technique as an antiviral strategy by the introduction of viral gene specific siRNAs in mammalian cells. Due to the potential emergence of a novel pandemic H5 virus from a low pathogenic avian influenza virus (LPAIV) and possible spread to the human population, LPAI subtype H5N2 will be chosen as a model of our study. In addition, this study will mainly focus on NP and three polymerase genes which are important in viral replication and transcription. Using a number of *in-vitro* assays, the efficacies of custom-designed siRNAs against these four genes of H5N2 will be evaluated.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

All general reagents used in the experiments were analytical/molecular biology grades. For the sterile works, the reagents were either autoclaved at 121°C for 20 min or passed through 0.2 µm filter-sterilization (Sartorius).

2.1.1. Cells

MDCK (Madin Darby Canine Kidney Cells)	ATCC
A549 (Human Adenocarcinomic Lung Cells)	ATCC
HEK 293T (Human Embryonic Kidney Cells)	ATCC
Myeloma Cells P3x63Ag8.653 (CRL 1580)	ATCC
Embryonated Chicken Eggs	Animal & Plant Health Centre, Singapore
One-shot® TOP10 Chemically Competent <i>E. coli</i> (Cat. # K450001)	Invitrogen
One-shot® BL21 (DE3) Chemically Competent <i>E. coli</i> (Cat. # C60000)	Invitrogen

2.1.2. Viruses

A/WSN/1933 (H1N1/WSN)	ATCC
A/Singapore/471/2009 (pH1N1/471)	DSO National Lab
A/Duck/Malaysia/F118/2004 (H5N2/F118)	DSO National Lab
A/Duck/Malaysia/1/2001 (H9N2)	AVA

2.1.3. Tissue culture media and solutions

Dulbecco Modified Eagle Medium (DMEM) + GlutaMAX (Cat. # 10569-010)	Gibco
Foetal Bovine Serum (FBS) (Cat. # 10270-106)	Gibco
Sterile 10× Phosphate Buffer Saline (pH 7.2) (Cat. # 70013-032)	Gibco
Penicillin-Streptomycin (100x) (Cat. # 15140-122) (10,000 U/ml Penicillin and 10,000 µg/ml Streptomycin)	Gibco
Trypsin-EDTA 0.25% (Cat. # 25200072)	Gibco

Bovine Serum Albumin (BSA) Fraction V 7.5% (Cat. # 15260037)	Gibco
Trypsin-TPCK (Cat. # LS03740)	Worthington
RPMI-1640 Medium (Cat. # 11875093)	Sigma-Aldrich
L-Glutamine (200 mM) (Cat. # G2150)	Sigma-Aldrich
MEM Non-essential Amino Acid Solution (100x) (Cat. # M7145)	Sigma-Aldrich
Sodium Bicarbonate 7.5% Solution (50x) (Cat. # S8761)	Sigma-Aldrich
Sodium Pyruvate Solution (100x) (Cat. # S8636)	Sigma-Aldrich
HAT Media (50x) Supplement (Cat. # H0262)	Sigma-Aldrich
HT Media (50x) Supplement (Cat. # H0137)	Sigma-Aldrich
DMSO (Hybrimax) (Cat. # D2650)	Sigma-Aldrich
BM Condimed H1 (Cat. # 11088947001)	Roche

2.1.4. Cell fusion and mouse immunization

PEG (Hybri-Max™), mol wt 1,300-1,600 (Cat. # P7777)	Sigma-Aldrich
Complete Freund's Adjuvant (Cat. # F5881)	Sigma-Aldrich
Incomplete Freund's Adjuvant (Cat. # F5506)	Sigma-Aldrich

2.15. Protein denaturation, purification and refolding

Urea (Cat. # 17-1319-01)	GE Healthcare
Guanidine HCl (Cat. # 50935)	Sigma-Aldrich
NaCl (Cat. # S3014)	Sigma-Aldrich
Imidazole (Cat. # 56749)	Sigma-Aldrich
Nonident P-40 (Cat. # 21-3277)	Sigma-Aldrich
PMSF (Cat. # P7626)	Sigma-Aldrich
Lysozyme (Cat. # L6876)	Sigma-Aldrich
Sodium Deoxycholate (Cat. # 30970)	Sigma-Aldrich
L-Arginine (Cat. # A5006)	Sigma-Aldrich
Polyethylene glycol (PEG) 6000 (Cat. # 81253)	Sigma-Aldrich
Tris (Cat. # H5135)	Promega
Triton X-100 (Cat. # 1610407)	Bio-Rad
TALON® Metal Affinity Resin (Cat. # 635503)	Clontech
Snakeskin Dialysis Tubing (Cat. # 68035)	Pierce

2.1.6. Antibodies

mAbs-8257 (mouse anti-influenza A nucleoprotein), usage 1:200 (IFA), 1:50 (IP), 1:5,000 (Western blotting)	Merck Millipore
mAbs-PA (mouse anti-PA pH1N1 9F5 and 2E2), usage neat (IFA, IP and Western blotting)	In-House
mAbs-PA (mouse anti-PA H5N2 4D7, 6A10, 6A11, 6B11, 7A4), usage neat (IFA and Western blotting)	In-House
mAb-PB2(mouse anti-PB2 4G3), usage neat (IFA and Western blotting)	In-House
Polyclonal anti-PB1mouse serum, usage 1:500 (IFA), 1:5,000 (Western blotting)	In-House
mAb-His (mouse anti-His), (Cat. # 27-4710-01) usage 1:3,000 (ELISA), 1:5,000 (Western blotting)	GE Healthcare
mAb- β actin (mouse anti- β actin), (Cat. # A2228) usage 1:5,000 (Western blotting)	Sigma Aldrich
mAb- β tubulin (mouse anti- β tubulin), (Cat. # sc-55529) usage 1:5,000 (Western blotting)	Santacruz
Polyclonal anti-FLAG (rabbit), (Cat. # F7425) usage 1:100 (IFA), 1:5,000 (Western blotting)	Sigma-Aldrich
Polyclonal anti-Histone H4 (rabbit), (Cat. # ab7311) usage 1:200 (IFA)	Abcam
Polyclonal anti-lamin A/C (rabbit), (Cat. # sc-20681) usage 1:5,000 (Western blotting)	Santacruz
Goat Anti-mouse IgG Alexa Fluor 488, (Cat. # A11001) usage 1:200 (IFA)	Invitrogen
Goat Anti-rabbit IgG Alexa Fluor 555, (Cat. # A21428) usage 1:2,000 (IFA)	Invitrogen
Goat Anti-mouse IgG Horseradish Peroxidase, (Cat. # A4416) usage 1:10,000 (ELISA and Western blotting)	Sigma-Aldrich
Goat Anti-rabbit Horseradish Peroxidase, (Cat. # A0545) usage 1:10,000 (Western blotting)	Sigma-Aldrich

2.1.7. Immunostaining, ELISA and transfection

Paraformaldehyde (Cat. # 158127)	Sigma-Aldrich
Methanol (Cat. # M/4000/17)	Fisher scientific
Acetone (Cat. # A/0600/17)	Fisher scientific
10× Phosphate Buffer Saline (pH 7.2) (Cat. # CUS-2040)	1 st Base
Triton X-100 (Cat. # 1610407)	Bio-Rad
Tween-20 (Cat. # VWRCM147)	Amresco
H ₂ O ₂ 30% (w/w) (Cat. # H1009)	Sigma-Aldrich
3,3',5,5'-Tetramethylbenzidine (TMB) table (Cat. # T5525)	Sigma-Aldrich
H ₂ SO ₄ (Cat. # 320501)	Sigma-Aldrich
Opti-MEM Reduced Serum Media (Cat. # 31985070)	Gibco
Lipofectamine 2000 (Cat. # 11668019)	Invitrogen
TransIT-LT1 (Cat. # MIR 2300)	MirusBio
Dharmafect 1 to 4 (Cat. # T-2005-02)	Dharmacon
Fluorescence Mounting Medium (Cat. # S3023)	Dako

2.1.8. Commercially available kits

RNeasy Mini Kit (Cat. # 74104)	Qiagen
QIAquick Gel Extraction Kit (Cat. # 28704)	Qiagen
QIAprep Spin Miniprep Kit (Cat. # 27104)	Qiagen
PCR Cloning ^{plus} Kit (Cat. # 231224)	Qiagen
Platinum [®] Taq DNA Polymerase (Cat. # 10966018)	Invitrogen
Platinum [®] Taq DNA Polymerase High Fidelity (Cat. # 11304011)	Invitrogen
SuperScript [®] II First Strand (Cat. # 18064014)	Invitrogen
BioLux [®] Gaussia Luciferase Assay Kit (Cat. # E3300S)	NEB
NE-PER Nuclear and Cytoplasmic Extraction Kit (Cat. # 78833)	Pierce
2-D Clean-Up Kit (Cat. # 80-6484-51)	GE Healthcare
2-D Quant Kit (Cat. # 80-6483-56)	GE Healthcare

2.1.9. Cloning vectors

pCR2.1-TOPO (Cat. # K450002)	Invitrogen
pCR4-TOPO (Cat. # 457502)	Invitrogen

pRSET A (Cat. # V351-20)	Invitrogen
pCAGGS	Gift from C.C. Broder
pPoLI	Gift from DSO National Lab
pIRES2-EGFP	Clontech
pNEBR-XGLuc	NEB

2.1.10. Cloning and *E. coli* growth medium

Restriction Enzymes	
T4 DNA ligase (Cat. # M1801)	NEB
SOC Medium (Cat. # 15544034)	Promega
LB Broth, Miller (Cat. # 244620)	Invitrogen
LB Agar, Miller (Cat. # 244520)	BD, Difco
Ampicillin (Sodium Salt) (Cat. # A-301-25)	BD, Difco
Kanamycin (Powder) (Cat. # K-120-25)	Gold Biotechnology
	Gold Biotechnology

2.1.11. DNA gel analysis

10× Tris-Borate-EDTA Buffer (pH 8.3) (Cat. # BUF-3010)	1 st Base
Ethidium Bromide (Cat. # 161-0433)	Bio-Rad
Agarose (Cat. # PC0701)	Vivantis

2.1.12. SDS-PAGE gel, loading buffer and Western blot analysis

30% Acryl/Bis Solution (37.5:1) (Cat. # 06144-05)	Bio-Rad
Glycine (Cat. # 1610718)	Bio-Rad
Ammonium Persulphate (APS) (Cat. # 1610700)	Bio-Rad
TEMED (Cat. # 1610800)	Bio-Rad
Sodium Dodecyl Sulfate (SDS) (Cat. # L3771)	Sigma-Aldrich
Skim Milk (Cat. # 70166)	Sigma-Aldrich
2-Mercaptoethanol (Cat. # M3148)	Sigma-Aldrich
Methanol (Cat. # 1.07018.2501)	Fisher scientific
10× Phosphate Buffer Saline (pH 7.2) (Cat. # CUS-2040)	1 st Base
Tween-20 (Cat. # VWRCM147)	Amresco
Glycerol (Cat. # H5433)	Promega
Tris (Cat. # H5135)	Promega

Bromophenol Blue (Cat. # US12370)	Affymetrix
Enhanced Chemiluminescent (ECL) substrate (Cat. # RPN 2106)	GE Healthcare
2.1.13. Radio-immunoprecipitation assay	
EasyTag™ L-[³⁵ S]-Methionine Label (Cat. # NEG709A005MC)	Perkin-Elmer
DMEM without L-methionine and L-cystine (Cat. # D0422)	Sigma-Aldrich
Amplify Fluorographic Reagent (Cat. # NAMP100)	GE Healthcare
Protein G-Sepharose Beads (Cat. # P3296)	Sigma-Aldrich
2.1.14. Two-dimensional analysis	
RIPA lysis and extraction buffer (Cat. # 89900)	Pierce
Urea (Cat. # 17-1319-01)	GE Healthcare
Thiourea (Cat. # RPN6301)	GE Healthcare
CHAPS (Cat. # 17-1314-01)	GE Healthcare
Dithiothreitol (DTT) (Cat. # 17-1318-01)	GE Healthcare
Immobilized pH gradient (IPG) gel strip (7 cm, pH 3-10) (Cat. # 17-6001-11)	GE Healthcare
IPG buffer (pH 3-10) (Cat. # 17-6000-87)	GE Healthcare
Immobiline DryStrip Cover Fluid (Cat. # 17-1335-01)	GE Healthcare
Protease Inhibitor (Cat. # 80-6501-23)	GE Healthcare
Iodoacetamide (IAA) (Cat. # I1149)	Sigma-Aldrich
Sodium Dodecyl Sulfate (SDS) (Cat. # L3771)	Sigma-Aldrich
Tris (Cat. # H5135)	Promega
Glycerol (Cat. # H5433)	Promega
Bromophenol Blue (Cat. # US12370)	Affymetrix
2.1.15. Purification of influenza virus particles and vRNP complexes	
Sucrose (Cat. # US21938)	Affymetrix
Glycerol (Cat. # H5433)	Promega
Tris (Cat. # H5135)	Promega
MES hydrate (Cat. # M88250)	Sigma-Aldrich
NaCl (Cat. # S3014)	Sigma-Aldrich
KCl (Cat. # P9541)	Sigma-Aldrich

MgCl ₂ hexahydrate (Cat. # 63068)	Sigma-Aldrich
Octylglucoside (Cat. # O8001)	Sigma-Aldrich
Lysolecithin (Cat. # L4129)	Sigma-Aldrich
Dithiothreitol (DTT) (Cat. # 17-1318-01)	GE Healthcare
DEPC-treated water (Cat. # 750024)	Invitrogen

2.1.16. Protease and detergent treatments on purified virions

Trypsin Gold, MS Grade (Cat. # V5280)	Promega
Tris (Cat. # H5135)	Promega
Triton X-100 (Cat. # 1610407)	Bio-Rad
NaCl (Cat. # S3014)	Sigma-Aldrich
CaCl ₂ (Cat. # 21115)	Sigma-Aldrich
PMSF (Cat. # P7626)	Sigma-Aldrich

2.1.17. Protein gel stains with Coomassie Blue and Sypro-Ruby

Coomassie Brilliant Blue G-250 Powder (Cat. # 1610406)	Bio-Rad
Sypro-Ruby Protein Gel Stain (Cat. # 1703125)	Bio-Rad
Methanol (Cat. # M/4000/17)	Fisher scientific
Acetic Acid (Glacial) 100% (Cat. # 100063)	Merck

2.2. Methods

2.2.1. Generation of monoclonal antibodies against polymerase subunits of influenza A virus

2.2.1.1. Plasmids and bacterial hosts

The sources of three polymerase genes: PA, PB1 and PB2 were plasmids pCR2.1 PA, pCR4 PB1 and pCR4 PB2 respectively and their DNA sequences had been verified in our laboratory (Yeo *et al.*, 2009; Sutejo *et al.*, 2012). Each plasmid contained a cDNA copy of the corresponding full length P gene of influenza A virus pH1N1/471 and H5N2/F118. These plasmids were then handed over to Protein Production Platform (PPP) for subsequent cloning, expression and purification of recombinant proteins to produce the soluble protein and utilize them as antigen for mice immunization.

It has been shown that “multi-construct approach” to protein production dramatically increases the likelihood of obtaining soluble and correctly folded protein (Gräslund *et al.*, 2008). To implement this approach, ten constructs per each polymerase subunit (a full length sequence as well as a series of truncated fragments) were firstly generated by PPP. All targets were fused with an N-terminal 6xHis tag to aid in downstream purification and a Tobacco Etch Virus (TEV)-protease cleavage site. The main expression vector in use was pNIC28-Bsa4 developed by Opher Gileadi at SGC Oxford (Figure 2.1).

Figure 2.1. Features of pNIC28-Bsa4 vector (GenBank accession EF198106), developed by Opher Gileadi at SGC Oxford.

All cloning was performed in 96-well format using ligation-independent cloning (LIC) technology in RecA-strain of *Escherichia coli* which was used for the initial transformation and plasmid production followed by DNA sequence analysis of each target. Then, a re-transformation of individual construct into an in-house expression strain (BL21-DE3 Rosetta T1-phage resistant) was performed.

2.2.1.2. Expression and analysis of plasmid-encoded proteins

A small-scale analytical expression screen was carried out from all clones to assess the likelihood of successful large-scale protein expression and purification. Once

the optical density at 600nm reached ~2, the temperature was lowered from 37°C to 18°C and protein production was induced with IPTG at a final concentration of 0.5 mM. After over-night cultivation, a small sample was harvested from the induced bacteria liquid culture and the presence of target protein was verified in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie brilliant blue staining.

2.2.1.3. Purification of soluble recombinant polymerase proteins

Clones that score sufficiently high in the small-scale expression analysis were put through large scale expression and purification via initial to immobilised metal affinity chromatography (IMAC) resin purification followed by gel filtration (GF). GF-fractions were then pooled and the proteins were concentrated. Sample purity was assessed on SDS-PAGE and the exact size of the protein was then determined by mass spectrometry (MS) analysis.

The detail procedures for cultivation and purification were as follow: Large scale cultivation was performed in a LEX system (Harbinger Biotech). Using glycerol stocks, inoculation cultures were started in 20 ml TB media supplemented with appropriate antibiotics. The cultures were incubated at 37°C, 200 rpm overnight. The following morning, bottles of 750 ml TB supplemented with appropriate antibiotics and 100 ul of antifoam 204 (Sigma-Aldrich) were inoculated with the inoculation cultures. The cultures were incubated at 37°C in the LEX system with aeration and agitation through the bubbling of filtered air through the cultures. The cultivation volumes mentioned above were for score 3 and 4 proteins. For score 1 and 2 proteins, the inoculation culture volume was increased to 40ml and the large scale cultivation volume was increased to 1.5 L. The volumes of the components supplementing the cultures were increased proportionately. When the OD 600 reached ~2, the temperature was reduced to 18°C

and the cultures were induced after 30 to 60 min with 0.5 mM IPTG. Protein expression was allowed to continue overnight. The following morning, cells were harvested by centrifugation at 4,200 rpm, 15°C for 10 min. The supernatants were discarded and the cells were resuspended in lysis buffer (1.5 times of the cell pellet weight) at 200 rpm, 4°C for approximately 30 min. The cell suspensions were stored at -80°C.

The re-suspended cell pellet suspensions were thawed and sonicated (Sonics Vibra-cell) at 70% amplitude, 3 s on/off for 3 min, on ice. The lysate was clarified by centrifugation at 47,000 x *g* 4°C for 25 min. The supernatants were filtered through 1.2 µm syringe filters and loaded onto AKTA Xpress system (GE Healthcare). The purification regime is briefly as follows. The lysates were loaded on IMAC columns. The columns were washed with 20 CV of wash 1 and 20 CV of wash 2 buffer or until a stable baseline was obtained respectively. The eluted proteins were collected and stored in sample loops on the system and then injected into Gel Filtration (GF) columns. Elution peaks were collected in 2 ml fractions and analyzed on SDS-PAGE. The entire purification was performed at 4°C. All IMAC columns are stripped of Ni²⁺, cleaned-in-place with 0.5 M NaOH, re-charged with Ni²⁺ (0.1 M NiCl₂) and stored in 20% ethanol in between purification runs. All GF columns are cleaned-in-place with 0.5 M NaOH and stored in 20% ethanol in between purification runs. Relevant peaks were pooled, TCEP was added to a total concentration of 2 mM. The protein sample was concentrated in Vivaspin 20 filter concentrators (VivaScience) at 15°C to approximately 15 mg/ml. The final protein concentration was assessed by measuring absorbance at 280 nm on Nanodrop ND-1000 (Nano-Drop Technologies). The final protein purity was assessed on SDS-PAGE. The final protein batch was then aliquoted into smaller fractions, frozen in liquid nitrogen and stored at -80°C.

2.2.1.4. Denaturation and purification of insoluble recombinant polymerase proteins

Bacteria cell pellet provided by PPP was resuspended in TN buffer (50 mM Tris.HCl-50 mM NaCl, pH 8.0) supplemented with 0.2 mM PMSF, and cells were lysed using lysozyme (200 µg/ml) at 4°C for 20 min. Then, sodium deoxycholate (0.1% w/v) was added to the cell lysate and incubated at 25°C for 10 min. Cells were further disrupted by sonication on ice until less viscous. Supernatant and pellet were separated by centrifugation at 5,000 x g for 20 min at 4°C. Pellet was then resuspended in TN buffer containing (0.5% v/v) Triton X-100 by sonication and the sample was gently agitated at 4°C for 20 min on a shaker followed by spinning at 5,000 x g for 20 min at 4°C. The washing step was repeated twice and small portion of samples from each step were set aside for Coomassie brilliant blue staining and Western blot analysis. Finally, the pellet containing insoluble protein (inclusion bodies) was subjected to denaturation and purification process.

To isolate and purify denatured protein, the pellet containing inclusion bodies was resuspended in denaturing buffer (6 M Guanidine HCl in TN buffer) by sonication. The sample was gently agitated on the shaker until it became translucent, followed by spinning at 12,000 x g for 20 min at room temperature (RT) to pellet any insoluble material. Clarified sample was carefully transferred to a clean tube to proceed with the purification process. Before protein binding to the resin, TALON[®] metal affinity resin beads were briefly equilibrated with denaturing buffer and spun at 700 x g for 2 min to pellet the resin. Then, clarified sample containing denatured protein was added to the equilibrated resin and gently agitated at RT for 20 min on the shaker to allow the polyhistidine-tagged protein to bind the resin. The mixture of sample and resin was then transferred to a gravity-flow column with an end-cap in place (Bio-Rad) and the resin was allowed to settle out of suspension. The end-cap was then removed, and the buffer

was allowed to drain until it reached the top of the resin bed, followed by washing column once with wash buffer (8 M urea in TN buffer). Lastly, the polyhistidine-tagged protein was eluted by adding elution buffer (TN buffer including 8 M urea, 200 mM imidazole, and 0.5% Nonidet P-40) to the column, and the protein was recovered in small volume fractions. Small portion of samples from each eluted fraction were run on 10% SDS polyacrylamide gel, and visualized by Coomassie brilliant blue staining. All eluted fractions containing protein were pooled together for refolding. Protein refolding was performed at 4°C by rapid dilution (10x) using a refolding buffer (TN buffer including 0.5 M L-Arginine). The refolded protein was then dialyzed and concentrated using dialysis tubing method with PEG 6000. Finally, the concentrated protein was analyzed on SDS-PAGE and the protein concentration was estimated by ImageJ program.

2.2.1.5. Immunization of mice and preparation of hybridoma cell lines

Purified soluble/insoluble recombinant polymerase proteins were used as immunogens for mice immunization. Five six-week-old female Balb/c mice were injected intraperitoneally (IP) with 50 µg of a purified recombinant protein mixed with equal volume of complete Freund's adjuvant at Day 1. Two booster immunizations (50 µg of same protein mixed with incomplete Freund's adjuvant) were given at Day 15 and Day 36. Bleeding of the mouse was done 10 days after each boost and the antibody response in the sera was determined by ELISA and Western blotting. At Day 57, a final booster injection, 50 µg of same protein in 1x sterile PBS, was given intravenously into the tail vein of one mouse with highest serum antibody response. Three days after the final boost, the mouse was euthanized and spleen was excised subsequently under aseptic conditions. Splenocytes isolated from the spleen were fused with myeloma cells using PEG and hybridoma cells were generated by standard protocol. Fused cells were

diluted in hybridoma growth medium (RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1x sodium bicarbonate, 1x sodium pyruvate, 1x MEM non-essential amino acid, 1x BM Condimed, 1x antibiotics) plus 1x HAT (hypoxanthine-aminopterin-thymidine) medium for hybridoma selection. Cells were then plated out in ten 96-well tissue culture plates. When small hybridoma colonies appeared approximately at Day 5 or 6 after fusion, the medium was gradually diluted to similar hybridoma growth medium with 1x HT (hypoxanthine-thymidine) instead of 1x HAT. Cells were then maintained in the growth medium with 1x HT till transfer to normal growth medium. The hybridoma culture supernatants from each well were then screened by ELISA about 10 days after fusion. Potentially reactive hybridoma clones which revealed the highest affinity to the target protein in ELISA were chosen for subcloning by limiting dilution. Finally, ELISA-positive subclones were propagated into larger scale and their supernatants were tested further with IFA and Western blotting.

2.2.1.6. Enzyme-linked immunosorbent assay (ELISA)

Purified protein was diluted in carbonate coating buffer (0.1 M Na₂CO₃, 0.1 M NaHCO₃, pH 9.6) to a final concentration of 10 µg/ml and applied 100 µl/well to the 96-well microtiter plates (MaxisorpTM, NUNC, Denmark). The plates were incubated at 4°C overnight, followed by blocking with 300 µl/well of 5% skim milk dissolved in 1x PBS + 0.05% TWEEN20 (PBS-T) at 37°C for 3 hr. Then, the plates were incubated with each hybridoma supernatant at 37°C for 1 hr. Pre-immunized and last bleed mouse sera (1:1,000 dilution) were used as negative and positive controls respectively. After 1 hr incubation, 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:10,000 dilution) was applied into each well and the plates were incubated at 37°C for another 1 hr. The plates were washed 3 times with PBS-T after each incubation step. Finally, 50 µl of TMB was added to each well and the plates were

incubated at room temperature for 30 min, followed by an equal volume of 10% H₂SO₄ to stop the reaction. The optical absorbance of the samples at 450 nm was read using Tecan microplate reader. ELISA screening was done thrice independently to minimize the false positive results.

2.2.2. Viral RNA extraction, Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)

This was performed using standard protocols. Briefly, RT-PCR cloning of the NP and polymerase genes was performed from the pH1N1/471, H9N2, and H1N1/WSN viruses. Total RNA was first extracted from 100 µl of allantoic fluid containing individual virus using the RNeasy mini kit and reversed transcribed using SuperScript II RT, both according to the manufacturer's instructions. 300 ng of total RNA was used for reverse transcription of the negative-sense vRNAs with a Uni12 primer 5'AGCAAAAGCAGG3' (Hoffmann *et al.*, 2001). Using the High Fidelity PCR system, each gene segment was PCR amplified with gene-specific primers as described in Hoffmann *et al.* (2001) (Table 2.1). The PCR mix per reaction (50 µl) was as follow: 5 µl 10x HiFi buffer, 5 µl of 2 mM dNTP, 2 µl of 50 mM MgSO₄, 2 µl of 10 µM forward primer, 2 µl of 10 µM reverse primer, 0.5 µl of HiFi Platinum Taq DNA polymerase enzyme 100 U/µl, 4 µl of cDNA template and 29.5 µl of nuclease free water. The PCR temperature run setting was set as follows: Initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and elongation at 68°C for 7 min. The final elongation was set at 68°C for 10 min, followed by cooling. The PCR product for each gene was purified using QIAquick gel extraction kit/QIAprep spin miniprep kit. Using TOPO TA Cloning, each purified gene amplicon was cloned into vectors pCR2.1-TOPO or pCR4-TOPO and positive clones were sent for sequencing.

Virus genes	Primers	Sequence (5'-3')
NP	Bm-NP-1FW	TATTCGTCTCAGGGAGCAAAAGCAGGGTA
	Bm-NP-1565RV	ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTT
PA	Bm-PA-1FW	TATTCGTCTCAGGGAGCGAAAGCAGGTAC
	Bm-PA-2233RV	ATATCGTCTCGTATTAGTAGAAACAAGGTACTT
PB1	Bm-PB1-1FW	TATTCGTCTCAGGGAGCGAAAGCAGGCA
	Bm-PB1-2341RV	ATATCGTCTCGTATTAGTAGAAACAAGGCATT
PB2	Bm-PB2-1FW	TATTGGTCTCAGGGAGCGAAAGCAGGTC
	Bm-PB2-2341RV	ATATGGTCTCGTATTAGTAGAAACAAGGTCGTTT

Table 2.1. Primer sequences: forward (FW) and reverse (RV) primers for PCR amplification of the full-length full-length NP and polymerase gene fragments (Hoffmann *et al.*, 2001). The underlined nucleotides at the 3' end represent the segment specific sequences and the bolded nucleotides represent the sequence complementary to the influenza sequences.

2.2.3. Construction of recombinant mammalian expression pCAGGS plasmids

For mammalian expression, the full-length NP and polymerase genes of H1N1/WSN, pH1N1/471, H5N2/F118 and H9N2 strains were inserted into pCAGGS plasmid (Figure 2.2) using gene specific primers (Table 2.2). The forward primer and the reverse primer with or without a FLAG (DYKDDDDK) tag at C-terminal of individual gene contained recognition sequences for the suitable restriction enzymes. The DNA amplification was performed with the High Fidelity PCR system. The pCAGGS plasmid and the amplified gene products were digested with their respective restriction enzymes according to manufacturer's protocol. After restriction digestion, the digested plasmid and inserts were purified QIAquick gel extraction kit/QIAprep spin miniprep kit, and ligated overnight at 16°C using T4 DNA ligase. The ligation mix was transformed into TOP10[®] competent cells and plated on LB agar plates containing 100 µg/ml ampicillin. After overnight incubation at 37°C, colonies on plates were checked by PCR screening and positive clones were sent for sequencing.

H1N1/WSN virus genes	Primers	Sequence (5'-3')
NP	NP-EcoRI-FW	CCGGAATTCCGGAGCAAAAGCAGGGTA
	NP-KpnI-RV	CGGGGTACCCCGAGTAGAAACAAGGGTATTTTT
PA	PA-KpnI-FW	CGGGGTACCCCGAGCGAAAGCAGGTAC
	PA-XhoI-RV	CCGCTCGAGCGGAGTAGAAACAAGGTACTT
PB1	PB1-KpnI-FW	CGGGGTACCCCGAGCGAAAGCAGGCA
	PB1-XhoI-RV	CCGCTCGAGCGGAGTAGAAACAAGGCATTT
PB2	PB2-KpnI-FW	CGGGGTACCCCG AGCGAAAGCAGGTC
	PB2-XhoI-RV	CCGCTCGAGCGGAGTAGAAACAAGGTCGTTT

pH1N1/471 virus genes	Primers	Sequence (5'-3')
NP	NP-EcoRI-FW	CCGGAATTCCGGAGCAAAAGCAGGGTA
	NP-KpnI-RV	CGGGGTACCCCGAGTAGAAACAAGGGTATTTTT
	NP-FLAG-KpnI-RV	CGGGGTACCCCGCTATTTATCGTCATCGTCTTT GTAATCACTGTCATACTCCTCTGC
PA	PA-KpnI-FW	CGGGGTACCCCGAGCGAAAGCAGGTAC
	PA-XhoI-RV	CCGCTCGAGCGGAGTAGAAACAAGGTACTT
	PA-FLAG-XhoI-RV	CCGCTCGAGCGGCTATTTATCGTCATCGTCTTT GTAATCCTTCAGTGCATGTGTGAG
PB1	PB1-KpnI-FW	CGGGGTACCCCGAGCGAAAGCAGGCA
	PB1-XhoI-RV	CCGCTCGAGCGGAGTAGAAACAAGGCATTT
	PB1-FLAG-XhoI-RV	CCGCTCGAGCGGCTATTTATCGTCATCGTCTTT GTAATCTTTTTGCCGTCTGAGTTC
PB2	PB2-XhoI-FW	CCGCTCGAGCGGAGCGAAAGCAGGTC
	PB2-XhoI-RV	CCGCTCGAGCGGAGTAGAAACAAGGTCGTTT
	PB2-FLAG-XhoI-RV	CCGCTCGAGCGGCTATTTATCGTCATCGTCTTT GTAATCATTGATGGCCATCCGAAT

H5N2/F118 virus genes	Primers	Sequence (5'-3')
NP	NP-EcoRI-FW	CCGGAATTCCGGAGCAAAAGCAGGGTA
	NP-KpnI-RV	CGGGGTACCCCGAGTAGAAACAAGGGTATTTTT
	NP-FLAG-KpnI-RV	CGGGGTACCCCGCTATTTATCGTCATCGTCTTT GTAATCATTGTCATACTCCTCTGC

PA	PA-KpnI-FW	CGGGGTACCCCGAGCGAAAGCAGGTAC
	PA-KpnI -RV	CGGGGTACCCCGAGTAGAAACAAGGTACTT
	PA-FLAG-KpnI -RV	CGGGGTACCCCGCTATTTATCGTCATCGTCTTT GTAATC TTTCAGTGCATGCGTGAG
PB1	PB1-KpnI-FW	CGGGGTACCCCGAGCGAAAGCAGGCA
	PB1-XhoI-RV	CCGCTCGAGCGGAGTAGAAACAAGGCATTT
	PB1-FLAG-XhoI-RV	CCGCTCGAGCGGCTATTTATCGTCATCGTCTTT GTAATC TTTTTGCCGCCTGAGCTC
PB2	PB2-KpnI-FW	CGGGGTACCCCG AGCGAAAGCAGGTC
	PB2-XhoI-RV	CCGCTCGAGCGGAGTAGAAACAAGGTCGTTT
	PB2-FLAG-XhoI-RV	CCGCTCGAGCGGCTATTTATCGTCATCGTCTTT GTAATC ATTGATGGCCATCCGAAT

H9N2 virus genes	Primers	Sequence (5'-3')
NP	NP-EcoRI-FW	CCGGAATTCCGGAGCAAAAGCAGGGTA
	NP-KpnI-RV	CGGGGTACCCCGAGTAGAAACAAGGGTATTTTT
	NP-FLAG-KpnI-RV	CGGGGTACCCCGCTATTTATCGTCATCGTCTTT GTAATC ATTGTCTACTCTCTGC
PA	PA-BglII-FW	GGAAGATCTTCCAGCGAAAGCAGGTAC
	PA-BglII-RV	GGAAGATCTTCCAGTAGAAACAAGGTACTT
	PA-FLAG-BglII -RV	GGAAGATCTTCCCTATTTATCGTCATCGTCTTT GTAATC CTTCAGTGCATGTGTGAG
PB1	PB1-KpnI-FW	CGGGGTACCCCGAGCGAAAGCAGGCA
	PB1-XhoI-RV	CCGCTCGAGCGGAGTAGAAACAAGGCATTT
	PB1-FLAG-XhoI-RV	CCGCTCGAGCGGCTATTTATCGTCATCGTCTTT GTAATC TTTTTGCCGTCTGAGCTC
PB2	PB2-XhoI-FW	CCGCTCGAGCGGAGCGAAAGCAGGTC
	PB2-XhoI-RV	CCGCTCGAGCGGAGTAGAAACAAGGTCGTTT
	PB2-FLAG-XhoI-RV	CCGCTCGAGCGGCTATTTATCGTCATCGTCTTT GTAATC ATTGATGGCCATCCGAAT

Table 2.2. Primer sequences: forward (FW) and reverse (RV) primers for cloning of NP, PA, PB1 and PB2 genes of H1N1/WSN, pH1N1/471, H5N2/F118 and H9N2 strains into pCAGGS cloning vector. The underlined nucleotides represented the restriction enzyme sites and the bolded nucleotides represented the FLAG tag sequences.

Figure 2.2. Features of pCAGGS vector map including multiple cloning site (MCS), adapted from Addgene. Curve arrows indicate the forward and reverse primer region for PCR screening and sequencing.

2.2.4. Cell culture

2.2.4.1. Primary cell culture

Primary chicken embryonic fibroblasts (CEF) was prepared from 10 day-old specific pathogen-free embryonated chicken eggs obtained from Animal & Plant Health Centre, Singapore. Eggs were checked for the alive embryos with an ultra-light box and wiped with 70% ethanol. The chick embryos were then detached from the eggs using sterile forceps. The heads, four limbs, internal organs were removed from the embryos. The intact body parts were then minced using forceps and scissors and washed with PBS for a few times to remove blood and yolk. The minced solid tissue pieces were then incubated with 0.1% trypsin-EDTA for 15 min at 37°C with frequent vortexing until the solution became slightly viscous. Supernatant containing trypsinized cells was transferred into a clean tube when solid pieces were settled down. Then, trypsin was deactivated by adding DMEM including 10% FBS. Finally, cells were pelleted by spinning at 2000 rpm for 15 min 4°C and resuspended in fresh DMEM media. After cells were counted, they were grown in DMEM supplemented with 10% FBS and 1x antibiotics (penicillin-streptomycin) and incubated in 37°C with 5% CO₂.

2.2.4.2. Immortalized cell culture

Immortalized Madin-Darby canine kidney (MDCK), human alveolar basal epithelial (A549) and human embryonic kidney (293T) cells obtained from ATCC were grown in DMEM supplemented with 10% FBS and 1x antibiotics (penicillin-streptomycin) and incubated in 37°C with 5% CO₂.

2.2.5. Transfection

Lipofectamine 2000 was used as a transfection reagent. First, plasmid DNA-Lipofectamine 2000 complexes were prepared in reduced serum medium Opti-MEM according to manufacturer's protocol. These complexes were then incubated at room temperature for 5 min and directly added to the cells in DMEM supplemented with 2% FBS culture medium in the absence of antibiotics. Cells were then incubated in 37°C with 5% CO₂ until appropriate transfection time was reached.

2.2.6. Infection

After cell propagation medium was completely removed from the cells, the virus inoculum prepared in PBS was directly applied to the cells. Then, the cells were incubated in 37°C for 1 hr. After the incubation was done, the virus inoculum was removed and replaced with medium. For the infection at multiplicity of infection (moi) of 1 or more, the cells were maintained by using DMEM supplemented with 2% FBS and 1x antibiotics (penicillin-streptomycin), and incubated at 37°C with 5% CO₂. For infection at moi lower than 1, the cells were maintained with DMEM supplemented with 0.21% BSA, 1 µg/ml trypsin-TPCK and 1x antibiotics (penicillin-streptomycin), and incubated at 37°C with 5% CO₂ until appropriate infection time was reached.

2.2.7. Immunological assays

Immunofluorescence assay and Western blotting were performed to identify mainly NP and polymerase subunits from transfected cells or virus infected cells or purified viruses.

2.2.7.1. Immunofluorescence assay (IFA)

Cells were seeded onto 12 mm circular coverslip (Deckglaser) in 24-well plates. At about 70-80% confluence, cells were either transfected with plasmids or infected with viruses. For IFA after transfection of 293T cells were transfected with single/multiple plasmids using Lipofectamine 2000 transfection reagent. At fixed time point post-transfection, medium was removed and cells were washed with 1x PBS twice. Cells were then fixed and permeabilized with methanol:acetone (1:1). For IFA after infection of MDCK, A549 and CEF cells were infected with different virus strains using a multiplicity of infection (moi) of >1 for high moi or <1 for low moi. At specific post-infection times, medium was removed and cells were washed with 1x PBS twice. Cells were then fixed with 4% paraformaldehyde (PFA) for 20 min and permeabilized with 0.1% Triton X-100 for 10 min. After fixing and permeabilization, cells were labelled with different primary antibodies for 1 hr followed by counterstaining with corresponding secondary antibodies for 1 hr. Cells were washed 3 times with 1x PBS on an orbital shaker after each labelling step. The coverslips were then mounted with fluorescence mounting medium and visualised under a Nikon Eclipse 80i Microscope with appropriate machine settings.

2.2.7.2. Western blotting

For Western blot analysis, both transfected and infected samples were prepared as mentioned in IFA to use as antigens. In addition to the above samples, purified proteins and virus particles were also used as antigens. First, samples were dissolved in

SDS-PAGE loading buffer containing Tris-HCl (pH 6.8), SDS, glycerol, 2-mercaptoethanol, bromophenol blue, then run onto 10% or 12 % SDS-PAGE gel with Bio-Rad Mini-PROTEAN electrophoresis system using 200 volt for about 50 min. After protein separation by SDS-PAGE, the proteins were transferred by Western blotting onto a PVDF membrane. Then, the membrane was blocked with 5% skim milk in PBS-T at 4°C overnight, and probed with primary and secondary antibodies. Each probing step was done for 1 hour with gentle shaking. The membrane was washed 3 times with PBS-T on an orbital shaker after blocking with milk and each probing step. Finally, the membrane was treated with enhanced chemiluminescent (ECL) substrate and exposed to X-ray film (Kodak) to detect protein bands.

2.2.8. Protein gel stains with Coomassie Blue and Sypro-Ruby

After SDS-PAGE, the gel was stained with Coomassie Blue solution [0.25% Coomassie Brilliant Blue G250, methanol: water (1:1), 10% acetic acid] for 1 hr and destained using destaining solution [methanol: water (1:1), 10% acetic acid]. The gel was then scanned and quantified using a GS-800 Calibrated Densitometer (Bio-Rad). Sypro-Ruby is a luminescent stain with higher sensitivity, used for the detection of proteins separated by SDS-PAGE. The protein bands in the gel were first fixed with fixing solution (10% methanol, 7% acetic acid) for 30 min. Then the gel was covered with Sypro-Ruby stain, and kept in the dark overnight with gentle shaking. The excess stain was removed, and the gel was washed with wash solution (10% methanol, 7% acetic acid) twice for 30 min. It was then rinsed twice with pure water, and scanned using a Typhoon Variable Mode Imager (Amersham Biosciences).

2.2.9. Epitope mapping

The experimental flow for epitope mapping was divided into 3 phases (Figure 2.3). In general, the first phase consisted of 100 amino acid truncations, while the second would be the narrowing down on that particular region to 20 amino acid truncations. Therefore, our procedure started with the truncations of PA with 100 amino acid deletions from the C-terminus. As for the forward primer, it was set at 240 FW as the anti-PA antibodies were generated from 240-716 region, and the reverse primers were designed for each truncation. In the second phase, 20 amino acid deletions from the identified 100 amino acid region was carried out by designing both forward and reverse primers. The primers used to generate all truncated fragments of PA for both first and second phases are listed in Table 2.3. PCR amplification was performed for each truncation by using the listed primers, and the purified PCR product was inserted into the plasmid vector by ligation. pRSET A plasmid (Figure 2.4) was chosen as the vector for cloning all PA truncations as the whole sequence of the plasmid was in frame with them at the site of insertion between two restriction enzymes (XhoI at 5' and KpnI at 3'). After cloning was completed, expression was subsequently performed for each truncation by using BL21 (DE3) cells, and serially expressed truncated PA products were probed with anti-PA antibodies.

For the truncations of PB2, the same experimental flow used in PA was also applied. However, the forward primer for phase 1 was set at 255 FW as the anti-PB2 antibody was generated from 255-759 region. The primers used to generate all truncated fragments of PB2 for both first and second phases were listed in Table 2.4. pRSET A plasmid was also chosen as the vector for cloning of all PB2 truncations. Cloning and expression procedures were then carried out as for PA, and serially expressed truncated PB2 products were probed with anti-PB2 antibody.

Figure 2.3. Showing the experimental flow for epitope mapping: first phase, second phase and final phase.

Primers for PA truncations	Sequence (5'-3')
PA-XhoI-240 FW	CCG <u>CTCGAG</u> CGGGGCTGCATTGAGGGC
PA-XhoI-256 FW	CCG <u>CTCGAG</u> CGGAAAATTGAACCATTC
PA-XhoI-276 FW	CCG <u>CTCGAG</u> CGGTGCCATCAGCGGTCA
PA-XhoI-296 FW	CCG <u>CTC GAG</u> CGGAGTCACGAGGGGGAG
PA-XhoI-316 FW	CCG <u>CTCGAG</u> CGGGGCTGGAAAGAGCCT
PA-KpnI-316 RV	CGGGGTAC <u>CCCGCT</u> AGCCAAAGAATGTCTT
PA-KpnI-416 RV	CGGGGTAC <u>CCCGCT</u> ATTCACATGCCTTATT
PA-KpnI-516 RV	CGGGGTAC <u>CCCGCT</u> AATCAGTATCATTTCT
PA-KpnI-536 RV	CGGGGTAC <u>CCCGCT</u> ATTTGTGTGGCTCCAG
PA-KpnI-556 RV	CGGGGTAC <u>CCCGCT</u> ATTTGGCCTATCGCAGT
PA-KpnI-576 RV	CGGGGTAC <u>CCCGCT</u> ATTTTCATCTTGATCTT
PA-KpnI-596 RV	CGGGGTAC <u>CCCGCT</u> AAATCATGCTCTCAAT
PA-KpnI-616 RV	CGGGGTAC <u>CCCGCT</u> ACGATTTGTTTTCAAA
PA-KpnI-716 RV	CGGGGTAC <u>CCCGCT</u> ACTTCAGTGCATGTGT

Table 2.3. Primer sequences: forward (FW) and reverse (RV) primers to generate all truncated fragments of PA for first and second phases. The underlined nucleotides represented the restriction enzyme sites (XhoI and KpnI).

Primers for PB2 truncations	Sequence (5'-3')
PB2-XhoI-255 FW	CCG <u>CTCGAG</u> CGGGTTGACCAAAGTTTG
PB2-XhoI-335 FW	CCG <u>CTCGAG</u> CGGGGATCATCAGTCAAG
PB2-XhoI-415 FW	CCG <u>CTCGAG</u> CGGAGGGGCGATCTGAAC
PB2-XhoI-495 FW	CCG <u>CTCGAG</u> CGGGTAGTGAGTATTGAC
PB2-XhoI-575 FW	CCG <u>CTCGAG</u> CGGATGGAATTTGAACCA
PB2-KpnI-359 RV	CGGGGTACCCCGCTACCCTTCATGTACTCT
PB2-KpnI-459 RV	CGGGGTACCCCGCTATCCCATCACATTGTC
PB2-KpnI-559 RV	CGGGGTACCCCGCTAAATTTCCCAGTTCCT
PB2-KpnI-659 RV	CGGGGTACCCCGCTAGTTGTAATTGAATAC
PB2-KpnI-679 RV	CGGGGTACCCCGCTATGGATCTTCAGTCAA
PB2-KpnI-699 RV	CGGGGTACCCCGCTATTTGCCCAAATGAG
PB2-KpnI-719 RV	CGGGGTACCCCGCTATCCTTTTGCAAGATT
PB2-KpnI-739 RV	CGGGGTACCCCGCTACCGTTTTCGTTTCAT
PB2-KpnI-759 RV	CGGGGTACCCCGCTAATTGATGGCCATCCG

Table 2.4. Primer sequences: forward (FW) and reverse (RV) primers to generate all truncated fragments of PB2 for first and second phases. The underlined nucleotides represented the restriction enzyme sites (XhoI and KpnI).

Figure 2.4. Features of pRSET vector map including multiple cloning site (MCS), adapted from Invitrogen.

2.2.10. Nuclear cytoplasmic extraction assay

MDCK cells were seeded in 35 mm dishes with 70-80% confluence and cells were infected with pH1N1/471 virus using a moi of 5. Mock infected cells were also prepared in parallel. After 16 hpi, culture medium was carefully removed from adherent cells. Cells were then harvested in 1x cold PBS and cell lysate was transferred to a

microcentrifuge tube. Then, the sample was centrifuged at 500 x g for 5 min to pellet down. Cell pellet was washed with 1x cold PBS twice and supernatant was discarded, leaving the cell pellet as dry as possible. Cell extraction was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents. Protease inhibitor was added to the reagents immediately before use. Ice-cold cytoplasmic extraction reagent I (CER-I) was then added to the cell pellet as instructed and proceeded to cytoplasmic and nuclear protein extraction. After CER-I was added, the tube was vigorously vortexed on the highest setting for 15 sec to fully resuspend the cell pellet. Then, CER-II was added to the tube after incubation on ice for 10 min. The tube was vigorously vortexed on the highest setting for 5 sec and incubated on ice for 1 min. The tube was vigorously vortexed again on the highest setting for 5 sec, then centrifuged for 5 min at maximum speed in a microcentrifuge (~16,000 x g). The supernatant (cytoplasmic extract) was immediately transferred to a clean pre-chilled tube and placed on ice. Insoluble pellet which contains nuclei was then suspended in ice-cold nuclear extraction reagent (NER) followed by vortex on the highest setting for 15 sec. The sample was placed on ice with continuous vortexing for 15 sec every 10 min, for a total of 40 min. Finally, the tube was centrifuged at max speed (~16,000 x g) in a microcentrifuge for 10 min. The supernatant (nuclear extract) fraction was immediately transferred to clean pre-chilled tube and placed on ice or stored at -80°C until use. Then, cytoplasmic extract, nuclear extract and insoluble nuclear pellet were dissolved in SDS-PAGE loading buffer and analyzed by Western blotting.

2.2.11. Radio-immunoprecipitation assay

2.2.11.1. [³⁵S]-methionine metabolic radiolabelling

MDCK cells were seeded into 60 mm-dishes until 70-80% confluence. Mock infected and infected with the H1N1/WSN virus using a moi of 5. At 8 hpi, the medium

was removed and cells were washed with 1x sterile PBS once, then replaced with methionine and cysteine free DMEM and incubated for 1 hr. At 9 hpi, mock infected and virus infected cells were radiolabelled for 1 hr with 100 μ Ci/ml of [35 S]-methionine by adding to each dish. At 10 hpi, the radiolabelled medium was removed completely and cells were washed with 1x PBS twice. Cells were harvested in SDS-PAGE loading buffer and cell lysate was transferred to a microcentrifuge tube. The lysates were homogenized using 10 mm syringe instead of sonication. The samples were then analysed by 12% SDS-PAGE. After the gel was fixed with 10% acetic acid, it was soaked in amplify fluorographic reagent and dried using a gel drier set at 80°C for 1 hr. The dried gel was put into radiolabelling cassette and exposed with a storage phosphor screen overnight/24 hr. Radiolabelled protein bands were then detected by scanning the phosphor screen using a Typhoon Variable Mode Imager (Amersham Biosciences).

2.2.11.2. Radiolabelling followed by immunoprecipitation

All the above procedures for cell infection and radiolabelling were the same. After the radiolabelled medium was removed completely at 10 hpi, cells were washed with 1x PBS twice and lysed with 500 μ l of RIPA lysis and extraction buffer on ice for 20 min. Cell lysate was then transferred to a microcentrifuge tube and the sample was centrifuged at $13,000 \times g$ for 10 min to pellet the cell debris. Aliquots of the clarified lysate (120 μ l) were added to 600 μ l of binding buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.25% BSA, pH 8.0) and incubated with immunoprecipitation antibodies at 4°C for 18 hr. The antigen-antibody complexes were isolated by adding 40 μ l of protein G-sepharose beads into each sample and rotating at 4°C for 2 hr. Then, the beads were washed thrice with wash buffer (150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate, 1% Triton-X-100, pH 7.0), and centrifuged at $13,000 \times g$ for 10 min to pellet them. Finally, the beads were resuspended in SDS-PAGE loading buffer and heated at

98°C for 5 mins. The proteins were then separated by 12% SDS-PAGE. The procedures for gel fixing, drying, exposing and scanning were as above.

2.2.12. Two-dimensional analysis

MDCK cells were seeded in 35 mm dishes with 70-80% confluence and cells were infected with pH1N1/471 virus using a moi of 5. Mock infected cells were also prepared in parallel. After 16 hpi, culture medium was carefully removed from adherent cells. Cells were then washed with 1x cold PBS twice. Cold RIPA lysis and extraction buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, pH 7.6) from Pierce was added to the cells directly and the dish was kept on ice for 20 min, swirling occasionally for uniform spreading. Importantly, protease inhibitor was added to the RIPA buffer immediately before use. Cells were extracted in 500 µl of buffer and cell lysate was transferred to a microcentrifuge tube. Then, the sample was centrifuged at $13,000 \times g$ for 10 min to pellet the cell debris. After centrifugation, supernatant was transferred to a new tube for further protein analysis.

Using 2-D Clean-Up kit, non-protein impurities, excess salts and buffers from sample preparation were removed according to the manufacture's protocol. Protein precipitation was then carried out to concentrate proteins from the sample using the same Clean-Up kit. Precipitated protein pellet was resuspended in a rehydration loading solution (7 M urea, 2 M thiourea, 4% CHAPS, 0.002% bromophenol blue) prepared in distilled water for first dimension isoelectric focusing (IEF). 2-D Quant kit was then used according to the manufacturer's instructions to estimate the protein concentration of the sample. After calculation of sample volume to be used, dithiothreitol (DTT) and IPG buffer were added to the sample immediately before loading the protein sample into the immobilized pH gradient (IPG) gel strip. According to the manufacturer's protocol, procedures for first dimension IEF were begun. First, sample mixed in rehydration

solution was pipetted into a clean and dry strip holder. Then, a 7 cm IPG gel strip with pH 3-10 was placed onto the sample with the dried gel side down. The strip was overlaid with cover fluid to prevent evaporation and urea crystallization, and strip holder was finally covered to allow strip for overnight rehydration. Next, first dimension separation was run with rehydrated IPG gel strip on the Ettan IPGphor II unit (Amersham) using a total of 6,500 Volt for 2:45 hrs. Prior to second dimension procedures, the strip was firstly equilibrated in SDS equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) containing dithiothreitol (DTT) for 15 min, followed by immersed in the same buffer containing iodoacetamide (IAA) for another 15 min. Second dimension separation was then run straight away by placing the strip onto 12% SDS-PAGE gel with Bio-Rad Mini-PROTEAN electrophoresis system using 200 Volt for about 50 min. After protein separation by SDS-PAGE, the Western blot procedures were the same as mentioned in Section 2.2.7.2.

2.2.13. Virus propagation

The virus stocks were propagated in 12 day-old specific pathogen-free embryonated chicken eggs obtained from Animal & Plant Health Centre, Singapore. Prior to inoculation, all eggs used were checked for the viability of the embryos using an ultra-light box. The eggs were then marked at the air sac layer and wiped with 70% ethanol. Then each egg was punctured to make a small hole about 2 mm above air-sac marking. Prepared virus inoculum in PBS ($\sim 10^4$ pfu) was inoculated into the egg through the hole using a syringe and needle. The holes were wiped again with 70% ethanol and sealed with sticky tapes. The eggs were then incubated at 37°C incubator. After incubation for 2 days, the eggs were firstly placed at 4°C and chilled for minimally 4 hours, then tapes and egg-shells were removed gently using scissors and forceps. By using a sterile Pasteur pipette, allantoic fluid was carefully harvested without rupturing

the egg yolk and veins. The fluid was then transferred into 50 ml falcon tubes and clarified from debris and blood clots by spinning down at 2,500 rpm for 20 min. Finally, the clarified fluid was aliquoted into cryovials and these virus stocks were stored at -80°C until use.

2.2.14. Purification of virus particles by sucrose density gradient centrifugation

Influenza A viral strains H1N1/WSN and pH1N1/471 were purified by sucrose density gradient centrifugation. Virus infected allantoic fluid was clarified at 2,500 rpm for 20 min at 4°C on a table top centrifuge. Virus particles were pelleted down from the clarified allantoic fluid by centrifugation at $50,000 \times g$ for 90 min at 4°C using P28S rotor. The virus pellet was soaked overnight in PBS at 4°C. Then, the pellet was resuspended in PBS and loaded onto 30–60% (w/v) sucrose gradient with a 15% (w/v) overlay. This was centrifuged at $140,000 \times g$ for 90 min at 4°C using P28S rotor. After spinning, the virus band at 35% sucrose was harvested and the virus was pelleted down by centrifugation at $140,000 \times g$ for 90 min at 4°C using P40ST rotor.

2.2.15. Purification of influenza vRNP complexes

Influenza vRNPs were purified using the well-established protocol of Kemler *et al.*, 1994. It included two parts: (1) disruption of the influenza virions with detergents to release the vRNP complexes from the enveloped virions, and (2) separation of the released vRNPs from the other components of the virions by velocity sedimentation on a 33-70% step glycerol gradient. In the first part, 0.5 mg of influenza A H1N1 virus (A/WSN/1933 strain) was diluted with 4 volume of MNT buffer (20 mM MES, 150 mM NaCl, 30 mM Tris-HCl, pH 7.5) and pelleted by spinning at 35,000 rpm in a P56ST rotor, for 40 min at 4°C. After spinning, the virus pellet was resuspended in 500 µl of disruption buffer (100 mM KCl, 5 mM MgCl₂, 5% (w/v) glycerol, 50 mM

octylglucoside, 10 mg/ml lysolecithin, 1.5 mM dithiothreitol, 100 mM MES, pH 5.5) and incubated at 31°C for 20 min with shaking. In the second part, RNPs were purified by centrifugation at 45,000 rpm for 4 hr at 4°C in a P56ST rotor through a step glycerol gradient (1 ml of 70%, 0.75 ml of 50%, 0.375 ml of 40%, and 1.8 ml of 33% (w/v) glycerol) prepared with NM buffer (150 mM NaCl, 50 mM MES, pH 5.5). After the completion of centrifugation, fractions (250 µl) were manually harvested from the glycerol gradient starting from the top of the tube. The collected fractions were then run on SDS-PAGE, analyzed by Sypro-Ruby staining and examined by Western blot.

2.2.16. Protease and detergent treatments on purified virions

Purified H1N1/WSN virus sample (2.5 µg of total protein) was diluted in buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl₂, pH 7.5). Four samples containing the same concentrations of purified virus were prepared and treated in parallel as follow: (i) one received without treatment, (ii) one was treated with TPCK-treated trypsin protease to a final concentration of 4 µg/ml, (iii) one was treated with Triton X-100 to a final concentration of 1%, and (iv) one was treated with both trypsin and Triton X-100. All four samples were then incubated at 37°C for 1 hr. Protease activities were terminated by adding a Trypsin inhibitor, PMSF, to a 0.5 mM final concentration and incubating at RT for 15 min. Samples were then mixed with SDS-PAGE loading buffer to run SDS-PAGE and analyzed by Coomassie brilliant blue staining and Western blot analysis.

2.2.17. siRNA design and transfections

All siRNAs used in our experiments were synthesized by Dharmacon (USA). siRNA sequences of siNP-H1N1 and siPA-H1N1 were used as positive controls and siGFP was used as a negative control (as described by Ge *et al.*, 2003; Table 2.5). Dharmacon Custom SMARTpool designed siRNAs (four siRNAs per gene) were

synthesized to target the NP, PA, PB1 and PB2 genes of LPAI H5N2. The approximate positions of all custom-designed siRNAs targeted against four genes are shown in a schematic diagram (Figure 2.5).

Positive controls	siNP-H1N1	GGAUCUUAUUUCUUCGGAG
	siPA-H1N1	GCAAUUGAGGAGUGCCUGA
Negative control	siGFP	GGCUACGUCCAGGAGCGCAUU

Table 2.5. Sequences of siRNAs adapted from Ge *et al.*, 2003 as controls. Sequences listed are of the sense strand (5' to 3' direction).

Figure 2.5. Schematic diagram showing the approximate positions of four custom designed siRNAs against the mRNA sequences of the viral NP, PA, PB1 and PB2 genes of H5N2.

To initially optimize the transfection efficiency of siRNAs, siGLO (Dharmacon) with different transfection reagents - Dharmafect 1 to 4 and Lipofectamine 2000 were tested separately in MDCK and 293T cells. Subsequently, experiments were performed with various siRNAs in MDCK and 293T cells using Lipofectamine 2000. First, siRNA was diluted in Opti-MEM to a final concentration of 100 nM and mixed with diluted Lipofectamine gently. This transfection mixture was incubated for 20 min at room temperature. It was then added to the cells in DMEM supplemented with 2% FBS culture medium in the absence of antibiotics, and the cells were incubated in 37°C with

5% CO₂. After the 6 hr incubation, medium was changed, and the cells were incubated for 16-24 hr until next procedures.

2.2.18. Real-time quantitative PCR (qPCR)

Confluent MDCK cells were infected with H5N2 at moi of 1 in 35 mm dishes. Cells were harvested at 16 hpi. At the time of harvesting, cells from two 35 mm dishes were scraped down in the RNeasy lysis solution (Qiagen) and PBS buffer (1:1) and pooled. Cells were pelleted at 14,000 rpm for 10 mins and stored at -80°C. The whole experiment was repeated in triplicate.

Virus	Gene	Sequence (5'-3')	Probe Sequence (UPL Probe #)
H1N1	NP FW	TGGAATCAAGTACCCTTGAAGT	TCTGGTCC (#93)
	NP RV	GCCCTCTGTTGATTGGTGT	
	PA FW	CGGAAAAGGCAATGAAAGAG	TCCTCTCC (#55)
	PA RV	CTGCAAATTTGTTTGTTCGAT	

Virus	Gene	Sequence (5'-3')	Probe Sequence (UPL Probe #)
H5N2	NP FW	TGCTTCAAATGAGAACATGGA	CTCCAGCA (#67)
	NP RV	GCCCAATATCTGCTTCTTAGTTCA	
	PA FW	GGTATAAACCCAAATTACCTCCTG	GGAAGCAG (#38)
	PA RV	AATGTCTTGGAGTTCTGCCAGT	
	PB1 FW	ATACAGGAGGCCGGTTGG	TGGTGGAG (#22)
	PB1 RV	ATTCGGGCCCTAGACACC	
	PB2 FW	AGAGCAACGGCCATTCTAAG	GCAACCAG (#164)
	PB2 RV	TTCAGCAATTGATTGTTCGTCT	

Table 2.6. Primer sequences and UPL probes (Roche) designed for real-time qPCR of influenza H1N1 and H5N2 gene segments.

Host	Gene	Sequence (5'-3')	Probe Sequence (UPL Probe #)
Canine	EF FW	GCTGGAAGATGGTCCCAAG	GGATGCTG (#89)
	EF RV	TGCCAGGAACCATATCAACA	

Table 2.7. Primer sequences and UPL probes (Roche) used for real-time qPCR of canine host gene.

Total RNA was extracted using RNeasy mini kit and reversed transcribed using SuperScript II RT, both according to the manufacturer's instructions. Reverse transcription of the positive-sense viral mRNAs was carried out with the use of the Oligo (dT) primer, whereas the negative-sense vRNAs were made using a Uni12 primer 5'AGCAAAAGCAGG3' (Hoffmann *et al.*, 2001). The design of primers for gene specific PCR was based on the Probefinder software from the Universal Probe Library (UPL) Design Center (Roche) (<http://qpcr.probefinder.com/organism.jsp>). Each primer pair was designed to contain a specific probe from the UPL located within the selected amplification sequence. Real-time qPCR was performed with the LightCycler 2.0 System, software version 5.32 (Roche) according to the protocol previously published (Spackman *et al.*, 2002). PCR products for each gene were validated for the presence of a single band of the correct size by 2% agarose gel electrophoresis. A list of primers and probe sequence for H5N2-NP and polymerase genes is elaborated in Table 2.6, whereas that for host gene is listed in Table 2.7.

2.2.19. Polymerase reporter assay

Construction of plasmids by cloning the three polymerase genes (PB1, PB2, PA) & the nucleoprotein (NP) gene from H1N1/WSN, pH1N1/471, H5N2/F118 and H9N2 strains into mammalian expression vector (pCAGGS). The sources of reporter genes: enhanced green fluorescent protein (EGFP) and luciferase (Luc) were the commercial plasmids pIRES2-EGFP and pNEBR-XGLuc respectively.

Figure 2.6. Schematic diagram illustrating the polymerase reporter assay in human 293T cells. vPoL represents the viral genes or proteins, PB2, PB1, PA and NP. The inverted Reporter represents either Luciferase or EGFP gene in an ambisense orientation. (-) represents negative sense RNA while (+) represents positive sense RNA. Rb represents ribozyme.

As shown in Figure 2.6, the vRNA-like reporter gene containing EGFP or Luc was cloned in the negative sense into a pPoL I plasmid, flanked by the 5' and 3' untranslated regions (UTRs) of the NS gene of influenza A/H1N1/PR8 virus. Expression of vRNA was driven by a truncated human RNA polymerase I (Pol I) promoter (nucleotide nt -250 to -1) positioned upstream of the vRNA-coding region. The sequence of the hepatitis delta virus genomic ribozyme (Rb) was positioned downstream of the vRNA-coding region in order to ensure that RNA processing gave the correct 3' end of the vRNA.

In the polymerase/luciferase reporter assay, combinations of four pCAGGS plasmids, each carrying a viral gene (PB1, PB2, PA or NP) from pH1N1/471 or H5N2/F118 or H9N2, together with a reporter plasmid (pPoL I-EGFP or pPoL I-Luc) were transfected into 293T cells in a 24-well format as initial reporter assay to analyze the functional activity of RNP complex from individual strain. In this assay,

H1N1/WSN strain was also used as a comparison. A total of 1 µg DNA (200 ng per construct) was transfected using TransIT-LT1 transfection agent according to the manufacturer's recommendations. As a negative control, pPoLI-EGFP plasmid or pPoLI-Luc plasmid alone was used to transfect into the cells. The assay was performed at 37°C. The transfected cells were observed for reporter gene expression and EGFP signal was visualized on the Nikon Eclipse TE2000 inverted fluorescence microscope. Supernatants of transfected cells were harvested at fixed time points post-transfection and luciferase activities were measured using a Gaussia luciferase assay kit and the signals were read by Fluroscanskan Ascent FL (Thermo). For the single-gene reassortment, NP and polymerase subunits between two different strains were reassorted. The individual reassorted complexes were created in parallel with their parental complexes. Luciferase reporter was used in this experiment and the method of transfection was the same as above. The assay was carried out at 37°C and luciferase activities of the parental and reassorted polymerase complexes were then measured at Day 4 post-transfection. Data were normalized to the activity RLU % in terms of respective parental complex and *p* values were calculated using an unpaired *t* test.

CHAPTER 3. GENERATION AND CHARACTERIZATION OF ANTIBODIES AGAINST POLYMERASE ACIDIC (PA) SUBUNIT

3.1. Generation of antibodies against PA protein from human 2009 pandemic H1N1 (pH1N1) and low pathogenic avian (LPAI) H5N2 subtypes of influenza virus

3.1.1. Cloning

Cloning of target PA protein was attempted as multiple truncated constructs in addition to the full length protein. Ten constructs per each polymerase subunit (Table 3.1) were cloned into *E. coli* expression vector using LIC-technology. Expression of the cloned gene and N-terminal 6xHis tag was driven by the T7 RNA polymerase promoter of plasmid pNIC28-Bsa4. The recombinant plasmids were then transferred to *E. coli* BL21-DE3 T1R cells for analysis of the expression of the target gene.

	PA subunits of pH1N1/471 or H5N2/F118		
Construct	AA-start	AA-stop	Molecular weight
vc001	240	550	38 kDa
vc002	240	600	44 kDa
vc003	300	550	32 kDa
vc004	300	600	37 kDa
vc005	350	550	26 kDa
vc006	350	600	32 kDa
vc007	240	716	57 kDa
vc008	300	716	51 kDa
vc009	350	716	45 kDa
vc010	2	716	85 kDa

Table 3.1. Ten constructs per PA subunit (a series of truncated fragments are shaded in blue and full length sequences are shaded in yellow) generated by protein production platform (PPP).

3.1.2. Protein expression

All cloned constructs that contained inserts of expected size were screened in small-scale for total expression and analyzed by SDS-PAGE (Figure 3.1).

Figure 3.1. Small scale screening for total expression of ten PA constructs (lane 1 to 10) from (A) pH1N1 and (B) H5N2 strains. Lanes M, molecular weight markers. Expressed target protein band from each construct is indicated by asterisks.

Induction with IPTG resulted in the accumulation of polypeptides with the molecular weights expected from the cloned genes and all the polypeptides expressed from the plasmid DNA could be observed in cell lysates. Then, the levels of total and soluble protein were tested and scored on a subjective scale from 0 to 4 (4 - a very strong band, 3 - a dominating band, 2 - a clear band, 1 - a barely visible band and 0 – no visible band). Constructs with an expression score of 3 or 4 were regarded to be clearly soluble and suitable candidates for large scale protein production. Constructs with an expression score of 2 or less may also be suitable for scale-up expression, but usually require more optimizations for procedures and larger cultivation volumes.

Of the all PA screened constructs, vc007 constructs from both pH1N1 and H5N2 strains were successfully scored as 4 in soluble expression (Table 3.2), and these soluble candidates were selected to carry out large-scale expression and purification. Schematic

representation of two selected PA constructs for scale-up production was illustrated in parallel with their full length respective genes (Figure 3.2).

Construct	AA-start	AA-stop	Molecular weight	Total expression	Soluble expression score
PA-pH1N1-vc007	G240	K716	57 kDa	4	4
PA-H5N2-vc007	G240	K716	57 kDa	4	4

Table 3.2. Summarizing the selected PA constructs with their respective expected protein sizes and soluble expression scores (Soluble score 4-Target protein dominates the gel; 3-Target protein present and significantly stronger than background; 2-Target protein present at approximately equal strength as background; 1-Target protein present but significantly weaker than background; and 0-No visible expression).

Figure 3.2. Schematic representation of two selected PA constructs from pH1N1 and H5N2 strains. The black line represents the full-length gene. Note that the green lines represent soluble constructs respectively.

3.1.3. Protein purification

After large scale expression, two soluble PA proteins (PA-pH1N1-vc007 and PA-H5N2-vc007) were purified using Immobilized-Metal Affinity Chromatography (IMAC) and gel filtration. The purified proteins were characterized by inspection of gel filtration profile and SDS-PAGE analysis to estimate the homogeneity, amount of protein, and purity of sample. As shown in Figure 3.3A, gel filtration chromatography of both PA proteins revealed single-peaked symmetric elution profile which reflect homogeneous or non-aggregated sample. When the protein concentrate was analyzed by SDS-PAGE and MS analyses, a purely distinct band was observed from each target protein with the size of 57 kDa (Figure 3.3B).

Figure 3.3. (A) Gel filtration profiles: PA-pH1N1-vc007 (top) and PA-H5N2-vc007 (bottom). (B) Coomassie blue staining of final proteins: PA-pH1N1-vc007 (left) and PA-H5N2-vc007 (right).

3.1.4. Mice immunization and antibody responses

Western blotting was performed to analyze the antibody responses of mice sera against corresponding target proteins which are purified pH1N1-PA_{His} protein (Figure 3.4) and purified pH1N1-PA_{His} protein (Figure 3.5). Pre-immune sera and post-immune sera containing the polyclonal antibodies from all five mice were used to probe the membranes. As shown in Figure 3.4 and 3.5, no signal was detected by pre-immune mice sera collected at Day 1 (1st column), while signals containing the target protein band at about 57 kDa were produced by post-immune mice sera collected at Day 25 and Day 46 (2nd and 3rd columns) indicating that mice immunization procedure by using purified PA protein as an antigen was successful with detectable antibody responses. Noticeably, increased intensity of signals could be picked up from the 2nd bleed sera and the level of antibody responses was found to be similar in all mice.

Figure 3.4. Western blot analysis of the mice antibody responses. Equal amount of samples containing purified pH1N1-PA_{His} antigen were loaded into each lane (Total 16 lanes). First, second and third columns were probed with different immune sera of five mice (labelled as 1st, 2nd, 3rd, 4th and 5th) collected at Day 1, Day 25 and Day 46 respectively. Last lane (labelled as C) probed with commercial anti-His antibody was used as positive control. PA_{His} protein was indicated by arrow at about 57 kDa and highlighted in red box. The asterisk indicates additional bands at 37 kDa.

Figure 3.5. Western blot analysis of the mice antibody responses. Equal amount of samples containing purified H5N2-PA_{His} antigen were loaded into each lane (Total 16 lanes). First, second and third columns were probed with different immune sera of five mice (labelled as 1st, 2nd, 3rd, 4th and 5th) collected at Day 1, Day 25 and Day 46 respectively. Last lane (labelled as C) probed with commercial anti-His antibody was used as positive control. PA_{His} protein was indicated by arrow at about 57 kDa and highlighted in red box. The asterisk indicates additional bands at 37 kDa.

Two strong additional bands which correspond to the molecular weight of 37 kDa were also noted in both experiments together with our protein of interest, but the exact characteristics of these bands are unclear. Hence, it is necessary to explore the nature of these protein bands in our subsequent experiments of PA protein.

3.1.5. Hybridoma production

Splenocytes from mice immunized with the corresponding recombinant PA proteins were fused with myeloma cells to produce hybridoma cell lines which are able to secrete antibodies. 10 days after the fusion, growing hybridoma could be observed around 50-60% in 96-wells and were ready to screen by ELISA for specific monoclonal antibody production as well as to rule out the negative clones.

Based on the ELISA screening results (Figure 3.6A & B), we obtained a total of 14 and 20 hybridoma cell lines which recognized the viral immunogens and secreted antibodies against PA(pH1N1) and PA(H5N2) respectively. Among these potentially positive hybridoma clones, two anti-PA(pH1N1): 9F5 and 2E2, and five anti-PA(H5N2): 4D7, 6A10, 6A11, 6B11 and 7A4 which possessed the highest affinity to the target PA protein in ELISA were chosen for subcloning by limiting dilution and ELISA-positive subclones were successfully propagated into larger scale. They were then further characterized by IFA and Western blotting. In the later part of results and discussion, the term of the monoclonal antibody against PA protein has been shortened as mAb-PA.

Figure 3.6. ELISA screening results of (A) pH1N1-PA and (B) H5N2-PA hybridoma clones. Those clones with the highest affinity to the target PA protein were highlighted by red circles.

3.2. Characterization of antibodies against PA protein from human 2009 pandemic H1N1 (pH1N1) and low pathogenic avian (LPAI) H5N2 subtypes of influenza virus

3.2.1. Transfection studies

3.2.1.1. Optimization of transfection

Prior to the characterization of mAbs, the pCAGGS vector expression system was utilized to express the full length PA protein with FLAG-tag at the C-terminus in 293T cells. The expression of protein was optimized at specific time points post-transfection by IFA and Western blotting using commercially available anti-FLAG antibody, and the results are shown in Figure 3.7 and 3.8 respectively. In IFA, we observed a positive staining in 293T cells transfected with pCAGGS expression vector containing FLAG-tagged full length PA gene (Figure 3.7). It indicates that FLAG-

tagged PA protein could be well-expressed in the mammalian 293T cells. In addition, we noted that the intensity of the signal was gradually increased from 8 hpt to 24 hpt, but it dropped at 48 hpt due to dying cells.

Figure 3.7. Optimization of transfection in 293T cells at specific time points post-transfection by IFA. (A) Mock transfection, and (B) FLAG-tagged full length PA gene transfection. Transfected cells were stained with anti-FLAG. Cells were then stained with the secondary antibody, anti-rabbit Alexa 488 (green). Bright field images reveal the cell density.

Figure 3.8 showed a protein product (at about 83 kDa) corresponding to the size of the FLAG-tagged full length PA protein from each time point post-transfection. Among different post-transfection time points, 24 hpt was found to sufficiently express the target protein. Hence, 24 hr was chosen as a fixed post-transfection time point for the characterization of generated mAbs in our further transfection studies. Additionally, smaller protein products at about 50 kDa, 37 kDa and 25 kDa were also noticed in this experiment. The time dependent expression pattern of these smaller products with the

target protein were found correspond to each other. It suggests that these smaller products are possible proteolytic products of the target protein.

Figure 3.8. Optimization of transfection in 293T cells at specific time points post-transfection by Western blot analysis. Equal amount of samples were loaded into each lane. Lane 1 was loaded with cells transfected with expression vector alone (mock transfection). Lane 2, 3, 4 and 5 were loaded with cells transfected with FLAG-tagged full length PA gene from 8 hpt, 16 hpt 24 hpt and 48 hpt respectively). Membrane was probed with anti-FLAG. The FLAG-tagged full length PA protein at about 83 kDa (PA) is indicated. The additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) are also indicated.

3.2.1.2. IFA and Western blot analysis in transfected cells

Firstly, we characterized the specificity of our generated mAbs-PA with 293T transfected cells at 24 hpt using IFA. Cells expressing FLAG-tagged full length pH1N1/471-PA protein were stained with mAbs-PA(9F5 and 2E2), and anti-FLAG. As shown in Figure 3.9A, the positive staining was obtained with both mAbs-PA, suggesting that both 9F5 and 2E2 recognize the target recombinant PA protein. Moreover, we could also see the positive cell staining when co-stained with anti-FLAG, reflecting the well-expressed FLAG-tagged PA protein in 293T cells with approximately 90% transfection efficiency. Although the two mAbs-PA showed positive results, the staining intensities observed between 9F5 and 2E2 were slightly different. It may be due to differences in binding region or affinity of these two mAbs.

In the mock transfection, there was no positive signal with the above corresponding antibodies (Figure 3.9B), indicating that both 9F5 and 2E2 specifically recognize the PA protein, but not the host cell protein.

Figure 3.9. Characterization of mAbs against pH1N1-PA reactivity in 293T transfected cells at 24 hpt by IFA. (A) FLAG-tagged full length PA gene transfection, and (B) Mock transfection. Transfected cells were stained with either mAb-PA(9F5) or mAb-PA(2E2), and co-stained with anti-FLAG. Cells were then stained with anti-mouse Alexa 488 (green) and anti-rabbit Alexa 555 (red).

Figure 3.10. Characterization of mAbs against pH1N1-PA reactivity in 293T transfected cells at 24 hpt by Western blot analyses. M represents mock transfection and T represents FLAG-tagged full length PA gene transfection. Western blotting using (A) mAb-PA(9F5), (B) mAb-PA(2E2), and (C) anti-FLAG. The FLAG-tagged full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) are also indicated.

We also carried out Western blot analysis with 293T transfected cells to further characterize the mAbs-PA(9F5 and 2E2). As seen in Figure 3.10, a prominent protein product at about 83 kDa which corresponds to the size of the full length PA protein was detected in FLAG-tagged full length pH1N1/471-PA gene transfected cells using either 9F5 or 2E2. However, Western blotting with 9F5 revealed additional smaller products corresponding in size to 50 kDa, 37 kDa and 25 kDa (collectively referred to as PA*). Moreover, Western blotting of cells expressing the recombinant PA protein with a C-terminal FLAG epitope with anti-FLAG showed a similar array of smaller protein products corresponding in size to PA*. This proved that PA* contains the C-terminal end of the PA protein, but does not contain the mAb-PA(2E2) binding region. In addition, there was no signal indicating the target protein in mock transfected cells, demonstrating the specificity of these immunological reagents.

Next, the reactivity of five selected mAbs against H5N2-PA(4D7, 6A10, 6A11, 6B11 and 7A4) were also characterized by IFA and Western blotting using 293T transfected cells, and the results are shown in Figure 3.11 and 3.12 respectively. Both IFA and Western blot analyses revealed the recognition of the FLAG-tagged H5N2/F118 PA protein expressed in mammalian 293T cells by all five mAbs with various intensities of the signals. It may be explained by various binding region or affinity among these antibodies. In addition, the presence of smaller protein products at about 50 kDa and 37 kDa were also noted in Western blotting with all five mAbs.

Figure 3.11. Characterization of mAbs against H5N2-PA reactivity in 293T transfected cells at 24 hpt by IFA. (A) FLAG-tagged full length PA gene transfection and (B) Mock transfection. Transfected cells were stained with mAb-PA(4D7) or (6A10) or (6A11) or (6B11) or (7A4). Cells were then stained with anti-mouse Alexa 488 (green), and Evans (red) to visualize all cells.

Figure 3.12. Characterization of mAbs against H5N2-PA reactivity in 293T transfected cells at 24 hpt by Western blot analyses. M represents mock transfection and T represents FLAG-tagged full length PA gene transfection. Western blotting using (A) mAb-PA(4D7), (B) mAb-PA(6A10), (C) mAb-PA(6A11), (D) mAb-PA(6B11), and (E) mAb-PA(7A4). The FLAG-tagged full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa and 37 kDa (*) are indicated.

3.2.2. Infection studies

3.2.2.1. IFA and Western blot analysis in infected cells

Further characterization of the antibodies was carried out by IFA and Western blot analysis to identify the specific viral protein from virus infected MDCK cells. MDCK cells possess both the NeuAc α 2,6Gal and NeuAc α 2,3Gal sialic acid receptors and can be infected by both human and avian influenza viruses, and is often the cell-line for isolation of both human and avian influenza viruses (Kumari *et al.*, 2007).

The IFA images of infected MDCK cells using a moi of 5 at specific post-infection time points were shown in Figure 3.13. We first studied the replication kinetics of A/Singapore/471/2009 human pandemic H1N1 virus in MDCK cells based on the detection and localization of the PA protein in parallel with the NP protein by IFA at different time-points. From this experiment, appearance of NP could be observed by earlier nuclear localization at 6 hpi. Subsequently, more cells had nuclear localization detected with minimal export at 8 hpi and 10 hpi. Then, extensive nuclear export of the NP protein was observed by 16 hpi (Figure 3.13), indicating virus assembly and budding by this time point.

However, the signal of PA protein in infected cells was observed only from 8 hpi onward by both mAbs-PA(9F5 and 2E2) with increasing intensity of the signal at later time points implying that the rate of PA signal development is slower than NP signal. It has been reported that three RNA polymerase proteins (PB1, PB2 and PA) are associated with NP protein to form the RNP complexes and these complexes are responsible for both virus transcription and replication in the infected cell nucleus (Resa-Infante *et al.*, 2010). Based from the above observation, it is possible that the rate of transcription/replication and nuclear transport of PA is different from NP. The factor responsible for delayed transcription and transport of PA protein into the nucleus remain unknown. Early reports by Akkina *et al.* suggested that the kinetics of nuclear transport

of PA could just be slower or that PA undergoes some post-translation modifications (Akkina *et al.*, 1987). Their group also observed delay in transport of PA protein in the cells infected with influenza virus which agrees with our finding. Moreover, use of a different virus and host cells as well as a different multiplicity of infection (moi) may have caused expression of PA earlier than that observed in our studies. Further work will be necessary to understand the kinetics of PA protein transport in the virus infected cells.

From the IFA results, the PA staining labelled with 9F5 was observed throughout the cell (both nucleus and cytoplasm), especially at 16 hpi (Figure 3.14A). In contrast, the PA protein labelled with 2E2 mainly localized in the nucleus at the same post infection time (Figure 3.14B). This demonstrated that two mAbs would have bound to different epitopes on a target protein or recognized a particular post-translational modification which is specific to the nuclear localization. Further investigation would be required to identify the specific antigen binding sites of these antibodies and their significance.

A similar picture was observed when we examined the replication kinetics of the MDCK infected cells by Western blot analyses using mAbs-PA(9F5 and 2E2). A protein product at 83 kDa corresponding to the expected size of the full length PA protein was detectable by both mAbs from 8 hpi onward (Figure 3.15A and B). Interestingly, the simultaneous appearance of the smaller products (PA*), together with the major product, were also detected by 9F5 in infected cells, suggesting that they must be related to our protein of interest, probably proteolytic products. The presence of PA* with or without virus infection suggests that PA* is unlikely to be generated as a result of the host response to virus infection, further suggesting that the formation of PA* may be an intrinsic property of the PA gene expression profile.

Figure 3.13. Characterization of mAbs against pH1N1-PA reactivity in pH1N1/471 virus infected MDCK cells at specific time points post-infection by IFA. Mock infected and pH1N1/471 virus-infected cells were stained with anti-NP or mAb-PA(9F5) or mAb-PA(2E2). Cells were then stained with anti-mouse Alexa 488 (green), and Evans (red) to visualize all cells. N: nuclear localization, N+C: both nuclear and cytoplasmic localization.

Figure 3.14. Confocal images of pH1N1/471 virus infected MDCK cells at 16 hpi. Infected cells stained with either (A) mAb-PA(9F5) or (B) mAb-PA(2E2) and co-stained with anti-Histone H4 to show cell nuclei. Cells were then stained with anti-mouse Alexa 488 (green) and anti-rabbit Alexa 555 (red). The patterns of protein distribution were visualized by confocal microscopy. N: nuclear localization, N+C: both nuclear and cytoplasmic localization.

Figure 3.15. Characterization of mAbs against pH1N1-PA reactivity in pH1N1/471 virus infected MDCK cells at specific post-infection time points by Western blot analyses using (A) mAb-PA(9F5) and (B) mAb-PA(2E2). The full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) are indicated. Loading control (β-actin) is also shown.

Subsequently, we also characterized the reactivity of the selected five mAbs against H5N2-PA in MDCK cells infected with LPAI H5N2/F118 virus using a moi of 5 at a fixed post-infection time point (16 hpi). As shown in Figure 3.16, all of them were able to detect the PA protein from the infected cells by IFA. In addition, the appearance of PA protein was observed throughout the cell (both nucleus and cytoplasm) and this type of appearance was found to be similar in all of them. It demonstrates that these mAbs may recognize the same epitope on the target protein. Moreover, the Western blot analyses on infected cells revealed the similar pictures observed in transfected cells (Figure 3.17).

Figure 3.16. Characterization of mAbs against H5N2-PA reactivity in H5N2/F118 virus infected MDCK cells at 16 hpi by IFA. (A) H5N2/F118 virus-infected cell and (B) Mock infected cells were stained with mAb-PA(4D7) or (6A10) or (6A11) or (6B11) or (7A4) and co-stained with anti-Histone H4 to show cell nuclei. Cells were then stained with anti-mouse Alexa 488 (green), and anti-rabbit Alexa 555 (red). N+C: both nuclear and cytoplasmic localization.

Figure 3.17. Characterization of mAbs against H5N2-PA reactivity in H5N2/F118 virus infected MDCK cells at 16 hpi by Western blot analyses. M represents mock infected cells and I represents H5N2/F118 virus infected cells. Western blotting using (A) mAb-PA(4D7), (B) mAb-PA(6A10), (C) mAb-PA(6A11), (D) mAb-PA(6B11), and (E) mAb-PA(7A4). The full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa and 37 kDa (*) are indicated.

We noted the appearance of both PA and PA* concomitantly during PA expression, indicating that PA* does not arise from an accumulation of excess PA protein. It is possible that PA* originates due to alternative translation initiation sites within mRNA generated from the segment 3, as has recently been described by Muramoto *et al.*, 2013. It is also possible that PA* may be generated by an alternative mechanism (e.g. via proteolytic processing of the PA protein). Although previous studies have reported a proteolytic activity associated with the PA protein (Sanz-Ezquerro *et al.*, 1996; Hara *et al.*, 2001; Rodriguez *et al.*, 2007), this proteolytic activity which may generate PA* from the PA protein has not been identified yet. In the structural model proposed for the polymerase complex the N and C-terminal domains are separated by a PA linker region which is located on the surface of the polymerase complex (Pflug *et al.*, 2014). It is possible that the PA linker region is prone to proteolytic processing and may play a role in promoting PA* formation. Previous *in vitro* studies using limited proteolysis of the PA protein has described the production of 25 kDa and 55 kDa PA protein fragments (Hara *et al.*, 2006), which provided evidence

for the existence of at least two stable domains with the PA protein. The 55 kDa domain is similar in size to the larger 50 kDa PA* species that we detected, and the formation of PA* in virus infected cells suggests that the different forms of PA* represent individual stable sub-domains of the PA protein.

3.2.3. Fractionation analysis

To examine the distribution of PA protein in the cytoplasm and nucleus of virus-infected cells, MDCK cells were mock infected or infected with pH1N1/471 using a moi of 5, and infected cells at 16 hpi were fractionated into cytoplasmic and nuclear fractions. Each fraction was then examined by Western blotting using mAbs-PA(9F5 and 2E2).

As shown in Figure 3.18, the PA protein was detected in both cytoplasmic and nuclear enriched fractions of virus-infected cells by 9F5. This observation is consistent with our previous IFA result at this particular time point infection (Figure 3.14A). Based on this single time-point experiment, it is likely that the relative amount of PA protein is more abundant in the cytoplasmic fraction compared to the nuclear fraction. To explore the kinetics of nuclear transport of PA in virus infected cells, multiple time-point experiments might need to be conducted. Moreover, the presence of smaller products (PA*) was also noted in both cytoplasmic and nuclear fraction. The PA protein contains two NLS sequences in the N-terminal domain (Nieto *et al.*, 1994), which we would expect to be absent in PA*. This suggests that either the PA* traffics into the nucleus by interaction with PB1 or other NLS are present with the PA protein.

Figure 3.18. Western blot analysis on nuclear cytoplasmic fractionation. Cytoplasmic fraction (CF), nuclear fraction (NF), and insoluble nuclear pellet (NP) from mock and pH1N1/471 virus-infected MDCK cells at 16 hpi were loaded as indicated above. Each fraction was then examined using mAb-PA(9F5). The full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) are indicated. Nuclear marker (Lamin A/C) and cytoplasmic marker (Tubulin) are also shown.

Figure 3.19. Western blot analysis on nuclear cytoplasmic fractionation. Cytoplasmic fraction and nuclear fraction from mock infected and pH1N1/471 virus infected MDCK cells at 16 hpi were loaded as indicated above. Each fraction was then examined using mAb-PA(2E2). The full length PA protein at about 83 kDa (PA) is indicated. Nuclear marker (Lamin A/C) and cytoplasmic marker (Tubulin) are also shown.

Unlike the distribution pattern given by 9F5, the PA protein recognized by the 2E2 was more intense in the nuclear fraction of virus-infected cells (Figure 3.19), consistent with our previous IFA result at this specific time point infection. This implied that 2E2 mainly targeted the full length PA protein located in the nucleus. Our observation also suggests that the linker region recognized by 2E2, which causes the conformational change in the two domains, may become fully exposed after the target protein was imported into the nucleus. Further investigation would be required to understand the significances of these two antibodies.

3.2.4. Epitope mapping

The two mAbs against pH1N1-PA (clones 9F5 and 2E2) recognized mammalian expressed PA protein in both immunofluorescence assay and Western blot analyses. Furthermore, these two mAbs showed different staining patterns of PA protein in pH1N1 virus infected cells by immunofluorescence assay. 2E2 recognized PA protein in the nuclear compartment only and 9F5 recognized PA protein throughout the cells. The results suggested that these mAbs might have different epitopes. To identify the binding region of these two mAbs on the target protein, epitope mapping was subsequently performed by serial expression of the truncated fragments of the target PA protein using two steps and antibody recognition was determined by Western blotting (Figure 3.20 and 3.21).

1st Step

Figure 3.20. 1st Step: A series of nested deletions of the PA region was created by 100 aa deletions from the C-terminal domain. The amino acid position and the location of the N-terminal 6xHis-tag are indicated (top). Western blot analyses (bottom) using (A) anti-His (loading control), (B) mAb-PA(9F5) and (C) mAb-PA(2E2). Serial truncated protein products are indicated by asterisks on top of each protein band. The 9F5 and 2E2 binding regions are found to be between aa517-616 and aa240-316 respectively.

2nd Step

Figure 3.21. 2nd Step: A further series of nested deletions of PA region was created by 20 aa deletions from the C-terminus of truncation 2. The amino acid position and the location of the N-terminal 6xHis-tag are indicated (top). Western blot analyses (bottom) using (A) anti-His (loading control), (B) mAb-PA(9F5) and (C) mAb-PA(2E2). Serial truncated protein products are indicated by asterisks on top of each protein band. The 9F5 and 2E2 binding regions are found to be between aa597-616 and aa240-255 respectively.

Final Step

Figure 3.22. A schematic showing the positions of binding regions of mAb-PA(9F5) and mAb-PA(2E2) on PA protein construct.

mAbs pH1N1-PA	Antibody recognition region
9F5	Amino acid positions 597-616 : EAESSVKEKDMTKEFFENKS (Total – 20 aa)
2E2	Amino acid positions 240-255: GCIEGKLSQMSKEVNA (Total – 16 aa)

Table 3.3. Identified amino acid positions and sequences recognized by mAb-PA(9F5) and mAb-PA(2E2).

The results revealed that the 9F5 bound to the PA protein between aa597-616 (within the PA-C domains that contain the PB1 binding domain), while the 2E2 bound to the PA protein between aa240-255 (within the PA-linker), as shown in Figure 3.22. The identified different amino acid sequences recognized by 9F5 and 2E2 are indicated in Table 3.3. This clearly demonstrated that two mAbs possessed different epitope regions. The linker region connecting the N and C-terminal domains has been found to provide the PA with conformational flexibility (Liu *et al.*, 2009). Hence, the 2E2 recognizing region may be masked in the cytoplasmic compartment and becomes exposed in the nuclear compartment by an unknown mechanism which needs to be further investigated.

After identifying binding regions of 9F5 and 2E2, we aligned the sequences of interest (20 amino acid residues recognized by 9F5 and 16 amino acid residues recognized by 2E2) from pH1N1/471 strain with other influenza strains involved in our studies. As shown in Figure 3.23A, we noted that there was one amino acid difference within the 9F5 recognized epitope region of H5N2 and H9N2 strains, but this region

was conserved in WSN strain. Again, based on the sequence alignment findings of the epitope region recognized by 2E2, we noticed only one amino acid difference in WSN sequence among four different strains (Figure 3.23B).

Figure 3.23. PA protein sequence alignment. (A) The 20 amino acid binding region (aa597-616) recognized by mAb-PA(9F5), and (B) the 16 amino acid binding region (aa240-255) recognized by mAb-PA(2E2) were aligned with the PA sequences of other influenza strains (H1N1/WSN, H5N2/F118 and H9N2) against pH1N1/471. Protein residues that match pH1N1/471-PA exactly were shown as ‘.’.

Next, epitope mapping was also carried out for five mAbs against H5N2-PA to identify their binding regions. Due to the time constraints, we performed only one step from the above strategy which is a series of nested deletions of the PA region created by cloning of the C-terminal domain using 100 aa deletion, and antibody recognition was determined by Western blotting (Figure 3.24). The background protein bands in the control from Figure 3.24 was found to be more apparent compared to the control from Figure 3.20 was due to different exposure times. The positions of 100 aa binding regions were shown as a schematic diagram on the PA protein construct (Figure 3.25) and identified amino acid positions recognized by five mAbs were listed in Table 3.4. Among five mAbs, the 4D7 and 7A4 binding regions are found to be between aa417-516, while the 6A10, 6A11 and 6B11 binding regions are found to be between aa317-416.

Figure 3.24. A series of nested deletions of the PA region was created by 100 aa deletions from the C-terminal domain. Western blot analyses using (A) anti-His (loading control), (B) mAb-PA(4D7), (C) mAb-PA(7A4), (D) mAb-PA(6A10), (E) mAb-PA(6A11), and (F) mAb-PA(6B11). Serial truncated protein products are indicated by asterisks on top of each protein band. The 4D7 and 7A4 binding regions are found to be between aa417-516, and the 6A10, 6A11 and 6B11 binding regions are found to be between aa317-416.

Figure 3.25. A schematic showing the positions of binding regions of five mAbs-PA(4D7, 7A4, 6A10, 6A11 and 6B11) on PA protein construct.

mAbs H5N2-PA	Antibody recognition region
4D7	Amino acid positions 417-516: (Total – 100 aa)
7A4	Amino acid positions 417-516: (Total – 100 aa)
6A10	Amino acid positions 317-416: (Total – 100 aa)
6A11	Amino acid positions 317-416: (Total – 100 aa)
6B11	Amino acid positions 317-416: (Total – 100 aa)

Table 3.4. Identified amino acid positions recognized by five mAbs-PA(4D7, 7A4, 6A10, 6A11 and 6B11).

Figure 3.26. PA protein sequence alignment. (A) The 100 amino acid binding region (position 317-416) recognized by mAbs-PA(6A10, 6A11 and 6B11), and (B) the 100 amino acid binding region (position 417-516) recognized by mAbs-PA(4D7 and 7A4) were aligned with the PA sequences of other influenza strains (H9N2, pH1N1/471 and H1N1/WSN) against H5N2/F118. Protein residues that match H5N2/F118-PA exactly were shown as '.'.

Furthermore, we also performed the sequence analyses on two different epitope regions recognized by five mAbs against H5N2-PA by aligning other strains against H5N2/F118 strain. Within the regions of interest, we noted differences in some amino acid residues in between the conserved PA sequences among avian and human strains (Figure 3.26A and B).

3.2.5. Specificity testing across various strains

As show in Figure 3.23, we had noted the highly conserved sequences recognized by mAb-PA(9F5) and mAb-PA(2E2) across four different strains. Therefore, we decided to determine the specificity and cross-reactivity of these two antibodies for the different strains. The specificity of the two mAbs against pH1N1/471 PA protein was tested with three other strains: A/WSN/33 (H1N1/WSN), A/Malaysia/F118/2004 (H5N2/F118) and A/H9N2/Malaysia/2001 (H9N2) infected MDCK cells by IFA and Western blot analyses. Based on the IFA results, 9F5 reacted equally well with PA proteins from all of the strains tested, while 2E2 did not react with any of them (Figure 3.27A, B and C).

Figure 3.27. Characterization on the specificity of mAbs against pH1N1-PA in the various strains of virus infected MDCK cells at 16 hpi by IFA. (A) H1N1/WSN, (B) H5N2/F118, and (C) H9N2 virus infected cells stained with anti-NP Ab (control for infection) or mAb-PA(9F5) or mAb-PA(2E2) and co-stained with anti-Histone H4 to show cell nuclei. Cells were then stained with anti-mouse Alexa 488 (green), anti-rabbit Alexa 555 (red), and Evans (red) to visualize all cells. N+C: both nuclear and cytoplasmic localization.

As shown in Figure 3.28A, C and E, the PA protein could be detected not only in IFA, but also in Western blot by mAb-PA(9F5) in the MDCK cells infected with H1N1/WSN, H5N2/F118 and H9N2 viruses.

Figure 3.28. Characterization on the specificity of mAbs against pH1N1-PA in the various strains of virus infected MDCK cells at specific post-infection time points by Western blot analyses using mAb-PA(9F5) and mAb-PA(2E2). (A and B) H1N1/WSN virus infection, (C and D) H5N2/F118 virus infection, and (E and F) H9N2 virus infection. The full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) are indicated. Loading control (β -actin) is also shown.

It is confirmed that 9F5 has cross reactivity with all the strains tested in our study. The simultaneous appearance of PA associated smaller protein products (PA*), together with the full length PA protein, were also detected in H1N1/WSN, H5N2 and H9N2 virus-infected cells indicating that the presence of PA* was not subtype-specific. Unlike the replication kinetics of three other strains, H1N1/WSN infected MDCK cells displayed the expression of PA protein, as early as 6 hpi, suggesting faster rate of viral entry and replication kinetic (Sutejo *et al.*, 2012).

Based on the protein sequence analysis, the PA protein sequences of these 4 different strains are highly conserved because they share relatively similar sequence homology (more than 95% sequence identity to each other). Therefore, it is not surprising to see the cross-reactivity of our mAb-PA(9F5) with other strains. Previous analysis of the specificity of the mAbs developed against PA protein of A/chicken/Nanchang/3-120/01 (H3N2) also exhibited the cross-reactivity with other influenza A virus strains which have more than 95% identity in their protein sequences (MacDonald *et al.*, 2012). Surprisingly, mAb-PA(2E2) did not react with other strains tested in our study (Figure 3.26B, D and F). This could be due to conformational changes which allow the epitope to expose in the pH1N1 strain only. Another factor such as post-translational modification which may play a role in the specificity of 2E2, while the certain reason for this is unknown. To predict any potential modification within the epitope region, various prediction programs from Center for Biological Sequence Analysis (<http://www.cbs.dtu.dk/index.shtml>) were used. No significant difference was observed across various strains used in our study. It is worth to note that the prediction programs are based on the nature of proteins from different organisms such as human.

Due to time constraints, specificity testing of five mAbs against H5N2-PA would be explored in the future.

3.2.6. Detection of PA protein in various infected cells

From the Section 3.2.5, we have observed the cross reactivity of mAb-PA(9F5) in MDCK cells infected with various strains tested in our study and the additional smaller products were recognized together with the major PA protein by 9F5. Hence, we decided to use it for further studies on the PA protein. We used MDCK cell line (canine origin) as a main model cell line throughout our viral infection studies and other antibody characterization processes because it was the most commonly used cell line to study for viral infection of cells in the flu research (Dukes *et al.*, 2011). However, it would be also interesting to examine the viral proteins by using other cell lines (e.g. A549 and CEF). A human lung epithelial cell line known as A549 (one of the permissive cell lines to influenza virus infection) has been widely used to study the viral replication kinetics and host response to virus infection (Farooqui *et al.*, 2012; Sutejo *et al.*, 2012). Moreover, using egg-derived CEF cell line in the study of viral infection could inform us about permissiveness of embryonated chicken eggs for propagation of our influenza viruses which would be conducted in our later study.

Figure 3.29. PA protein detection in various infected cells by Western blot analyses. (A) MDCK, (B) A549 and (C) CEF cells were mock-infected (M), infected with pH1N1/471 (pH1) and H1N1/WSN (WSN) using a moi of 5 at 16 hpi. Cells were then examined by Western blotting using mAb-PA(9F5). The full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) are indicated. Loading control (β-actin) is also shown.

First, we performed the infection simultaneously in three cell lines (MDCK, A549 and CEF) with pH1N1/471 and H1N1/WSN viruses at moi of 5 and harvested the samples at 16 hpi. Then, we carried out the PA protein detection from these samples by using mAb-PA(9F5). In general, the target protein was detected in all three infected cell lines with both viruses (Figure 3.29). This suggested that infection could be established with our viruses in these hosts. Furthermore, the smaller products (PA*) were also noted in virus-infected A549 and CEF cells although their intensities were slightly lower than those in virus-infected MDCK cells. This indicated that PA* expression was not cell type-specific, and since PA* have been detected in avian cells, we also expected them to be expressed in virus-infected embryonated eggs.

3.2.7. Radio-immunoprecipitation of PA protein

Figure 3.30. (A) [³⁵S]-methionine metabolic radiolabelling image of MDCK cells with mock infection (M) and H1N1/WSN virus infection (I) using a moi of 5 after 10 hpi, with 1 hr radiolabelling at 9 hpi. (B) [³⁵S]-methionine metabolic radiolabelling image of MDCK cells with mock infection (M) and H1N1/WSN virus infection (I) using a moi of 5 after 10 hpi, with 1hr radiolabelling at 9 hpi, followed by immunoprecipitation without Ab (control) or with anti-NP or mAb-PA(9F5). The samples were run on 12% SDS-PAGE gel and visualized after 24 hr exposure time.

To detect total protein expressions in virus-infected cells, [³⁵S]-methionine metabolic radiolabelling method was used in our study. First, MDCK cells were either mock-infected or infected with H1N1/WSN virus using a moi of 5. Then, the cells were examined 1 hr after labelling with [³⁵S]-methionine for the incorporation of radiolabelled ³⁵S methionine into newly synthesized viral proteins. As shown in radiolabelling image (Figure 3.30A), three prominent bands were observed only in the virus-infected sample with the sizes of approximately 85 kDa, 56 kDa and 28 kDa. These bands corresponded to P proteins (PB2, PB1 and PA), NP and M1 proteins respectively.

Next, we needed to enrich the PA protein by immunoprecipitation (IP) method to further study the PA associated proteins. Therefore, mock-infected and H1N1/WSN virus-infected MDCK cells were labelled with [³⁵S] followed by IP of target proteins, NP with anti-NP and PA with mAb-PA(9F5). As shown in Figure 3.30B, a large amount of NP protein could be enriched by IP with anti-NP from the infected sample. Besides, IP with mAb-PA(9F5) showed a single band in the infected sample at about 83 kDa corresponding to the full length PA protein. This indicated that 9F5 could be useful in the study of the PA associated proteins with IP method. However, PA* could not be detected together with the PA protein in this study. This could be due to relatively low amount of PA* at this time point and IP conditions.

In addition, the appearance of two bands corresponding to the NP and M1 proteins was noted in all infected samples with or without IP antibody (control). The amounts of NP and M1 proteins in the influenza virus have been known to be relatively higher than other viral proteins (Fodor and Brownlee, 2002). This could be one possible reason for non-specific detection of these two proteins in all infected samples. Another reason could be non-specific binding to the IP reagents or beads, leading to NP and M1

proteins present in the infected samples after the IP. Moreover, a number of additional factors such as binding and wash conditions must be also considered.

3.2.8. siRNA showing smaller protein products are PA-related

To confirm that the smaller protein products were generated by PA protein expression, we used siRNA to silence PA gene expression. MDCK cells were infected with H1N1/WSN at a moi of 5, using cells either treated with a siPA-H1N1 to inhibit PA protein expression or control cells treated with siGFP (targeted against the GFP) or non-treated-cells. The siPA-H1N1 treatment correlated with a 60-70% reduction in the level of PA mRNA compared to that in siGFP-treated and non-treated virus-infected cells (Figure 3.31).

Figure 3.31. Relative quantification of knockdown of PA mRNA level in siPA-H1N1 treated or siGFP-treated or no siRNA-treated H1N1/WSN virus-infected MDCK cells. (* $p < 0.05$).

Figure 3.32. IFA showing the level of PA protein expression in siPA-H1N1 treated or siGFP-treated or no siRNA-treated H1N1/WSN virus infected MDCK cells.

Figure 3.33. Western blot analysis on MDCK cells infected with H1N1/WSN using a moi of 5 at 10 hpi after treated with siPA-H1N1 or siGFP or no siRNA. Mock infected MDCK cells (M) is shown. The full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) are indicated. Loading control (β -actin) is also shown.

The IFA using mAb-PA(9F5) also revealed the absence of PA protein staining in a large number of infected cells treated with siPA-H1N1 when compared to siGFP-treated cells and non-treated cells (Figure 3.32). In addition, Western blot analysis of cell lysates using mAb-PA(9F5) showed similar levels of the PA and PA* proteins in siGFP-treated cells and non-treated cells, while siPA-H1N1 treatment resulted in a 90% reduction in the PA protein and PA* protein levels (Figure 3.33), confirming the smaller protein products (PA*) are related to target PA protein.

3.2.9. Two-dimensional analysis on PA protein and PA related products in virus-infected cells

To obtain a better separation of PA protein and PA* from infected cells, we used two-dimensional (2-D) electrophoresis followed by Western blot analysis. Initially, mock-infected MDCK cell lysate was used to optimize the parameters of 2-D electrophoresis with immobilized pH gradients (IPG). As shown in Figure 3.34, multiple protein spots were detected in areas of the 2-D gel after Coomassie blue staining, indicating the proper 2-D separation of protein sample.

Figure 3.34. Coomassie blue staining of SDS-PAGE gel after two-dimensional electrophoresis with total cell lysate from mock-infected MDCK cells. The pH gradient from 3 to 10 is acidic to basic. Multiple protein spots detected in areas of the 2-D gel are shown.

Then, MDCK cells lysates prepared from either mock-infection or pH1N1/2009 virus infection using a moi of 5 at 16 hpi were analyzed by 2-D electrophoresis combined with Western blotting using mAbs-PA(9F5 and 2E2). From the experiment with infected cell lysate, 9F5 revealed specific protein spots corresponding to the expected molecular weights of the full length PA protein and PA* (Figure 3.35B). Meanwhile, we also noted that the range of isoelectric points (pI) for both PA and PA* were between 5 and 6. In the case of 2E2 with infected cell lysate, we observed only a single protein species at about 83 kDa which was also corresponding to the size of PA protein. pI of that protein species was also found between 5 and 6 (Figure 3.36B). In the

experiments with mock-infected cell lysates, we failed to detect these PA and PA related protein spots, thus confirming the specificity of above findings (Figure 3.35A and 3.36A).

Figure 3.35. Two-dimensional electrophoresis combined with Western blotting using mAb-PA(9F5). Samples were mock-infected or infected MDCK cells with pH1N1/471 virus using a moi of 5 at 16 hpi. Total cell lysates were analyzed by 2-D gel electrophoresis. The pH gradient from 3 to 10 is acidic to basic. Western blots of (A) mock infected sample and (B) pH1N1/471 virus-infected sample. The position of the PA specific spot and PA related spots from the virus-infected sample are indicated by arrows. Non-specific spots present in both mock infection and virus infection are indicated by circles.

Figure 3.36. Two-dimensional electrophoresis combined with Western blotting using mAb-PA(2E2). Samples were mock-infected or infected MDCK cells with pH1N1/471 virus using a moi of 5 at 16 hpi. Total cell lysates were analyzed by 2-D gel electrophoresis. The pH gradient from 3 to 10 is acidic to basic. Western blots of (A) mock infected sample and (B) pH1N1/471 virus-infected sample. The position of the PA specific spot and PA related spots from the virus-infected sample are indicated by an arrow.

The isoelectric point (pI) is defined as the pH where the net charge of a protein molecule is neutral, indicating stability of the protein in that particular pI value. Previous study has analyzed the physicochemical properties of all seven proteins of influenza H1N1 virus including their isoelectric points (Sharma and Kumar, 2010). As expected, pI value of PA protein was revealed as 5.42 in their study, consistent with the pI value of PA protein observed in our study. Moreover, the presence of several PA isoforms in infected cells with different isoelectric points, all within the acidic range, has been previously reported with 2-D gel analysis (Sanz-Ezquerro *et al.*, 1998), which also supported our results. In addition, 2-D gel coupled with mass spectrometry analysis have been widely used to identify influenza virus proteins and to detect virus-associated host proteins in infected cells (Privalsky and Penhoet, 1978; Kosai *et al.*, 2008; Ohman *et al.*, 2009; Vester *et al.*, 2010; Zou *et al.*, 2010). Using these above methods, it would be interesting to identify the nature of our PA related proteins and study their properties as our future work.

3.3. Summary

The PA monoclonal antibodies (mAbs) against pH1N1/471 strain (namely 9F5 and 2E2), and H5N2/F118 strain (namely 4D7, 6A10, 6A11, 6B11 and 7A4) were successfully developed by expressing the respective virus PA protein between aa240-716. The binding regions of these antibodies were determined by expressing nested truncations of the PA protein and determining antibody recognition by Western blotting. Using this procedure, we determined that the 9F5 bound to the PA protein between aa597-616 (within the PA C-terminal domain), while the 2E2 bound to the PA protein between aa240-255 (within the PA-linker). Moreover, we also identified that the 4D7 and 7A4 bound to the PA protein between aa417-516, while the 6A10, 6A11, 6B11

bound to the PA protein between aa317-416. The results revealed these mAbs possessed different epitope regions on their target protein.

These mAbs were also extensively characterized using various approaches including IFA and Western blotting. Interestingly, two mAbs-PA against pH1N1/471 (9F5 and 2E2) showed different staining pattern of PA protein when we examined the PA protein expression in pH1N1 virus-infected MDCK cells by IFA. 2E2 recognized PA protein in nuclear compartment only, while 9F5 recognized PA protein throughout the cells (both nuclear and cytoplasmic compartment). A similar picture was seen in fractionation analysis when we analyzed the infected cells by Western blotting. Due to their different binding regions on the target protein, they may recognize specific forms of PA protein from the infected cells. We also examined the expression profile of the PA protein in pH1N1 virus-infected MDCK cells using 9F5 and 2E2. Western blotting with 9F5 revealed the presence of full-length PA protein from 8 hpi, together with the simultaneous appearance of a 50 kDa, 37 kDa and 25 kDa PA protein species (collectively referred to as PA*). However, Western blotting with 2E2 only recognized the full-length PA protein from 8hpi. This provided evidence that PA* contains the C-terminus end of the PA protein, but does not contain the 2E2 binding region. Both full length PA and PA* were also detected in H1N1/WSN, H5N2/F118 and H9N2 virus-infected MDCK cells by 9F5, demonstrating the cross reactivity of 9F5. It also indicated that the presence of PA* was not subtype-specific. In addition to virus-infected MDCK cells, the PA* was also observed in virus-infected A549 and CEF cells, inferring that the presence of PA* was not cell type-specific. Besides, the appearance of PA protein detected in H1N1/WSN-virus infected cells at 6hpi was earlier than in pH1N1/471 virus-infected cells, consistent with faster replication characteristics of the H1N1/WSN

virus. Based on the radio-immunoassay finding, 9F5 was also found to be useful for studying PA associated proteins.

Furthermore, siRNA targeted against the PA was used to confirm that PA* was generated by PA protein expression in virus-infected cells and the result revealed that siPA treatment caused a significant reduction in the PA protein and PA* protein levels, confirming that PA* was generated by PA gene expression. Not only in virus-infected cells, PA* was also detected in cells expressing the recombinant PA protein. This suggested that the appearance of PA* is an intrinsic property of PA gene expression, and not due to a peculiarity of virus infection e.g. host-response. To identify the PA* and associated host proteins, better resolution of protein separation and mass spectrometry analysis would be valuable. In our preliminary study, both PA and PA* from virus-infected cells were successfully resolved using 2-D gel combined with Western blot analysis and their pI values were found within 5 and 6 (acidic range). Based on the radio-immunoassay finding, 9F5 was found to be useful for studying PA associated proteins.

CHAPTER 4. GENERATION AND CHARACTERIZATION OF ANTIBODIES AGAINST POLYMERASE BASIC 2 (PB2) SUBUNIT

4.1. Generation of antibodies against PB2 protein from human 2009 pandemic H1N1 (pH1N1) and low pathogenic avian (LPAI) H5N2 subtypes of influenza virus

4.1.1. Cloning

Cloning of target PB2 protein was attempted as multiple truncated constructs in addition to the full length protein. Ten constructs per each polymerase subunit (Table 4.1) were cloned into *E. coli* expression vector using LIC-technology. Expression of the cloned gene and N-terminal 6xHis tag was driven by the T7 RNA polymerase promoter of plasmid pNIC28-Bsa4. The recombinant plasmids were then transferred to *E. coli* BL21-DE3 T1R cells for analysis of the expression of the target gene.

Construct	PB2 subunits of pH1N1/471 or H5N2/F118		
	AA-start	AA-stop	Molecular weight
vc001	255	525	33 kDa
vc002	255	600	42 kDa
vc003	300	525	28 kDa
vc004	300	600	37 kDa
vc005	350	525	23 kDa
vc006	350	600	32 kDa
vc007	255	759	60 kDa
vc008	300	759	55 kDa
vc009	350	759	49 kDa
vc010	2	759	88 kDa

Table 4.1. Ten constructs per PB2 subunit (a series of truncated fragments are shaded in blue and full length sequences are shaded in yellow) generated by protein production platform (PPP).

4.1.2. Protein expression

All cloned constructs that contained inserts of expected size were screened in small-scale for both total and soluble expression, and analyzed by SDS-PAGE (Figure 4.1).

Figure 4.1. Small scale screening for total expression of ten PB2 constructs (lane 1 to 10) from (A) pH1N1 and (B) H5N2 strains. Lanes M, molecular weight markers. Expressed target protein band from each construct is indicated by asterisks.

In the case of PB2, none of the constructs could be scored as a soluble fraction although they were scored as 3 in total expression of the cell lysates. Therefore, as listed in Table 4.2, a truncated construct with the highest score from each group of PB2 subunit of pH1N1 and H5N2 strains was chosen to start with larger cultivation for denaturation and purification of protein. Schematic representation of two selected PB2 constructs for scale-up production was illustrated in parallel with their full length respective genes (Figure 4.2).

Construct	AA-start	AA-stop	Molecular weight	Total expression	Soluble expression score
PB2-pH1N1-vc007	V255	N759	60 kDa	3	0
PB2-H5N2-vc007	I255	N759	60 kDa	3	0

Table 4.2. Summarizing the selected PB2 constructs with their respective expected protein sizes and soluble expression scores (Soluble score 4-Target protein dominates the gel; 3-Target protein present and significantly stronger than background; 2-Target protein present at approximately equal strength as background; 1-Target protein present but significantly weaker than background; and 0-No visible expression).

Figure 4.2. Schematic representation of two selected PB2 constructs from pH1N1 and H5N2 strains. The black line represents the full-length gene. Note that the red lines represent insoluble constructs respectively.

4.1.3. Protein denaturation and purification

As mentioned previously, it was expected that the PB2 protein of both pH1N1 and H5N2 would form inclusion bodies in the bacteria cells, when the *E.coli* expression system was being used. As shown in Figure 4.3A & B, the small scale expression of recombinant PB2 proteins from both pH1N1 and H5N2 strains could be observed in the total expression (lane 1). After cell lysis, the target proteins at their corresponding sizes were present in the pellet (lane 2) and absent in the supernatant (lane 3). Inclusion bodies were advantageous in this case, as they were easily isolated as pellets by centrifugation. It has been shown that the recombinant proteins are also expressed very highly in inclusion bodies, and inclusion bodies are expected to be composed of 40-90% of protein of interest (Cabrita and Bottomley, 2004). Furthermore, proteins expressed in inclusion bodies are easier to purify as there is higher homogeneity of the target proteins within them (Singh and Panda, 2005). Therefore, we generated our own in-house protocol for denaturation and purification of these insoluble proteins.

Figure 4.3. Coomassie blue staining of two constructs: (A) PB2-pH1N1-vc007 and (B) PB2-H5N2-vc007. The total expressions (1:10 dilution) serve as controls (lanes 1). Induced cells are lysed and fractionated into pellet (lanes 2) and supernatant (lanes 3) fractions by centrifugation. Lanes M, molecular weight markers. Target proteins are indicated by arrows between 50 kDa and 75 kDa markers.

Firstly, we started with one of the insoluble constructs, PB2-pH1N1-vc007, to carry out a small scale protein denaturation and purification. As shown in Figure 4.4A, overexpression of recombinant PB2_{His} protein could be observed from total cell lysate (lane 1) on SDS-PAGE. After cell lysis and centrifugation, it can be seen that the target recombinant protein at about 60 kDa was only present in the pellet (lane 3), but not in the supernatant (lane 2). The inclusion body-containing pellet was then washed consecutively with buffer containing detergent Triton X-100, by centrifugation. This washing step was designed to remove bacteria cell debris and other contaminants adsorbed onto the hydrophobic patches of the inclusion bodies during processing (Clark, 1998). The resulting insoluble protein pellet changed from a creamy yellow color, before wash, to creamy white, after wash. Previous studies also showed that Triton X-100 has been successfully used not only to remove contaminants from inclusion bodies, but also to recover inclusion bodies with high purity, decrease aggregation and increase the process yield (Mosaabadi *et al.*, 2007; Dasari *et al.*, 2008).

Figure 4.4. PB2-pH1N1-vc007 construct: (A) Coomassie blue staining, and (B) Western blotting using anti-His antibody. Lanes M, molecular weight markers; lanes 1, total cell lysates; lanes 2, supernatants after cell lysis; lanes 3, pellets after cell lysis; lanes 4, supernatants after 1st wash with Triton-X 100; lanes 5, pellets after 1st wash with Triton-X 100; lanes 6, supernatants after 2nd wash with Triton-X 100; and lanes 7, pellets after 2nd wash with Triton-X 100. pH1N1-PB2_{His} protein is indicated by arrows at about 60 kDa.

From the Coomassie analysis of lysates before wash and after wash with Triton X-100 (Figure 4.4A), it can be seen that the lysate after Triton wash (lane 5 & 7) had thinner and less well-defined bands than the lysate before Triton wash (lane 3), indicating that washing steps helped to increase the purity of the lysate. In addition, the supernatant after Triton wash (lane 4 & 6) did not contain the protein of interest which showed that the protein could not be solubilized in a buffer containing 0.5% Triton X-100. Western blot analysis using commercial anti-His antibody (Figure 4.4B) also confirmed that the band at about 60 kDa was the protein of interest (PB2_{His}). Besides, smaller-sized products recognized by anti-His antibody could be some breakdown products containing N-terminal His tag caused by the proteolytic degradation.

After washing, 6 M Guanidine HCl was chosen to solubilize the inclusion bodies. As shown in Figure 4.5, larger amount of denatured protein at its corresponding size could be observed from the clarified sample by centrifugation (lane 1) whereas no target protein was found in the pellet (lane 2). It indicated that the target recombinant proteins were successfully and completely solubilized in Guanidine HCl. Moreover, as seen in lane 3, only a trace amount of denatured proteins was being left in the flow

through after resin binding step showing that Guanidine HCl did not affect protein binding to the resin and the amount of resin used could be also sufficiently bind to almost all of the proteins from the clarified sample.

Figure 4.5. Coomassie blue staining of PB2-pH1N1-vc007 construct after denaturation in 6 M Guanidine HCl. Lanes M, molecular weight markers; lane 1, clarified supernatant after denaturation; lane 2, pellet after denaturation; lane 3, flow through after resin binding. pH1N1-PB2_{His} protein is indicated by arrow at about 60 kDa.

It is generally accepted that Guanidine HCl is a stronger denaturant than urea (Cabrita and Bottomley, 2004). However, due to its high salt content, protein samples containing high Guanidine concentrations form precipitates when loaded on SDS-PAGE, resulting in poor running of the sample. It would therefore be ideal for the samples in Guanidine HCl to be dialyzed overnight in urea buffer, to remove the Guanidine HCl from the samples, and yet preserve the proteins in their denatured state.

After protein denaturation, the resin with bound protein was washed once with wash buffer containing 8 M urea. It was known that low concentration of imidazole can also be included in the wash buffer to reduce the number of proteins that bind nonspecifically to the resin (Bornhorst and Falke, 2000). In our study, imidazole was not added to the wash buffer, as it appeared to be eluting the bound proteins in addition to washing off the unbound and weakly bound proteins. As shown in Figure 4.6, only a small amount of protein was washed off in the wash buffer without imidazole (lane W)

and the proteins of interest were able to be successfully eluted as small volume fractions (lane 1 to 15) in elution buffer containing 8 M urea, 200 mM imidazole, and 0.5% Nonidet P-40). Under this elution condition, we observed that the majority of the polyhistidine-tagged protein was recovered in the first three fractions (lane 1, 2 and 3) while no significant amount of protein was being bound onto the resin (lane R). In our study, EDTA was not used in elution buffer as it was found to be incompatible with TALON resin and it could remove the bound cobalt ions from the resin, causing the eluted protein to precipitate.

Figure 4.6. Coomassie blue staining of PB2-pH1N1-vc007 construct after elution as fractions. Lanes M, molecular weight markers; lane W, flow through wash buffer; lane 1 to 15, small volume elution fractions in elution buffer; lane R, resin after elution. pH1N1-PB2_{His} protein is indicated by arrows at about 60 kDa.

All eluted fractions were pooled together and protein was refolded using the refolding buffer (TN buffer including 0.5 M L-Arginine). The refolded protein was then dialyzed and concentrated. The concentrated protein was then analyzed on SDS-PAGE (Appendix 7), and the protein concentration was estimated by ImageJ program. Finally, the purified and concentrated PB2 protein was used as an antigen for mice immunization.

4.1.4. Mice immunization and antibody responses

Western blotting was performed to examine the antibody responses of different mice sera against target protein (pH1N1-purified PB2_{His} protein) with pre-immune sera and post-immune sera collected at the respective days. As shown in Figure 4.7, no signal was detected by pre-immune mice sera collected at Day 1 (1st column). However, signals containing the target protein band at about 60 kDa were produced by post-immune mice sera collected at Day 25 and Day 46 (2nd and 3rd columns) indicating that mice immunization procedure by using purified PB2 protein as an antigen was successful with detectable antibody responses. Noticeably, increased intensity of signals could be picked up from the 2nd bleed sera and the level of antibody responses was found to be similar in all mice.

Figure 4.7. Western blot analysis of the mice antibody responses. Equal amount of samples containing purified pH1N1-PB2_{His} antigen were loaded into each lane (Total 16 lanes). First, second and third columns were probed with different immune sera of five mice (labelled as 1st, 2nd, 3rd, 4th and 5th) collected at Day 1, Day 25 and Day 46 respectively. Last lane (labelled as C) probed with commercial anti-His antibody was used as positive control. PB2_{His} protein is indicated by arrow at about 60 kDa and highlighted in red box.

4.1.5. Hybridoma production

Splenocytes from one of the immunized mice were fused with myeloma cells to produce antibody secreting hybridoma cell lines. 10 days after the fusion, growing hybridoma could be observed around 50-60% in 96-wells and they were ready to screen by ELISA for specific monoclonal antibody production as well as to rule out the negative clones.

Based on the ELISA screening results (Figure 4.8), we obtained a total of 15 hybridoma cell lines which recognized the viral immunogens and secreted antibodies against PB2(pH1N1) protein. Among these potentially positive hybridoma clones, 4G3 which showed the highest affinity to the target protein in ELISA was chosen for subcloning by limiting dilution and the ELISA-positive subclone was successfully propagated into larger scale. It was then further characterized by IFA and Western blotting. In the later part of results and discussion, the term of the monoclonal antibody against PB2 protein has been shorten as mAb-PB2.

Figure 4.8. ELISA screening results of pH1N1-PB2 hybridoma clones. A clone with the highest affinity to the target PB2 protein is highlighted by red circle.

4.2. Characterization of antibody against PB2 protein from human 2009 pandemic H1N1 (pH1N1) subtypes of influenza virus

4.2.1. Transfection studies

4.2.1.1. IFA and Western blot analysis in transfected cells

We firstly characterized the specificity of our mAb-PB2(4G3) in transfected cells by IFA. After 24 hpt, 293T cells with mock transfection and FLAG-tagged full length pH1N1/471-PB2 gene transfection were stained with mAb-PB2(4G3) and anti-FLAG. Cells were then viewed under a fluorescence microscope after staining. As shown in Figure 4.9A, 4G3 gave positive staining in the nuclei of FLAG-tagged PB2 transected cells, indicating its recognition of the mammalian expressed PB2 protein. Moreover, we could also observe positive nuclear staining when we co-stained with anti-FLAG, reflecting that FLAG-tagged PB2 protein is well-expressed in 293T cells with nearly 90% transfection efficiency. However, no positive signal was detected in the mock transfection when stained with the above antibodies (Figure 4.9B). It signifies that the generated mAb-PB2(4G3) specifically recognizes the PB2 protein, but not host cell proteins.

Figure 4.9. Characterization of mAb against pH1N1-PB2 reactivity in 293T transfected cells at 24 hpt by IFA. (A) FLAG-tagged full length PB2 gene transfection, and (B) Mock transfection. Transfected cells were stained with mAb-PB2(4G3), and co-stained with anti-FLAG. Cells were then stained with anti-mouse Alexa 488 (green) and anti-rabbit Alexa 555 (red).

Western blot analysis of 293T cell lysates prepared from mock transfection and FLAG-tagged full length pH1N1/471-PB2 gene transfection was carried out after 24 hpt

(Figure 4.10). No signals were observed in the mock transfection, but a protein product corresponding to the size of full length PB2 protein at about 85 kDa (PB2) was detected by mAb-PB2(4G3) in FLAG-tagged full length PB2 gene transfection. Together with target PB2 protein, two additional smaller protein products at about 40 kDa and 25 kDa (collectively referred to as PB2*) were also simultaneously detected by 4G3. Interestingly, a similar picture was observed with Western blotting of cells expressing the recombinant PB2 protein with a C-terminal FLAG epitope with anti-FLAG, suggesting that PB2* contains the C-terminus end of the PB2 protein.

Figure 4.10. Characterization of mAb against pH1N1-PB2 reactivity in 293T transfected cells at 24 hpt by Western blot analyses. M represents mock transfection and T represents FLAG-tagged full length PB2 gene transfection. Western blotting using (A) mAb-PB2(4G3), and (B) anti-FLAG. The FLAG-tagged full length PB2 protein at about 85 kDa (PB2), and the additional smaller products at about 40 kDa and 25 kDa (*) are indicated.

4.2.2. Infection studies

4.2.2.1. IFA and Western blot analysis in infected cells

Further characterization on the mAb-PB2(4G3) was carried out by IFA and Western blot analyses to identify the specific viral protein from virus infected MDCK cells. The IFA images of infected MDCK cells using a moi of 5 at specific post infection time points were shown in Figure 4.11.

Figure 4.11. Characterization of mAb against pH1N1-PB2 reactivity in pH1N1/471 virus infected MDCK cells at specific time points post-infection by IFA. Mock infected and pH1N1/471 virus-infected cells were stained with either anti-NP or mAb-PB2(4G3), and co-stained with anti-Histone H4 to show cell nuclei. Cells were then stained with anti-mouse Alexa 488 (green) and anti-rabbit Alexa 555 (red). N: nuclear localization, N+C: both nuclear and cytoplasmic localization.

We firstly studied the replication kinetics of A/Singapore/471/2009 human pandemic H1N1 virus in MDCK cells based on the detection and localization of the PB2 protein in parallel with the NP protein by IFA at different time-points. From this experiment, appearance of NP could be observed by earlier nuclear localization at 6 hpi. Subsequently, more cells had nuclear localization detected with minimal export at 8 hpi and 10 hpi. Then, extensive nuclear export of the NP protein was observed by 16 hpi (Figure 4.11), indicating virus assembly and budding by this time point.

Likewise in PA, the rate of PB2 signal development is slower than NP signal. The appearance of PB2 protein in infected cells was observed only from 8 hpi onward by mAb-PB2(4G3) and the signal was mainly localized in the nucleus with increasing intensity of the signal at later time points. Figure 4.12 shows the confocal images indicating nuclear labelling of PB2 protein, together with Histone H4 (nuclear marker), on MDCK cells infected with pH1N1/471 virus at 16 hpi. It is expected to observe the signal of PB2 mainly in the nucleus since previous studies have demonstrated the nuclear accumulation of three polymerase subunits in the nucleus of infected cells (Jones *et al.*, 1986; Akkina *et al.*, 1987; Mukaigawa and Nayak, 1991).

Figure 4.12. Confocal images of pH1N1/471 virus infected MDCK cells at 16 hpi. Infected cells stained with mAb-PB2(4G3) and co-stained with anti-Histone H4 to show cell nuclei. Cells were then stained with anti-mouse Alexa 488 (green) and anti-rabbit Alexa 555 (red). The patterns of protein distribution were visualized by confocal microscopy. N: nuclear localization.

Similar pictures were observed when we examined the replication kinetics by Western blot analyses using our novel mAb-PB2(4G3) in parallel with the commercially

available anti-NP antibody. NP protein could be detected earlier from 6 hpi onward (Figure 4.13A), while PB2 protein was detectable only from 8 hpi onward (Figure 4.13B).

Figure 4.13. Characterization of mAb against pH1N1-PB2 reactivity in pH1N1/471 virus infected MDCK cells at specific post-infection time points by Western blot analyses using (A) anti-NP and (B) mAb-PB2(4G3). The full length NP and PB2 proteins at about 56 kDa (NP) and 85 kDa (PB2) are indicated respectively. The additional smaller products at about 40 kDa and 25 kDa (*) are indicated. Loading control (β -actin) is also shown.

In addition, time dependent expression patterns of the smaller products (PB2*) were also observed together with our target PB2 protein. This suggests that these smaller proteins must have relationships with the major protein. The identification of PB2* in our study could be due to alternative reading frame or proteolytic processing. Although the study of additional PB2 product has not been previously reported yet, the identified smaller products from our study possibly interact with the major protein which has been observed in the nucleus of infected cells. Hence, these small products may play a significant role in the viral transcription and replication which take place in the nucleus of the cells.

4.2.3. Epitope mapping

To identify the binding region of mAb-PB2(4G3) on the target protein, epitope mapping was subsequently performed by serial expression of the truncated fragments

1st Step

Figure 4.14. 1st Step: A series of nested deletions of the PB2 region was created by 100aa deletions from the C-terminal domain. The amino acid position and the location of the N-terminal 6xHis-tag are indicated (top). Western blot analyses (bottom) using (A) anti-His (loading control) and (B) mAb-PB2(4G3). Serial truncated protein products are indicated by asterisks on top of each protein band. The mAb-PB2(4G3) binding region is found to be between aa659-759.

2nd Step

Figure 4.15. 2nd Step: A further series of nested deletions of PB2 region was created by 20aa deletions from the C-terminus of truncation 1. The amino acid position and the location of the N-terminal 6xHis-tag are indicated (top). Western blot analyses (bottom) using (A) anti-His (loading control) and (B) mAb-PB2(4G3). Serial truncated protein products are indicated by asterisks on top of each protein band. The mAb-PB2(4G3) binding region was found to be between aa720-739.

of the target PB2 protein using two steps and antibody recognition was determined by Western blotting (Figure 4.14 and 4.15). Finally, the mAb-PB2(4G3) binding region was determined to be between aa720-739 (Figure 4.16), which is within the NLS region and determining amino acid sequence was indicated in Table 4.3.

Final Step

Figure 4.16. A schematic showing the position of binding region of mAb-PB2(4G3) on PB2 protein construct.

Anti-PB2 (H1)	Antibody recognition region
4G3	Amino acid position 720-739 : EKANVLIGQGDVVLVM <u>KRKR</u> (Total – 20 aa)

Table 4.3. Identified amino acid positions and sequence recognized by mAb-PB2(4G3). The underlined nucleotides represents the linear NLS sequence present on the C-terminal domain of PB2.

The C-terminal domain of PB2 protein contains a classic bipartite nuclear localization sequence (NLS) and a cap-binding domain (Blaas *et al.*, 1982; Mukaigawa and Nayak, 1991). Moreover, four basic residues (⁷³⁶KRKR⁷³⁹) at the end of the identified binding region of mAb-PB2(4G3) has been shown to have an important role in the nuclear translocation of PB2 and this linear NLS sequence was also found to interact with α -importin in a crystal structure of PB2 C-terminal domain (Mukaigawa and Nayak, 1991; Tarendeau *et al.*, 2007). The interaction between the NLS and α/β -importin pathway mediates nuclear transport of the PB2 independently of the other polymerase proteins (Gabriel *et al.*, 2008; Gabriel *et al.*, 2011). Based on the above findings, the mAb-PB2(4G3) generated against the pH1N1/471 strain was confirmed to

interact with the C-terminus of the PB2 subunit. The C-terminal NLS, which is responsible for the localization of the protein into the nucleus of host cells, explains the observation of the nuclear staining pattern of PB2 protein detected by mAb-PB2(4G3) in the virus infected cells (referred to Section 4.2.2).

Figure 4.17. PB2 protein sequence alignment. The 20 amino acid binding region (aa720-739) recognized by mAb-PB2(4G3) was aligned with the PB2 sequences of other influenza strains (H1N1/WSN, H5N2/F118, and H9N2) against pH1N1/471. Protein residues that match pH1N1/471-PB2 exactly are hidden (as '.').

Moreover, when the identified 20 amino acid binding region was aligned with the PB2 sequence of other influenza strains (H1N1/WSN, H5N2/F118, and H9N2) against pH1N1/471, it was found to be conserved among these strains (Figure 4.17). This suggested that the mAb-PB2(4G3) most likely interact with three other strains used in our study and potentially be suitable to study the PB2 protein of these strains. In addition, the generation of specific antibodies against H5N2-PB2 protein would be omitted if this antibody had been found to cross-react with H5N2-PB2 protein.

4.2.4. Specificity testing across various strains

Next, the specificity of the mAb-PB2(4G3) against pH1N1/471 PB2 protein was also tested across other strains (H1N1/WSN, H5N2/F118 and H9N2) used in our study by IFA and Western blotting. As shown in the IFA results, mAb-PB2(4G3) reacted equally with PB2 protein from all tested strains (Figure 4.18A, B and C). In this experiment, anti-NP antibody was used as a control to ensure that the virus infection which occurred successfully in MDCK cells and access the level of infection at 16 hpi. Most of the NP staining was observed throughout the infected cells at this time point.

However, PB2 staining was prominently found in the nuclei of infected cells at the same time point from all three strains.

Figure 4.18. Characterization on the specificity of mAb against pH1N1-PB2 in the various strains of virus infected MDCK cells at 16 hpi by IFA. (A) H1N1/WSN, (B) H5N2/F118, and (C) H9N2 virus infected cells stained with either anti-NP (control for infection) or mAb-PB2(4G3). Cells were then stained with anti-mouse Alexa 488 (green). N: nuclear localization; N+C: both nuclear and cytoplasmic localization.

In Western blot analyses, mAb-PB2(4G3) could also detect the full length PB2 protein in the MDCK cells infected with three other strains such as H1N1/WSN, H5N2/F118 and H9N2 (Figure 4.19A, B and C). Therefore, it is confirmed that 4G3 has cross reactivity with other strains tested in our study. As seen in pH1N1 virus, we noted the appearance of the full length PB2 protein from 8 hpi, together with the simultaneous appearance of two smaller protein products (PB2*) in H5N2/F118 and H9N2 virus infection (Figure 4.17B and C). However, the full length PB2 protein was detected as early as 6 hpi, together with PB2* in H1N1/WSN virus infection (Figure 4.19A). This was earlier than we observed in pH1N1/471 virus-infected cells, consistent with faster

replication characteristics of the H1N1/WSN virus (Sutejo *et al.*, 2012). This finding was also supported by previous study in which the initial detection of PB2 at the nucleus of H1N1/WSN virus-infected cells was noted from 3 hpi in their time-course experiment (Carr *et al.*, 2006). Moreover, the presence of PB2* in all strains tested indicated that PB2* expression was not subtype-specific. However, there were very strong PB2* bands (particularly the 25 kDa band) in the H9N2 virus infection. Possible explanations are a stronger proteolytic activity associated with sequence variations in the particular subtype and defective interfering particles. Further investigations such as amino acid sequence analysis would be required to better understand these smaller products,

Figure 4.19. Characterization on the specificity of mAb against pH1N1-PB2 in the various strains of virus infected MDCK cells at specific post infection time points by Western blot analyses using mAb-PB2(4G3). (A) H1N1/WSN virus infection, (B) H5N2/F118 virus infection, and (C) H9N2 virus infection. The full length PB2 protein at about 85 kDa (PB2), and the additional smaller products at about 40 kD and 25 kDa (*) are indicated. Loading control (β-actin) is also shown.

4.2.5. Detection of PB2 protein in various infected cells

As mentioned in Section (3.2.5) from previous chapter, we have noted that PA* expression was not cell type-specific. Therefore, we also wanted to examine the presence of PB2* in A549 and CEF cells infected with pH1N1/471 and H1N1/WSN viruses at a moi of 5. After 16 hpi, mock infected and virus-infected samples were then analyzed by Western blotting using mAb-PB2(4G3).

Figure 4.20. PB2 protein detection in various infected cells by Western blot analyses. (A) MDCK, (B) A549 and (C) CEF cells were mock-infected (M), infected with pH1N1/471 (pH1) and H1N1/WSN (WSN) using a moi of 5 at 16 hpi. Cells were then examined by Western blotting using mAb-PB2(4G3). The full length PB2 protein at about 85 kDa (PB2), and the additional smaller products at about 40 kDa and 25 kDa (*) are indicated. Loading control (β-actin) is also shown.

As shown in Figure 4.20, both full length PB2 and PB2* could be detected by 4G3 in all three cell lines infected with both viruses, while a lower level of PB2* at 25 kDa was noted in H1N1/WSN virus infection. This indicated that the expression of PB2* was also not cell type-specific. However, their significance and variations among different viruses have not been well-characterized yet.

For all subsequent analyses, the mAb-PB2(4G3) was used to study the target PB2 protein due to its above mentioned properties.

4.3. Summary

The PB2 monoclonal antibody mAb-PB2(4G3) was prepared by expressing the pH1N1/471 virus PB2 protein between aa255-759, and its binding region was determined to be between aa720-739 which is within the NLS region. Using IFA and Western blotting, mAb-PB2(4G3) was characterized. The specificity of the 4G3 was firstly determined in 293T transfected cells by IFA and the result revealed that 4G3 could specifically recognize the target PB2 protein. In addition to the expression of full length PB2 with FLAG epitope at the C-terminus, the concomitant appearance of smaller truncated species corresponding in size to 40 kDa and 25 kDa (PB2*) were detected by both 4G3 and anti-FLAG respectively in Western blot analyses. This suggested that PB2* contains the C-terminal end of the PB2 protein and the NLS region.

Moreover, the appearance of PB2 protein in pH1N1/471 infected MDCK cells detected by 4G3 was from 8 hpi (later than in H1N1/WSN virus-infected cells which produce the PB2 at 6 hpi), and the PB2 signal was found to be mainly localized in the nucleus. The time appearance of the PB2 protein determined by Western blotting was consistent with a similar analysis using IFA. Furthermore, mAb-PB2(4G3) also showed the full-length PB2 protein and PB2* from cells expressing the PB2 protein using H1N1/WSN, H5N2/F118 and H9N2 viruses, indicating its cross reactivity. Additionally, PB2* that we noted in virus-infected MDCK cells was also produced in virus-infected A549 and CEF cells. Similar to PA*, PB2* was also found to be neither subtype-specific nor cell type-specific.

CHAPTER 5. GENERATION AND CHARACTERIZATION OF ANTIBODIES AGAINST POLYMERASE BASIC 1 (PB1) SUBUNIT

5.1. Generation of antibodies against PB1 protein from human 2009 pandemic H1N1 (pH1N1) and low pathogenic avian (LPAI) H5N2 subtypes of influenza virus

5.1.1. Cloning

Cloning of target PB1 protein was attempted as multiple truncated constructs in addition to the full length protein. Ten constructs per each polymerase subunit (Table 5.1) were cloned into *E. coli* expression vector using LIC-technology. Expression of the cloned gene and N-terminal 6xHis tag was driven by T7 RNA polymerase promoter of plasmid pNIC28-Bsa4. The recombinant plasmids were then transferred to *E. coli* BL21-DE3 T1R cells for analysis of the expression of the target gene.

	PB1 subunits of pH1N1/471 or H5N2/F118		
Construct	AA-Start	AA-stop	Molecular weight
vc001	255	525	33 kDa
vc002	255	600	42 kDa
vc003	300	525	28 kDa
vc004	300	600	37 kDa
vc005	350	525	23 kDa
vc006	350	600	32 kDa
vc007	255	757	60 kDa
vc008	300	757	55 kDa
vc009	350	757	49 kDa
vc010	2	757	88 kDa

Table 5.1. Ten constructs per PB1 subunit (a series of truncated fragments are shaded in blue and full length sequences are shaded in yellow) generated by protein production platform (PPP).

5.1.2. Protein expression

All cloned constructs that contained inserts of expected size were screened in small-scale for both total and soluble expression, and analyzed by SDS-PAGE (Figure 5.1).

Figure 5.1. Small scale screening for total expression of ten PB1 constructs (lane 1 to 10) from (A) pH1N1 and (B) H5N2 strains. Lanes M, molecular weight markers. Expressed target protein band from each construct is indicated by asterisks.

After induction with IPTG, the expression levels of total and soluble protein were tested and scored on a subjective scale from 0 to 4. Similar to PB2, none of the PB1 constructs could be scored as a soluble fraction although they were scored as 3 in total expression of the cell lysates. The choice of the N- and C-terminal boundaries plays an important role in expression of a protein domain because even small differences can dramatically influence both solubility and expression (Graslund *et al.*, 2008). Previous studies suggested that expression of the full-length PB1 and PB2 subunits could not yield a soluble form (Boivin *et al.*, 2010). Hence, as shown in Table 5.2, a truncated construct with the highest score from each group of PB1 subunit of pH1N1 and H5N2 strains was chosen to start with larger cultivation for denaturation and purification of protein. Schematic representation of two selected PB1 constructs for scale-up production was illustrated in parallel with their full length respective genes (Figure 5.2).

Construct	AA-start	AA-stop	Molecular weight	Total expression	Soluble expression score
PB1-pH1N1a-vc008	F300	K757	55 kDa	3	0
PB1-H5N2a-vc007	V255	K757	60 kDa	3	0

Table 5.2. Summarizing the selected PB1 constructs with their respective expected protein sizes and soluble expression scores (Soluble score 4-Target protein dominates the gel; 3-Target protein present and significantly stronger than background; 2-Target protein present at approximately equal strength as background; 1-Target protein present but significantly weaker than background; and 0-No visible expression).

Figure 5.2. Schematic representation of two selected PB1 constructs from pH1N1 and H5N2 strains. The black line represents the full-length gene. Note that the red lines represent insoluble constructs respectively.

5.1.3. Protein denaturation and purification

Figure 5.3. Coomassie blue staining of two constructs: (A) PB1-pH1N1-vc008 and (B) PB1-H5N2-vc007. The total expressions (1:10 dilution) serve as controls (lanes 1). Induced cells are lysed and fractionated into pellet (lanes 2) and supernatant (lanes 3) fractions by centrifugation. Lanes M, molecular weight markers. Target proteins are indicated by arrows between 50 kDa and 75 kDa markers.

Figure 5.3A & B showed the small scale expression of recombinant PB1 proteins from both pH1N1 and H5N2 strains. Although the total expression of PB1 proteins could be observed in lanes 1 before cell lysis, the target proteins at their corresponding

sizes were present only in the pellet (lanes 2) and absent in the supernatant after cell lysis (lanes 3). From these findings, we confirmed that our PB1 proteins were also insoluble and they formed inclusion bodies in the bacteria cell pellets like our PB2 proteins. Therefore, we proceeded to denaturation and purification of the PB1-pH1N1-vc008 protein using the same protocols from the PB2 protein. The protein was then refolded, dialyzed and concentrated. The concentrated protein was then analyzed on SDS-PAGE (Appendix 7), and the protein concentration was estimated by ImageJ program. Finally, the purified and concentrated PB1 protein was used for mice immunization.

5.1.4. Mice immunization and antibody responses

We started the mice immunization with purified PB1_{His} protein from pH1N1 strain according to the immunization schedule. Then, the antibody responses of mice sera against target protein were analyzed by Western blotting by using pre-immune sera collected on Day 1, and post-immune sera collected on Day 25 and 46 from all 5 mice.

Figure 5.5. Western blot analysis of the mice antibody responses. Equal amount of samples containing purified pH1N1-PB1_{His} antigen were loaded into each lane (Total 16 lanes). First, second and third columns were probed with different immune sera of five mice (labelled as 1st, 2nd, 3rd, 4th and 5th) collected at Day 1, Day 25 and Day 46 respectively. Last lane (labelled as C) probed with commercial anti-His antibody was used as positive control. PB1_{His} protein was indicated by arrow at about 55 kDa and highlighted in red box.

As shown in Figure 5.5, there was no signal detected from pre-immune mice sera (1st column), whereas signals at about 55 kDa which corresponded to the size of the target protein were able to be detected from post-immune mice sera (2nd and 3rd columns). It inferred that mice immunization was achieved by using purified PB1 protein as an antigen. However, we noted that the high signal could be picked up only from the serum of third mouse and it became more intense in the 2nd bleed. Therefore, we aimed to start the hybridoma production using the third mouse.

5.1.5. Hybridoma production

Using the same protocol from the two previous proteins, PA and PB2, we carried out the fusion of splenocytes from the third mouse with myeloma cells. Despite being expected to see growing hybridoma at Day 10 after the fusion, no hybridoma was found unfortunately even after five weeks. Again, the same result was obtained from the repeated attempt of fusion. Therefore, the fusion was considered unsuccessful in obtaining antibody producing hybridomas against PB1 protein for both attempts. Failure to obtain the hybridomas could be due to unsuitable fusion partners resulting in loss of chromosomes or repression of gene involving in growth (Gravekamp *et al.*, 1985). Hence, we decided to use anti-PB1 serum from the third mouse (polyclonal in nature) for further studies of PB1 protein.

5.2. Characterization of antibody against PB1 protein from human 2009 pandemic H1N1 (pH1N1) subtype of influenza virus

5.2.1. Transfection studies

5.2.1.1. IFA and Western blot analysis in transfected cells

Using IFA, we started our transfection study in 293T cells. Mock transfected and FLAG-tagged full length pH1N1/471-PB1 gene transfected cells were stained with anti-

PB1(mouse serum) and co-stained with anti-FLAG after 24hpt. As shown in Figure 5.6, both anti-PB1 and anti-FLAG showed the positive staining in FLAG-tagged PB1 transfection, but not in mock transfection, indicating that both antibodies specifically recognize mammalian expressed FLAG-tagged PB1 protein.

Figure 5.6. Characterization of antibody against pH1N1-PB1 reactivity in 293T transfected cells at 24 hpt by IFA. (A) FLAG-tagged full length PB1 gene transfection, and (B) Mock transfection. Transfected cells were stained with anti-PB1(serum), and co-stained with anti-FLAG. Cells were then stained with anti-mouse Alexa 488 (green) and anti-rabbit Alexa 555 (red).

Figure 5.7. Characterization of antibody against pH1N1-PB1 reactivity in 293T transfected cells at 24 hpt by Western blot analyses. M represents mock transfection and T represents FLAG-tagged full length PB1 gene transfection. Western blotting using (A) anti-PB1(serum) and (B) anti-FLAG. The FLAG-tagged full length PB1 protein at about 85 kDa (PB1) is indicated.

Next, cell lysates prepared from 24 hpt of mock transfection and FLAG-tagged full length pH1N1/471-PB1 gene transfection were analyzed by Western blotting using anti-PB1 and anti-FLAG (Figure 5.7). No signal was observed in the mock transfection,

while a single protein product at about 85 kDa corresponding to the size of full length PB1 protein was detected by both anti-PB1 and anti-FLAG in the FLAG-tagged PB1 transfection. In addition, one thing that we noted in both IFA and Western blot analyses was the high background, likely due to the polyclonal nature of anti-PB1 mouse serum.

5.2.2. Infection studies

Further characterization was carried out with infected cells. To study the viral replication kinetic with PB1 protein, MDCK cells were mock infected and infected with pH1N1/471 virus using a moi of 5. At specific post-infection time points, infected cells were analyzed by IFA and Western blotting using anti-PB1(serum). Figure 5.8 showed the IFA images of virus-infected MDCK cells using a moi of 5 at specific post-infection time points. In this experiment, the PB1 protein at different time-points was studied by IFA using anti-PB1(serum). As we have seen previously, the NP protein was observed by earlier nuclear localization at 6 hpi and more cells with minimal export from 8 to 10 hpi. Subsequently, widespread NP protein throughout the cell was observed by 16 hpi. However, the initial appearance of PB1 protein in the nuclei of infected cells was observed only at 8 hpi and the signal was found to spread throughout the cells at 16 hpi.

In the Western blot analysis, anti-PB1 serum detected only a single protein product (at about 85 kDa) from the infected cells (Figure 5.9) and the product's size corresponded to the expected size of the PB1 protein. Similar to IFA, the first protein signal was detected from the sample harvested at 8 hpi and the intensity of the signal became increased at the later time points. Unlike the Western blot pictures seen in time-point infections with either mAb-PA(9F5) or mAb-PB2(4G3), we did not observe any additional smaller product with anti-PB1 serum (except the high background given by mouse serum).

Figure 5.8. Characterization of antibody against pH1N1-PB1 reactivity in pH1N1/471 virus infected MDCK cells at specific time points post-infection by IFA. Mock infected and pH1N1/471 virus-infected cells were stained with anti-PB1 (serum), and co-stained with anti-Histone H4 to show cell nuclei. Cells were then stained with anti-mouse Alexa 488 (green) and anti-rabbit Alexa 555 (red). N: nuclear localization, N+C: both nuclear and cytoplasmic localization.

Figure 5.9. Characterization of antibody against pH1N1-PB1 reactivity in pH1N1/471 virus infected MDCK cells at specific post-infection time points by Western blot analyses using anti-PB1(serum). The full length PB1 proteins at about 85 kDa (PB1) is indicated. Loading control (β -actin) is also shown.

As mentioned early, the antibody that we used in our study for the detection of PB1 protein was from mouse serum (polyclonal in nature). Therefore, we decided to omit two tests (epitope mapping and specificity testing across various strains) from the characterization processes of anti-PB1 antibody.

5.2.3. Detection of PB1 protein in various infected cell lines

Figure 5.10. PB1 protein detection in various infected cells by Western blot analyses. (A) MDCK, (B) A549 and (C) CEF cells were mock-infected (M), infected with pH1N1/471 (pH1) and H1N1/WSN (WSN) using a moi of 5 at 16 hpi. Cells were then examined by Western blotting using anti-PB1(serum). The full length PB1 protein at about 85 kDa (PB1) is indicated. Loading control (β -actin) is also shown.

After studying the replication kinetics of pH1N1/471 with the vial PB1 protein using anti-PB1(serum), we also examined this antibody with different cell lines infected with two viruses (pH1N1/471 and H1N1/WSN) by Western blotting. In addition to MDCK cells, the single target PB1 protein was also detected by anti-PB1(serum) in both viruses-infected A549 and CEF cells (Figure 5.10). Thus, we decided to use this polyclonal antibody in our further analyses.

5.3. Summary

In this study, the anti-PB1 is a mouse immune serum prepared using bacterial expressed truncated pH1N1/471 virus PB1 protein between aa300-757. Using IFA, the recombinant PB1 protein was able to detect by anti-PB1(serum) in 293T transfected cells. Western blotting with recombinant protein (expressing the pH1N1/471 PB1 protein with a C-terminal FLAG epitope) using anti-PB1(serum) and anti-FLAG respectively showed a single protein product corresponding to the size of the full length PB1-FLAG protein.

Similar to the time appearance of PA and PB2, the appearance of PB1 protein in pH1N1/471 infected MDCK cells detected by anti-PB1(serum) was from 8 hpi in both IFA and Western blot analysis. Although anti-PB1(serum) was polyclonal in nature, it recognized the PB1 protein from infected cells as a single product, suggesting that it was able to detect the full length target protein only. Additionally, it could detect the PB1 not only in pH1N1/471 and H1N1/WSN infected MDCK cells, but also in the same viruses-infected A549 and CEF cells, indicating neither subtype nor cell type-specific.

CHAPTER 6. CHARACTERIZATION OF POLYMERASE SUBUNITS FROM PURIFIED INFLUENZA A VIRUS PARTICLES

6.1. Virus purification gradient profile

To further confirm the specificity of the mAbs, a proteomics approach was used to analyze the target protein from purified virus particles. Virus particles were purified by sucrose density gradient centrifugation as described in the materials and methods section. Initially, virions of pH1N1/471 strain were used for purification and an opalescent band containing majority of mature virus particles was observed at 35% (w/v) sucrose as shown in Figure 6.1A.

Figure 6.1. (A) Virus purification by sucrose density gradient centrifugation and observation of an opalescent band at 35% (w/v) sucrose. (B) Fractions taken from each sucrose concentration was resolved by SDS-PAGE and viewed by Coomassie blue staining. Control mock from non-infected allantoic fluid and purified virions concentrated from 35% (w/v) sucrose were loaded in lane M and V respectively. NP protein band at ~56 kDa and M1 protein band at ~28 kDa were observed in both lane V and 35% sucrose.

For sample analysis, fractions collected from each sucrose concentration were resolved by SDS-PAGE and viewed by Coomassie blue staining (Figure 6.1B). Meantime, purified virions concentrated from 35% (w/v) sucrose was also analyzed with control mock from non-infected allantoic fluid in the same gel. Two abundant proteins of influenza virus, NP protein at ~56 kDa and M1 protein at ~28 kDa, were observed in the fraction taken from 35% (w/v) sucrose gradient. Likewise, more intense

NP and M1 protein bands were also detected in the purified virions concentrated from 35% (w/v) sucrose gradient.

6.2. Detection of polymerase subunits (PA, PB2, and PB1) and NP from purified viruses

6.2.1. PA protein

First, distribution of the PA protein was tested with the samples harvested from each sucrose concentration by Western blot analysis using mAb-PA(9F5). As shown in Figure 6.2, target viral PA protein at about 83 kDa was detected in 35% (w/v) sucrose concentration. Moreover, additional smaller products (PA*) were also detected in the same concentration. It implies that these smaller products were co-purified with the target PA protein from virions at the same sucrose concentration.

Figure 6.2. Western blot analysis of PA protein from virions of pH1N1/471 strain purified from sucrose density gradients (15% to 60% (w/v)) using mAb-PA(9F5). Control mock from non-infected allantoic fluid was shown in lane M. The full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) at 35% (w/v) sucrose concentration are indicated.

Then, mAbs against pH1N1-PA were tested after concentration of purified virions pH1N1/471 strain from 35% (w/v) sucrose concentration. As shown in Figure 6.3A and B, both 9F5 and 2E2 detected the target PA protein in purified concentrated virus particles. The PA protein was not detected in a similar gradient fraction that was

prepared using non-infected allantoic fluid, thus confirming the specificity of these mAbs. In addition, smaller products (PA*) were also detected by 9F5 in purified virus sample in addition to transfected and infected samples shown in previous sections. This indicated that they were PA-related and co-existed with their major protein in the virus particles.

Figure 6.3. Western blot analyses of the PA protein from purified concentrated virions of pH1N1/471 strain using (A) mAb-PA(9F5) and (B) mAb-PA(2E2). Purified concentrated virions were loaded in lane V-pH1 and control mock from non-infected allantoic fluid was loaded in lane M. The full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) are indicated.

These smaller products had been observed throughout our characterization processes. It indicated that these additional novel protein products must be related to our protein of interest although the nature of these proteins had remained unclear. Similar findings of truncated additional PA-related proteins (62 kDa and 60 kDa) have been identified by Muramoto and his group (Muramoto *et al.*, 2013). This concept was supported in early reports by Akkina *et al.* (Akkina, 1990; Akkina *et al.*, 1991) that various influenza A virus strains (i.e., all 18 strains belonging to H1 to H13) expressed shorter PA-related polypeptides.

As we have seen in Section 3.2, mAb-PA(9F5) was able to recognize the PA protein from H1N1/WSN virus-infected cells. To identify its cross-reactivity on viral PA protein of WSN strain, we also tested it with purified concentrated virions of

H1N1/WSN strain in parallel with pH1N1/471 strain by Western blot analysis. Figure 6.4 showed that 9F5 well-recognized target viral PA protein as well as PA* from both strains, confirming its ability of cross-reactivity. Hence, we would also use it to study the PA protein in our further experiments which related purified WSN virions.

Figure 6.4. Western blot analysis of the PA protein from purified concentrated virions with mAb-PA(9F5). Purified concentrated virions of pH1N1/471 and H1N1/WSN strains were loaded in lane V-pH1 and V-WSN respectively. Control mock from non-infected allantoic fluid was loaded in lane M. The full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) are indicated.

6.2.2. PB2 protein

Next, the same purified concentrated virions of pH1N1/471 and H1N1/WSN strains from the above experiment were also used to analyse the viral PB2 protein by our mAb-PB2(4G3).

Figure 6.5. Western blot analysis of the PB2 protein from purified concentrated virions with mAb-PB2(4G3). Purified concentrated virions of pH1N1/471 and H1N1/WSN strains were loaded in lane V-pH1 and V-WSN respectively. Control mock from non-infected allantoic fluid was loaded in lane M. The full length PB2 protein at about 85 kDa (PB2) is indicated.

As shown in Figure 6.5, 4G3 could detect the target viral PB2 protein band at about 85 kDa in the virus particles of both strains, and the intensity of the bands appeared to be low. Unlike the recombinant plasmid transfected and virus-infected samples as shown in Section 4.2, PB2* were not seen in the virus samples. One possible reason could be due to the low abundance of PB2 and its related products in infectious virus particles. Another possible reason of being unable to detect the PB2* may be playing roles during the replication phases rather than being incorporated into mature virus particles. It is also possible that PB2 protein itself in the infectious virus particles might not have isoforms.

6.2.3. PB1 protein

After detection of PA and PB2 proteins from purified virions using our novel mAbs-PA and mAb-PB2 respectively, we used the same virus samples from both strains and performed Western blotting with anti-PB1 mouse serum (polyclonal) for the detection of PB1 protein.

Figure 6.6. Western blot analysis of the PB1 protein from purified concentrated virions with anti-PB1 mouse serum. Purified concentrated virions of pH1N1/471 and H1N1/WSN strains were loaded in lane V-pH1 and V-WSN respectively. Control mock from non-infected allantoic fluid was loaded in lane M. The full length PB2 protein at about 85 kDa (PB1) is indicated.

As expected, there was a high background given by the nature of mouse serum. A single particular faint band at approximately 85 kDa was detected in both virus

samples (Figure 6.6), but not in the mock. We assumed that these particular faint bands reflected PB1 protein as they corresponded to the size of PB1 protein (~85 kDa) and their exclusive presence in the virus samples.

According to the above PB1 and PB2 findings, low intensity of the protein bands was not due to the poor binding affinities of the antibodies. These antibodies had been tested in the transfected samples and their affinities were proved to be strong as shown in previous Sections 4.2 and 5.2. The RNA polymerase complex have been found in low copy numbers in virions compared to that of NP (Fodor and Brownlee, 2002). Therefore, the compositions of both PB1 and PB2 proteins in this study was also found to be present at low levels in the infectious virus particles.

6.2.4. NP protein

Next, it has been shown that NP is one of the abundant proteins in virus particles (Fodor and Brownlee, 2002) and plays several functions in the viral infectious cycle (Biswas *et al.*, 1998). Therefore, we also identified the NP protein in our purified concentrated virus samples using a commercially available anti-NP. Figure 6.7 showed a very intense band corresponded to the size of NP protein (~56 kDa) from each sample.

Figure 6.7. Western blot analysis of the NP protein from purified concentrated virions with anti-NP (MAB8257). Purified concentrated virions of pH1N1/471 and H1N1/WSN strains were loaded in lane V-pH1 and V-WSN respectively. Control mock from non-infected allantoic fluid was loaded in lane M. The full length NP protein at about 56 kDa (NP) is indicated.

6.3. Verification of PA related smaller products (PA*) packaged into mature virus particles

The PA protein is packaged into mature virus particles (Palese and Shaw, 2007), and we were interested to determine if PA* was also packaged into mature infectious virus. We examined this with the purified virus particles using an established protease protection assay. In this experiment, we used the H1N1/WSN virus since the PA protein profile was similar to that of the pH1N1/471 virus, and it possessed significantly higher virus titers than the pH1N1/471 virus. First, we examined the protein profile in Coomassie blue-stained SDS polyacrylamide gels under each experimental condition. As shown in Figure 6.8, two abundant proteins, NP at ~56 kDa and M1 at ~28 kDa, were resistant to trypsin digestion in the absence of detergent. However, we observed an absence of M1 protein and a smaller product beneath the NP protein when the trypsin digestion was performed in the presence of triton X-100. This inferred that triton X-100 removed the viral envelope and allowed trypsin to expose viral matrix. As a result, M1 matrix protein was completely digested and NP core protein was almost completely digested by trypsin.

Figure 6.8. Coomassie blue staining of SDS-PAGE gel after protease and detergent treatments on purified virions.

Next, we performed the Western blot analysis with the same samples from above using mAb-PA(9F5). As seen in Figure 6.9, the PA protein and PA* were still present following protease treatment alone, indicating that they were resistant to trypsin digestion. However, we failed to detect both PA protein and PA* after trypsin and triton X-100 treatment, thus suggesting that the PA* was inside the virion, protected by a lipid envelope and packaged into the mature virus particles. Other methods to support this finding are yet to be identified.

Figure 6.9. Western blot analysis of PA protein and PA related smaller products packaged into purified virions by protease and detergent treatments. The full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) are indicated.

6.4. Association of PA related smaller products (PA*) with the RNP within virus particles

The NP protein is the major structural component of the RNP complex, and its presence can therefore be used as a marker to detect the presence of the RNP. The virus RNP complex can be isolated from the virus particles using an established procedure that involves disruption of the purified virus particle using detergent. The RNP complex has a higher buoyant density compared to the soluble virus structural proteins, and it can therefore be isolated from the virus-detergent extract using glycerol gradient

ultracentrifugation (Kemler *et al.*, 1994; Wu *et al.*, 2007). We used this procedure to isolate the RNP and examine if PA* was associated with the RNP complex. In this analysis, we also used the purified H1N1/WSN virus because of its significantly higher titer.

The purified virus particles were firstly dissociated using mildly acidic conditions to disrupt the interaction of the NP and M1 proteins, and the RNP was isolated using glycerol gradient centrifugation. The gradient was fractionated, and the individual gradient fractions examined using SDS-PAGE. The polyacrylamide gel was Sypro Ruby Red-stained (Figure 6.10), which revealed major protein species of approximately 50 kDa (HA1 and NA proteins) and 28 kDa (HA2 and M1 proteins) in the fractions at the top of the gradient. A single 56 kDa protein band (the expected size for the NP protein) was also detected within the glycerol gradient, and the presence of the NP protein in the gradient was confirmed by Western blotting using anti-NP (Figure 6.11). This analysis confirmed the fractionation of the virus structural proteins in the glycerol gradient, and also indicated the location of the RNP in the glycerol gradient.

Figure 6.10. Fractionation profile of the purification of RNP complexes: purified H1N1/WSN virus particles were disrupted and separated by glycerol gradient centrifugation. Glycerol fractions collected from the top to the bottom (lane 1 to 15) were analyzed by Sypro Ruby staining. Lane C represents purified virus sample before loading onto the glycerol gradient. Arrows indicates the positions of the viral proteins NP, NA, M1 and the two cleavage forms of HA (HA₁ and HA₂). Square box highlights the fractions containing the NP protein separated from M1 protein.

Figure 6.11. Western blot analysis of the fractions collected from the top to the bottom (lane 1 to 15) using anti-NP. Lane C represents purified virus sample before loading onto the glycerol gradient. The full length NP protein at about 56 kDa (NP) is indicated.

Figure 6.12. Western blot analysis of the fractions collected from the top to the bottom (lane 1 to 15) using mAb-PA(9F5). Lane C represents purified virus sample before loading onto the glycerol gradient. The full length PA protein at about 83 kDa (PA), and the additional smaller products at about 50 kDa, 37 kDa and 25 kDa (*) are indicated.

Figure 6.13. Western blot analysis of the fractions collected from the top to the bottom (lane 1 to 15) using mAb-PB2(4G3). Lane C represents purified virus sample before loading onto the glycerol gradient. The full length PB2 protein at about 85 kDa (PB2) is indicated.

The gradient fractions were further analyzed by Western blotting using mAb-PA(9F5) (Figure 6.12) and mAb-PB2(4G3) (Figure 6.13) which showed that the PA and PB2 proteins were located within the same fractions that contained the NP protein. This was consistent with the presence of the PA and PB2 proteins within the RNP (Coloma *et al.*, 2009). Western blotting with mAb-PA(9F5) also revealed the presence of PA* within these fractions (Figure 6.12), indicating co-migration of PA* with the RNP and suggesting an interaction between PA* and the RNP. The levels of PA* accounted for between 15 and 20% of the total PA protein detected, indicating that although PA* was a minor proportion of the total PA protein detected, it was still present at significant levels.

The finding that PA* is associated with the RNP within infectious virus particles was not expected, and the molecular mechanism that mediates this association is currently unknown. The region of the RNP and the full length PA protein interaction has been defined using high resolution imaging analysis (Coloma *et al.*, 2009), but it is not clear if the PA* associates with the same region of the RNP. Several structures of the complete polymerase complex have been solved, providing the interaction of the polymerase complex with the NP to structural assembly of the RNP. However, only the full length polymerase proteins were present in these structures and there was no indication of the truncated PA proteins interacted with either the polymerase complex. The high resolution structural studies have largely used purified recombinant polymerase proteins expressed in insect cells (Pflug *et al.*, 2014; Reich *et al.*, 2014; Chang *et al.*, 2015). This may partly account for the apparent difference in the protein profile of the recombinant expressed PA protein and the PA protein that we observed in virus-infected cells and that is associated with the RNP in virus particles.

Since the PA protein C-terminal domain contains the PB1 binding site (He *et al.*, 2008; Obayashi *et al.*, 2008), it is also possible that PA* may interact with the RNP complex via the PB1 protein. However, the fully infectious influenza virus contains eight individual RNPs and it is currently unclear if the PA* is associated with all the eight RNPs or some of the packaged RNPs within the virus particle. Although PA* interacts with the RNP complex, it is possible that it plays a different role than the PA protein in the virus replication cycle. The truncated PA proteins from previous study were found not to be required for polymerase activity, but viruses that failed to express these proteins exhibited less efficient virus replication (Muramoto *et al.*, 2013), suggesting their alternative role during virus replication. It is however unknown if these truncated PA proteins play a direct role in the RNP packaging process during virus assembly, and this will be for future studies.

6.5. Summary

In this work, analyses of purified influenza virus particles by Western blotting were carried out using the generated reagents. The influenza virus particles purified from egg-cultured virus (either pH1N1/471 or H1N1/WSN strain) using sucrose gradient ultracentrifugation were used for protein analysis of purified virus. We then compared purified pH1N1/471 and H1N1/WSN virus preparations by Western blotting using mAb-PA(9F5), mAb-PB2(4G3), anti-PB1(serum) and anti-NP. These showed single protein species of the expected size for the PB2, PB1 and NP proteins, and the presence of both PA and PA* protein in each virus preparation. This indicated that while PA* was associated with the virus particles, PB2* was not virus-associated, suggesting the specificity of the incorporation of PA* into virus particles. Using a protease protection assay, we also determined that PA* was packaged into the mature virus particles, together with their major PA protein. In a final analysis, we examined the RNP

complex from purified influenza virus particles using glycerol gradient centrifugation. The result demonstrated that PA* was associated with the RNP complex, providing the evidence for the association of truncated smaller PA protein species with the functional RNP complex in influenza virus particles.

CHAPTER 7. FUNCTIONAL ANALYSIS OF VIRAL POLYMERASE COMPLEX VIA POLYMERASE REPORTER ASSAY SYSTEM

7.1. Verification of protein expression of plasmid constructs

To verify the expression of recombinant plasmid constructs involved in the polymerase reporter assay, we initially performed transfection of plasmids in HEK 293T cells. These plasmids composed of pCAGGS vectors expressing polymerase subunits (PB2, PB1 and PA) and NP gene from two human strains (pH1N1/471 and H1N1/WSN), and two avian strains (H5N2/F118 and H9N2). Then, the cells expressing the individual proteins from each of the four viruses were examined and viral proteins were detected by IFA and Western blotting with their respective antibodies.

Figure 7.1. (A) IFA and (B) Western blotting using mAb-PB2(4G3) to verify the PB2 protein expression from four different strains (pH1N1/471 ‘pH1’, H1N1/WSN ‘WSN’, H5N2/F118 ‘H5’ and H9N2 ‘H9’). pCAGGS vector alone was used as a negative control. PB2* at about 40 kDa and 25 kDa are also indicated.

As shown in the Figure 7.1A, the cells transfected with pCAGGS vectors of PB2 showed similar levels of expression in all four strains. Not only in IFA, but also in Western blotting, almost the same levels of expressed PB2 protein at about 85 kDa (Figure 7.1B) were observed for all four strains. These findings suggested that the recombinant plasmids carrying PB2 gene of both human and avian strains were

well-expressed in 293T cells and they provided similar expression levels regardless of the origins of virus strains. In addition, our novel mAb-PB2(4G3) also recognized PB2* in the cells expressing the recombinant PB2 protein from all four strains although lower level of PB2* at 25 kDa was noted in H1N1/WSN-PB2 transfected cells.

Figure 7.2. (A) IFA and (B) Western blotting using anti-PB1(serum) to verify the PB1 protein expression from four different strains(pH1N1/471 ‘pH1’, H1N1/WSN ‘WSN’, H5N2/F118 ‘H5’ and H9N2 ‘H9’). pCAGGS vector alone was used as a negative control.

Figure 7.3. (A) IFA and (B) Western blotting using anti-NP (MAB8257) to verify the NP protein expression from four different strains (pH1N1/471 ‘pH1’, H1N1/WSN ‘WSN’, H5N2/F118 ‘H5’ and H9N2 ‘H9’). pCAGGS vector alone was used as a negative control.

Based on the IFA and Western blot results of either recombinant expressed PB1 protein (Figure 7.2) or NP protein (Figure 7.3), we also discovered the similar PB1 or

NP levels for each of the four strains. Moreover, the anti-PB1 mouse serum (a polyclonal antibody) and anti-NP (a commercially available antibody) revealed single species of PB1 and NP proteins from all strains tested in this study. Although we could not predict the epitope regions of these two antibodies, the above analyses provided us more information of our plasmid constructs in addition to their sequence data.

From the above findings, PB2, PB1 and NP proteins from four different strains could be expressed almost equally in 293T cells and the protein profiles of these recombinant expressed proteins were similar to those detected in virus infected cells.

Figure 7.4. (A) IFA and (B) Western blotting using mAb-PA(9F5) to verify the PA protein expression from four different strains (pH1N1/471 ‘pH1’, H1N1/WSN ‘WSN’, H5N2/F118 ‘H5’ and H9N2 ‘H9’). pCAGGS vector alone was used as a negative control. PA* at about 50 kDa, 37 kDa and 25 kDa are also indicated.

Next, we also examined the recombinant PA protein expressions from the above four different stains by IFA and Western blot analysis using our novel mAb-PA(9F5) and compared the expression levels among them. Interestingly, unequal levels of PA signal were noted in both IFA and Western blotting (Figure 7.4A and B). While similar PA protein levels for two human strains (pH1N1 and WSN) were noted; reduced PA protein levels for the two avian strains (H5N2 and H9N2) were observed. It could be due to two possible reasons: (1) Poor binding affinity on the PA protein derived from

H5N2 and H9N2 strains by mAb-PA(9F5) generated against PA gene of pH1N1 strain, or (2) Poor PA protein expression of two avian strains in human 293T cells. Based on the findings of epitope study on this antibody described in Section 3.2, we noted there was only one amino acid difference in between the epitope region of H5N2 or H9N2 aligned with pH1N1.

To exclude the possibility of poor antigen-antibody recognition, we examined the PA protein expressions of pH1N1/471, H9N2 and H5N2/F118 with a FLAG-tag inserted at the C-terminus. The FLAG-epitope tag is a linear epitope which allows protein expression detection independent of antibody recognition, thus allowing the expression levels of different recombinant proteins to be assessed. Reduced PA-FLAG protein levels for the H9N2 and H5N2 strains compared to the pH1N1/471 strain were noted when both anti-PA (Figure 7.5A) and anti-FLAG (Figure 7.5B) were used in Western blotting, suggesting that the reduced PA protein detection was primarily due to low PA protein expression of H9N2 and H5N2 strains, and not due to poor anti-PA recognition. These findings reflected that PA protein of the two avian strains would have incompatibility with human 293T cells, resulting in low level of expression. Moreover, a previous study found that PA protein had been involved in host range restriction and pathogenicity of influenza viruses (Neumann and Kawaoka, 2006).

Figure 7.5. Western blot analyses using (A) mAb-PA(9F5) and (B) anti-FLAG to verify the PA protein expression of the FLAG-tagged full length PA gene from pH1N1/471 'pH1', H9N2 'H9' and H5N2/F118 'H5'.

After testing the expressions of all plasmids involved in our studies, we moved on to the optimization and quantification of polymerase assay with our plasmids of interest using EGFP and luciferase reporters respectively.

7.2. Optimization of polymerase assay using EGFP

To study the functional activity of viral polymerase complex, polymerase reporter assay was developed by transfecting four plasmids expressing the influenza RNP (vPoL) - PB2, PB1, PA and NP, together with a reporter plasmid, into 293T cells. The reporter plasmid contained either EGFP or luciferase gene. Using a combination of the polymerase subunits and NP gene together with the reporter, the earliest time point of noticeable EGFP fluorescence from either pH1N1/471 or H5N2/F118 or H9N2 was at Day 2 post transfection, while the EGFP signals started appearing in H1N1/WSN at Day 1 post transfection (Figure 7.6).

Figure 7.6. EGFP signals of cells transfected with pCAGGS plasmids carrying vPoL complex of H1N1/WSN, pH1N1/471, H5N2/F118 and H9N2 together with pPOLI-EGFP plasmid over a period from Day 1 to 4 at 10x magnification. Cells transfected with only pPOLI-EGFP plasmid were used as a negative control.

Moreover, cells transfected with WSN polymerase complex showed extensive expression of EGFP among all strains tested throughout from Day 1 to Day 4. It indicated that the NP associated polymerase complex activities seems to be slower in pH1N1/471, H5N2/F118 and H9N2 strains compared to H1N1/WSN strain. The high polymerase activity would enhance viral replication, and hence this finding may partly explained high virulence of WSN strain.

7.3. Quantification of polymerase assay using Luciferase

Based on the visualization results from the polymerase assay using EGFP, we repeated the assay using Gaussia Luciferase as a means to quantify the expression of reporter gene, which in turns reflects the viral polymerase (vPoL) activity.

Figure 7.7. The luciferase activities of vPoL complex from pH1N1/471, H5N2/F118, H9N2 & H1N1/WSN over a period from Day 1 to Day 6. Green circle indicates the luciferase activities of polymerase complexes from pH1N1/471, H5N2/F118 and H9N2 at Day 4 post transfection.

The culture media were harvested over a period from Day 1 to Day 6 and the luciferase activities were measured in relative light unit (RLU) and a time course was plotted (Figure 7.7). The luciferase activity showed similar trend as EGFP in the

polymerase activities of these strains. On one hand, polymerase activity of WSN complex sharply increased from Day 1 to Day 2 and peaked at Day 3, then maintained the higher activity till Day 4, then decreased on the following days. However, it still maintained the significant polymerase activity until Day 6. On the other hand, the polymerase activities of pH1N1, H5N2 and H9N2 exhibited the similar trend as WSN, but significantly low level of activities. They showed very low initial activities at Day 1, but progressively increased over a period of study. The comparable and peak level of polymerase activities of pH1N1, H5N2 and H9N2 was observed at Day 4. Overall, polymerase complex of H1N1/WSN strain was more active than that of other strains tested in terms of temporal and quantitative dimensions indicating a difference in their transcriptional activities.

7.4. Reassortment studies

With the established polymerase/luciferase assay together with our plasmids of interest, various transcriptional activities of polymerase complexes have been observed in different strains. Next, to determine the compatibility among NP and polymerase subunits of different strains used in our studies, we performed the reassortment studies. This involved single-gene reassortment of NP and polymerase subunits: (i) between human H1N1/WSN and avian H5N2/F118 strains, (ii) between human H1N1/WSN and avian H9N2 strains, and (iii) between two human (H1N1/WSN and pH1N1/471) strains. In these studies, we chose to measure the luciferase activities at Day 4 post-transfection where the peak levels of parental complexes were observed from all strains tested (shown in Section 7.3). Then, we investigated the ability of NP and polymerase subunits to restore an avian influenza virus polymerase activity which is normally impaired in human 293T cells.

7.4.1. Single-gene reassortment of NP and polymerase subunits between human H1N1/WSN and avian H5N2/F118

Figure 7.8. Polymerase assays were carried out in 293T cells by transfecting vectors expressing human H1N/WSN or avian H5N2/F118 NP, polymerase subunits and a vNA-based luciferase reporter. Luciferase activities were measured at Day 4 post-transfection. (A) Reassortants were created by the substitution of one of the polymerase subunits or NP with that from the avian H5N2 strain. (B) Reassortants were created by the substitution of one of the polymerase subunits or NP with that from the human WSN strain. Data were normalized to the activity RLU % in terms of (A) H1N1/WSN or (B) H5N2/F118 parental complex. Error bars represent the standard deviation of triplicate experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Firstly, we performed the reassortment of NP and polymerase subunits between human H1N/WSN and avian H5N2/F118 strains and measured the luciferase activities of parental and reassorted polymerase complexes (Figure 7.8). Our data revealed that substitution of the H5N2-PA or PB2 into the WSN-PA or PB2 led to a significant decrease (70-80%) in the activity of the WSN polymerase complex (Figure 7.8A). However, we observed the reverse activities in the parallel analysis. Substitution of the WSN-PA or PB2 into the H5N2-PA or PB2 resulted in a nearly 12-fold increase in the activity of H5N2 polymerase complex (Figure 7.8B). Moreover, in comparison to the polymerase activity of corresponding parental complex, we noted a higher activity of WSN polymerase complex in the replacement of WSN-PB1 with H5N2-PB1 (Figure 7.8A), while a lower activity of H5N2 polymerase complex was found in the replacement of H5N2-PB1 with WSN-PB1 (Figure 7.8B). Although we observed no statistically significant difference in the substitution of H5N2-NP into WSN-NP (Figure

7.8A), there was a small increase in the activity in the substitution of the WSN-NP into the H5N2-NP (Figure 7.8B).

7.4.2. Single-gene reassortment of NP and polymerase subunits between human H1N1/WSN and avian H9N2

Figure 7.9. Polymerase assays were carried out in 293T cells by transfecting vectors expressing human H1N1/WSN or avian H9N2 NP, polymerase subunits and a vNA-based luciferase reporter. Luciferase activities were measured at Day 4 post-transfection. (A) Reassortants were created by the substitution of one of the polymerase subunits or NP with that from the avian H9N2 strain. (B) Reassortants were created by the substitution of one of the polymerase subunits or NP with that from the human WSN strain. Data were normalized to the activity RLU % in terms of (A) H1N1/WSN or (B) H9N2 parental complex. Error bars represent the standard deviation of triplicate experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

We also performed the reassortment experiment between human H1N1/WSN and another avian strain H9N2 (Figure 7.9). We noted that substitution of H9N2-NP or PB2 into the WSN-NP or PB2 led to an approximate 80% reduction in the activity of the WSN polymerase complex, while the WSN polymerase in which the WSN-PA was replaced with the H9N2-PA exhibited nearly 1% of the polymerase activity of the parental WSN polymerase complex (Figure 7.9A). Similar to the finding of H5N2-PB1 substitution, the increase in activity of WSN polymerase complex was noticed when WSN-PB1 was replaced with H9N2-PB1 (Figure 7.9A). In the parallel analysis, replacement of the H9N2 polymerase complex with the WSN-NP or PB1 or PB2 showed only minor effects on the activity of the H9N2 polymerase complex

(Figure 7.9B). However, replacement of the H9N2-PA with the WSN-PA resulted in a 35-fold increase in the activity of the parental H9N2 complex, to levels that were comparable with the activity of the parental H1N1/WSN polymerase complex (Figure 7.9B).

Based on the findings of the above reassortment experiments, we generally observed that substitution of the human influenza PA or PB2 subunit into the avian polymerase complex and vice versa showed drastic changes in polymerase activity. This suggested that substitution of the human-origin PA or PB2 subunit into the avian polymerase complex alleviated species restriction and enhanced the activity of the avian polymerase in human cells. This finding was supported by previous groups (Mehle and Doudna, 2009; Mehle *et al.*, 2012; Yamayoshi *et al.*, 2014). In addition, the importance of residue 627 of PB2 has been proposed as a major host range determinant by many other reverse genetic studies (Hatta *et al.*, 2001; Mase *et al.*, 2006; Mehle and Doudna, 2009; Steel *et al.*, 2009; Zhang *et al.*, 2014; Sang *et al.*, 2015). They showed the contribution of lysine residue (K) at position 627 of PB2 from human isolates to high polymerase activity, while the glutamic acid (E) at this position of avian isolates exhibited low polymerase activity. In our study, this E627K mutation was introduced when H5N2-PB2 or H9N2-PB2 was reassorted with WSN-PB2. This provided a proof for observation of high avian polymerase activity in human 293T cells. Unexpectedly, a finding from a reassortant carrying WSN-PB2 gene with H9N2 avian polymerase background seem to contradict a role of human PB2 in polymerase activity in mammalian cells. It could be an issue of incompatibility to form a heterotrimer among polymerase subunits between different viruses (Li *et al.*, 2008). Reassortment and several mammalian-adapting mutations at various residues of avian-origin PA also enhanced the activity of avian influenza polymerases in mammalian cells and overcome

species barriers (Gabriel *et al.*, 2005; Song *et al.*, 2009; Kim *et al.*, 2010a; Bussey *et al.*, 2011; Mehle *et al.*, 2012). One of the known adaptive mutations, T552S, was found to be present in our study with the substitution of WSN-PA into H5N2-PA or H9N2-PA. Hence, it would be a major contributing factor which allows the avian polymerase activities significantly increase in mammalian cells.

In addition to PA and PB2, PB1 and NP are also suggested to be associated with host range restriction. In our study, we noted that substitution of the avian-origin PB1 subunit into the background of human polymerase restored the activity of the human polymerase complex. Previous studies also showed that human virus polymerase complex was more active with avian than with human PB1 protein in mammalian cells (Kawaoka *et al.*, 1989; Naffakh *et al.*, 2000; Mänz *et al.*, 2013). These findings suggested that avian PB1 may enhance the kinetic of polymerase complex in mammalian systems. Moreover, the finding in our study also revealed that human virus polymerase activity was significantly reduced with the replacement of NP from H9N2 although no significant difference was seen with the NP from H5N2. Avian virus NP gene has been shown the restriction of virus replication in mammalian cells and reassortment of NP segment from human virus with avian virus resulted in attenuation of reassortant viruses for monkeys (Snyder *et al.*, 1987). However, due to the limited data available, the contribution of NP gene to host range restriction and incompatibility among the polymerase genes remained unclear.

7.4.3. Single-gene reassortment of NP and polymerase subunits between human H1N1/WSN and human pH1N1/471

Figure 7.10. Polymerase assays were carried out in 293T cells by transfecting vectors expressing human H1N1/WSN or pH1N1/471 NP, polymerase subunits and a vNA-based luciferase reporter. Luciferase activities were measured at Day 4 post-transfection. (A) Reassortants were created by the substitution of one of the polymerase subunits or NP with that from the pH1N1/471 strain. (B) Reassortants were created by the substitution of one of the polymerase subunits or NP with that from the human WSN strain. Data were normalized to the activity RLU % in terms of (A) H1N1/WSN or (B) pH1N1/471 parental complex. Error bars represent the standard deviation of triplicate experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

From the initial quantification of polymerase activities, we have seen low transcriptional activities of polymerase complexes in two avian strains as well as in a human pH1N1/471 strain (compared to human H1N1/WSN strain). Although pH1N1 2009 was known as a human strain due to human to human spread, it was actually a triple reassortant virus (i.e. it contains genes derived from avian, swine and human). Its RNP complex composed of PB1 (H3N2 human-origin), PA and PB2 (North American avian-origin), and NP (classical swine-origin). Hence, to further explore the changes in polymerase activities between two human strains (H1N1/WSN and pH1N1/471), we also carried out the reassortment experiment of NP and polymerase subunits between these two strains.

As shown in Figure 7.10A, an introduction of either PA or PB2 subunit of pH1N1/471 virus into the H1N1/WSN polymerase complex significantly decreased the

WSN polymerase activity (nearly 70% and 50% for PA and PB2 substitutions respectively). When the pH1N1-NP gene was introduced into the WSN polymerase complex, the polymerase activity was reduced by about 30% in comparison to the activity of WSN parental complex. These data indicated that disruption of the parental WSN's functional polymerase complex, by introducing the avian-origin PA or PB2 subunit and the swine-origin NP gene of pH1N1/471 strain, decreased the transcriptional activities of its parental complexes. However, an alternate pattern was noted in the parallel analysis (Figure 7.10B). Replacement of the pH1N1-PA with the WSN-PA produced an approximate 9-fold increase in polymerase activity, while replacement of the pH1N1-PB2 with the WSN-PB2 led to a 2-fold increase in activity of the pH1N1 polymerase complex. Since 2009 pH1N1-PA and PB2 were originated from an avian subtype lacking the mammalian adaptive mutations (Guang-Wu and Shin-Ru, 2009), as we expected, the substitution of PA or PB2 of H1N1/WSN virus enhanced the activity of pH1N1/471 polymerase complex in human cells. It suggested that PA and PB2 subunits (derived from human-origin) may have greater activity that could provide an advantage in mammalian systems. Given the heterodimer nature of PA and PB1 (Deng *et al.*, 2005), the increase in WSN polymerase activity with pH1N1-PB1 substitution observed in our study could be due to a better compatibility of WSN-PA with pH1N1-PB1 (both derived from human-origin) and vice versa.

Collectively, our findings were in line with previous reassortment studies on H1N1, 2009 pH1N1, H5N1 and H9N2 subtype viruses (Li *et al.*, 2010; Hsieh *et al.*, 2011; Lam *et al.*, 2011; Octaviani *et al.*, 2011; Liu *et al.*, 2014), where PA and PB2 were found to be important host range determinants responsible for the increased polymerase activities. Not only PA and PB2 proteins, but also PB1 and NP proteins appeared to be involved in host range restriction and required for gene reassortment in influenza

viruses. However, there may be possibility of discrepancy in the finding between polymerase activity found in polymerase reporter assay and the virus growth of the corresponding viruses. Due to the experimental purpose and time constraint, we conducted the reassortment studies with the polymerase reporter assay in only one cell line (293T). Therefore, it would be worthwhile to further study in different cell types (e.g. respiratory epithelial cells) where influenza infection occurs.

7.5. Summary

Using recombinant plasmid constructs of the respective polymerase complexes (PB2, PB1, PA and NP) from each of the four different viruses (H1N1/WSN, pH1N1/471, H5N2/F118 and H9N2), we examined their expression levels in 293T cells by both IFA and Western blotting. Similar levels for PB2, PB1 and NP of the four viruses were noted, while low levels of PA protein for the H9N2 and H5N2 viruses were observed. Next, the activity of the recombinant complex of each virus was assessed using the polymerase/luciferase reporter assay. We noted a gradual increase in the detection of luciferase activity from day 1 to day 4 which reflected the activity of polymerase complex from each of the four viruses although the polymerase activities of pH1N1/471, H5N2/F118 and H9N2 were significantly lower than that of H1N1/WSN polymerase complex. Then, we moved onto genetic reassortment with the polymerase complexes of the above viruses and measured the activities of the parental and corresponding reassorted polymerase complexes. Overall, a single reassortant bearing a human-origin PA or PB2 subunit against an avian polymerase background could be sufficient to overcome the specie restriction of avian polymerase in human 293T cells, indicating the PA or PB2 as a major host range determining factor. Activity differences in avian/human polymerase reassortants can be partially explained by (1) host restriction/host specificity (e.g. altered effect of reassortant polymerase from avian-

origin in human 293T cells), and (2) incompatibility between human and avian NP/polymerase subunits (e.g. the multiple unconserved amino acid substitutions may have resulted in altered catalytic sites and/or binding affinities). Future studies would be essential for a better understanding of the factors contributing genetic reassortment in influenza viruses in order to predict and control the future influenza epidemic and pandemic.

CHAPTER 8. SILENCING OF INFLUENZA POLYMERASE GENES via RNAi

8.1. Development and optimization of assays to evaluate siRNAs

The emergence of new influenza A viruses with their high mutation rates possibly makes anti-influenza drugs ineffective. Therefore, RNAi as an alternative antiviral strategy allows one to rapidly design and screen more siRNAs against multiple gene targets of a sequenced influenza virus strain.

For the initial development and optimization of assays to evaluate siRNAs in a BSL2 laboratory setting, we used two siRNA sequences, siNP-H1N1 and siPA-H1N1, which demonstrated effective against human influenza H1N1 (Ge *et al.*, 2003). Firstly, the direct effect of the siRNA in gene silencing was assessed by the measurement of its ability to reduce mRNA expression of the target gene. To determine the relative level of mRNA expressed in the target NP and PA viral genes, a RT-qPCR assay was performed with H1N1 infected samples treated with siNP-H1N1, siPA-H1N1, and siGFP (a control). As shown in Figure 8.1, we noted that the levels of NP and PA mRNA expression for siNP-H1N1 and siPA-H1N1 were significantly decreased by almost 70% and 60% respectively in comparison to the control.

Figures 8.1. Relative quantification of knockdown of NP & PA mRNA levels by the use of different siNP-H1N1 and siPA-H1N1 in MDCK cells infected with H1N1. Error bars represent the standard deviation of triplicate experiments. (* $p < 0.05$).

Then, we performed IFA on MDCK cells treated with siRNA and infected with H1N1 virus to visualize the effect of the gene knockdown in terms of protein expression. We clearly observed that NP protein expression was absent in a large number of H1N1-infected cells treated with siNP-H1N1 (Figure 8.2B) compared to control siGFP (Figure 8.2A). Again, infected cells, treated with siPA-H1N1 and stained with anti-NP antibody, revealed a significant delay in export of the NP protein from the nucleus even after 16 hpi (Figure 8.2C). This indicated the slowed virus replication and/or assembly, or that the PA protein was involved in export of the NP protein from the nucleus in some way.

Figures 8.2. Immunostaining (anti-NP antibody) of MDCK cells treated with various siRNAs: (A) siGFP, (B) siNP-H1N1, and (C) siPA-H1N1 followed by infected with H1N1 (fixed and stained at 16 hpi).

Next, the ability of siRNA to slow down the production and release of progeny viruses in multiple cycles of infection was also one of the indicators of its effectiveness as an antiviral. MDCK cells, treated with siRNA, were infected at a low moi of 0.01, and the amount of released viruses was measured by virus titration over a period of 48 hpi. We found a significant decrease in virus titers in H1N1-infected cells after treatment with siNP-H1N1 at 10 and 24 hpi (Figure 8.3) in comparison to the control. At the later time-points of post infection, virus titers in siNP-H1N1 and siGFP treated samples showed almost equal levels. This could be due to increased viral loads at the later time-points whereby the effect of a single dose of siRNA was not able to sustain a significant decrease in virus titers.

Figure 8.3. The antiviral effect of siNP-H1N1 in MDCK cells infected with H1N1 at low moi of 0.01. The amount of progeny viruses released into the cell culture supernatant was measured by virus titration over a period of 48 hpi.

8.2. *In vitro* Evaluation of siRNAs targeting genes (NP, PA, PB1 and PB2) of LPAI H5N2

In this section, we chose an AIV subtype H5 as a model because of its potential to emerge a novel pandemic H5 virus and spread to the human population. Having established reliable *in vitro* assays to evaluate the efficacy of siRNAs in a BSL2 setting, we screened a panel of custom designed siRNAs targeting viral RNP genes (NP, PA, PB1, PB2) of the LPAI H5N2 virus in the BSL3 and evaluated the effectiveness and specificity of those siRNAs.

8.2.1. Quantification of mRNA knockdown by RT-qPCR

Firstly, we observed the siRNA knockdown effect on mRNA levels of NP, PA, PB1 and PB2 by RT-qPCR method. Figure 8.4A showed the relative quantification of NP mRNA knockdown in H5N2 infected cells after transfected with each of the four custom-designed siNPs-H5N2. We observed that siNP-1 and siNP-3 were the most effective at gene silencing, with mRNA knockdown efficiencies of 60-70%. This was followed by siNP-2 and siNP-4 with knockdown efficiencies of 55% and 40% respectively. NP is a major structural protein in influenza virus and is involved in several

functions. It primarily occurs in the processes of virus genome encapsidation for RNA transcription, replication and packaging (Portela and Digard, 2002). By silencing the NP gene, it would result in disruption of viral transcription, replication and packaging of influenza virus.

Figures 8.4. Relative quantification of knockdown of (A) NP, (B) PA, (C) PB1 and (D) PB2 mRNA levels by the use of different siRNAs in MDCK cells infected with H5N2. Error bars represent the standard deviation of triplicate experiments. (* $p < 0.05$).

From the measurement of PA mRNA levels after treated with four custom designed siPAs-H5N2, we noted that H5N2 virus infected cells showed >60% knockdown in PA mRNA levels with siPA-3 and siPA-4 (Figure 8.4B). PA is one of the three polymerase proteins. It is involved in multiple functions of the polymerase complex including endonuclease activity, protein stability, cap binding and virion RNA promoter binding activity (Hara *et al.*, 2006). Hence, viral replication and transcription would be inhibited as consequences of silencing of the PA gene.

Moreover, we also tested the custom-designed siPB1-1 to 4 and siPB2-1 to 4 for PB1 and PB2 mRNA knockdown in H5N2 infected cells. As shown in Figure 8.4C and D, we found that siPB1-1, siPB1-3, siPB2-1 and siPB2-2 could efficiently knockdown their respective mRNA about 70-80%. Like PA, PB1 and PB2 are also important subunits to form a RNA polymerase complex. They play essential roles within the polymerase and are also crucial for viral transcription and replication. Previous study has shown that the PB1-PB2 interface is required for transcription initiation (Sugiyama *et al.*, 2009). Silencing of the PB1 or PB2 gene would cause disruption of this interaction and possibly restriction of viral replication.

8.2.2. Immunofluorescence detection of viral protein expression

Figures 8.5. Immunostaining of MDCK cells treated with various siRNAs: (A) siGFP, (B) siNPs, (C) siPAs, (D) siPB1s & (E) siPB2s followed by infected with H5N2 at moi 1 (fixed and stained at 16 hpi). Anti-NP Ab was used for NP protein staining (green) and evans-blue dye was used for background cell staining (red). Widespread of NP throughout the cell was highlighted in the insert of Figure (A), whereas a pattern of *delay* in the *export of NP* out of the nuclei was highlighted in the insets of Figure (B, C, D and E). The results from the other siRNAs treated samples which did not give effective knockdown effect on cells are not shown here.

To determine the specific siRNA knockdown effect on protein level, immunostaining with anti-NP antibody was performed in MDCK cells treated with various siRNAs followed by H5N2 infection at moi of 1. We found clearly the presence of non-stained cells in siNP-1 and siNP-3 treated samples (Figure 8.5B) in comparison to control sample treated with siGFP (Figure 8.5A). This suggests that siNP-1 and 3 had potent knockdown effect on the NP protein expression. Based on this result, we hypothesize that it is necessary to achieve a certain level of mRNA knockdown in order to effectively abolish the protein expression which was the case in siNP-1 and 3, but not in siNP-2 and 4.

Again, virus infected cells after treatment with siRNAs against PA, PB1 and PB2 were also stained with anti-NP antibody. As shown in Figure 8.5A, nuclear export of NP was widespread in almost all cells in siGFP treated sample, while that of NP is much more limited and localized to the nucleus in those siPA-3 & 4, siPB1-1 & 3, siPB2-1 & 2 treated samples (Figure 8.5C, D & E). We interpret this as an indication of slowed export of new vRNPs from the nucleus. This indicates that not only in viral replication/transcription, but also in nuclear export/assembly of newly synthesized vRNPs would be affected by knocking down one of the polymerase proteins essential for the formation of polymerase complex.

8.2.3. Measurement of antiviral effect by virus titration

Next, antiviral effect of custom designed siRNAs was measured by titration of progeny infectious viruses from supernatants of MDCK cells treated with various siRNAs followed by H5N2 infection at a low moi of 0.01 at various time points. As shown in Figure 8.6A, a significant decrease in H5N2 virus titers (> 90% reduction) was observed at 1.5 and 2 dpi with siNP-1 and siNP-3 as compared to control siGFP.

Figure 8.6. The antiviral effect of various siRNAs: (A) siNP, (B) siPA, (C) siPB1 and siPB2 reflected as a percentage of virus titers from siGFP treated cells harvested at 1.5 and 2 dpi. MDCK cells were transfected with siRNA prior to H5N2 infection at low moi of 0.01. Error bars represent the standard deviation from triplicate experiments.

Moreover, a significant reduction in H5N2 virus titers by 80% was found at 1.5 and 2 dpi with siPA-4, while siPA-3 and siPA-H1 showed a moderate reduction in virus titers by 30 to 50% at the same time points (Figure 8.6B). Again, virus titer of cell culture supernatant at 1.5 dpi from MDCK cells treated with siPB1-3 and siPB2-1 revealed a significant reduction by nearly 80% followed by siPB1-1 and siPB2-2 with a reduction of 70% and 60% respectively (Figure 8.6C). Previous studies have shown a marked decrease in virus titer and suppression of virus replication *in vitro* by treatment with siRNAs targeting the NP and PA genes of HPAI H5N1 virus (Zhou *et al.*, 2007; Zhou *et al.*, 2008; Zhang *et al.*, 2009). In addition, other previous groups have also demonstrated the inhibitory effect of siRNAs specific for PB1 and PB2 genes of influenza A H1N1, 2009 pH1N1 and H5N1 subtypes (Ge *et al.*, 2003; Cheng *et al.*, 2009; Zhiqiang *et al.*, 2010; Mahmoud *et al.*, 2014).

8.2.4. Specificity assessment by polymerase reporter assay

The polymerase reporter assay was conducted to assess the specificity of the siRNAs (Figure 8.7A). Firstly, we used a combination of three polymerase and NP genes from a human influenza H1N1 strain in our initial reporter assay system to measure the knockdown effect of siRNAs in terms of the RNP activity as reflected by relative luciferase activity as shown in Figure 8.7B. Both siNP-H1N1 and siPA-H1N1 revealed a 50% reduction in relative luciferase activity compared to a control siGFP. This baseline reporter activity is attributed to transfection efficiencies of both siRNAs and plasmids.

Figure 8.7. (A) A schematic diagram illustrating the polymerase reporter assay to assess the specificity of the siRNAs. (B) The effect of siNP-H1N1 and siPA-H1N1 in a polymerase assay containing genes from a human influenza H1N1 (PR8) strain as reflected by reduction in relative light units (RLU) compared to a control siGFP. (* $p < 0.05$).

Then, we moved onto the combination of four plasmids expressing the avian influenza RNP – PB2, PB1, PA and NP from H5N2 strain together with a reporter plasmid. These plasmids were transfected in 293T cells alongside with one of the effective custom designed siRNAs from each set of gene resulted from previous assays and the RNP activity was measured. As shown in Figure 8.8, the highest knockdown effect on RNP activity was seen with siPB1-3 (about 70%) followed by the modest effect with siNP-3, siPA-4 and siPB2-1 (about 50% to 60%). However, we noted that siNP-H1N1 and siPA-H1N1 which had moderate effect on luciferase activity with RNP from H1N1 strain showed very little effect on luciferase activity with RNP from H5N2 strain. It indicates ineffectiveness of the siNP and siPA against human H1N1 strain to knockdown the NP and PA genes of avian H5N2 strain. This could be due to mismatched nucleotides between human and avian strains or different off-target effects which might come from the responses of different strains.

Figure 8.8. The effect of custom designed siNP-3, siPA-4, siPB1-3 and siPB2-1 against H5N2 in a polymerase reporter assay containing NP, PA, PB1 and PB2 genes from an avian H5N2 strain as reflected by reduction in relative luciferase units (RLU) compared to three controls (siNP-H1N1, siPA-H1N1 and siGFP). (* $p < 0.05$).

The results from this polymerase assay show that sequence specificity is required to achieve effective gene silencing in RNAi machinery. Although the polymerase

reporter assay can only be applied to assess siRNAs designed against four genes (NP, PA, PB1 and PB2) involved in the polymerase complex, a major advantage of this assay is only requiring the genetic sequences of the virus, but not the actual live virus. This would be useful especially in the case of a pandemic situation where the live virus may not be readily available.

8.3. Summary

In this study, we have designed a total of sixteen siRNAs against NP and polymerase gene segments of the LPAI subtype H5N2 and tested their efficacies by different virological assays. Eight out of sixteen (siNP-1 and 3, siPA-3 and 4, siPB1-1 and 3, siPB2-1 and 2) were identified as potentially effective siRNAs by observing effective knockdown on the mRNA and protein levels although the reductions in virus titers achieved were rather modest. Moreover, sequence specificity of the siRNAs was also further confirmed with one of the effective siRNAs from each gene segment by the reporter system. Therefore, we have demonstrated that the designing of siRNA against a new virus could be rapid as only the knowledge of the virus's genetic sequence is required to design siRNAs. Moreover, both the virological and reporter assays showed that the siRNAs were effective in inhibiting the replication of the targeted H5N2 virus. Our findings revealed RNAi as a potential antiviral strategy which can be rapidly deployed in crisis situations such as pandemic outbreak involving the emergence of a novel viral disease.

CHAPTER 9. CONCLUSION

We have immunized mice with purified soluble PA protein (2009 pH1N1/471 and LPAI H5N2/F118 strains), insoluble PB2 and PB1 proteins (2009 pH1N1/471 strain). Monoclonal antibodies (mAbs) directed against PA subunit of both strains were successfully generated. Similarly, mAb against PB2 subunit of 2009 pH1N1 strain was successfully developed, while mAb production against PB1 protein was not successful at fusion stage for two attempts.

Two mAbs (9F5 and 2E2) against PA subunit of pH1N1/471 strain were selected based on the ELISA screening and extensively characterized using various approaches. They could recognize the target PA protein specifically in both PA-transfected cells and virus-infected cells. In the analysis of epitope mapping, 9F5 recognized the amino acid region of 597-616 (within the PA C-terminal domain), while 2E2 recognized the region of 240-255 (within the PA-linker) shown in Appendix 8. Interestingly, these two mAbs-PA showed different staining pattern of PA protein in virus-infected cells by IFA. 9F5 recognized PA protein throughout the cells (both nuclear and cytoplasmic compartment), while 2E2 recognized PA protein in nuclear compartment only. In fractionation analysis, a similar picture was observed.

After the examination of PA expression profile with different strains and cell lines, 9F5 showed cross-reaction with other strains tested in our study, but not in the case of 2E2. In addition to the full length PA protein, PA related smaller protein products (PA*) were recognized by 9F5 in all the strains and cell lines tested. PA* proteins were therefore neither subtype nor cell type-specific. Using siRNA targeting PA gene, PA* proteins were confirmed to be related to the full length PA protein. Moreover, both PA protein and PA* from virus-infected cells have been successfully resolved with 2-D electrophoresis according to their molecular weights and isoelectric points. Besides the

transfected and infected cells, PA and PA* were detected in infectious virus particles, suggesting that PA* was virus-associated. Analysis with protease protection assay also provided evidence that PA* was packaged into the mature virus particles. Additionally, PA* was found to be associated with the functional RNP complex in influenza virus particles.

Next, five mAbs against PA subunit of H5N2/F118 strain were also selected and characterized by the same approaches. They could detect the protein of interest with a similar recognition pattern. Among five mAbs, two (4D7 and 7A4) recognized the amino acid region of 417-516, while the rest (6A10, 6A11 and 6B11) recognized the region of 317-416 displayed in Appendix 8.

To study PB2 protein expression, mAb(4G3) against PB2 subunit of pH1N1/471 was successfully generated and it was found to recognize the amino acid region 720-739 (within the NLS region) shown in Appendix 8. 4G3 mainly recognized the nuclear PB2 protein. It also demonstrated cross-reactivity with the PB2 protein from other strains tested. Similar to PA*, 4G3 identified PB2 related smaller products (PB2*) in both infected cells and recombinant expressed PB2 protein, but not in infectious virus particles. Moreover, PB2* appeared to be not subtype-specific and cell type-specific. In the case of PB1 subunit, the anti-PB1 (a mouse immune serum) was used in the study of PB1 protein expression. Due to the polyclonal nature, it recognized PB1 expression of different virus strains in different cell types.

For the functional analysis of viral polymerase complex, NP and three polymerase subunits of H1N1/WSN, pH1N1/471, H5N2/F118 and H9N2 strains have been successfully constructed using a mammalian expression system. After testing the expression of these recombinant plasmid constructs, polymerase complex activities were evaluated through a reporter EGFP and Gaussia Luciferase assay. It was observed

that H1N1/WSN polymerase complex was more active than polymerase complexes of other strains tested in terms of temporal and quantitative aspects. Our gene reassortment experiments showed that a single reassortant bearing a human-origin PA or PB2 subunit against an avian polymerase background could overcome the host range barrier of avian polymerase in human 293T cells, indicating the PA or PB2 as a determinant of species tropism and pathogenicity.

Recently, RNAi emerged as a potential antiviral strategy which can be rapidly used in the situation of influenza outbreak. Together with our *in vitro* assays, eight out of sixteen siRNAs (siNP-1 and 3, siPA-3 and 4, siPB1-1 and 3, siPB2-1 and 2) revealed potent inhibition of the H5N2 virus replication by reduction in mRNA levels, protein expressions and virus titers. Finally, sequence specificity of the siRNAs was further demonstrated with one of the effective siRNAs from each gene segment by the reporter system.

CHAPTER 10. FUTURE WORK

The observed PA* and PB2* fragments will be extensively characterized by various methods. Mass spectrometry analysis will be primarily used to determine the nature of proteins and any associated modification such as phosphorylation. Previous studies have reported a proteolytic activity associated with the PA protein. To determine any involvement of proteolytic processing for the smaller products, various protease inhibitors will be used. From the analyses of sequence and mass spectrometry data, potential important residues will be identified and assessed with the method of site-directed mutagenesis to investigate the truncation and post-translational modification.

Previous studies have identified a number of host proteins which are known to be associated with the viral polymerase complex and influence the viral replication, transcription and pathogenicity. For example, importin α acts as an import factor for PB2 and RanBP5 acts as a cofactor for the nuclear import of the PB1-PA dimer. Moreover, Hsp90 interacts with PB2 and it involves in nuclear import and assembly of RNA polymerase subunits (Naito *et al.*, 2007). Recently, ANP32A identified as an essential host partner facilitates vRNA synthesis and contributes to species-specific polymerase activity (Long *et al.*, 2016). Therefore, identification of other host factors and studying virus-host interactions (e.g. protein-protein and protein-RNA interactions) will be performed. These will be achieved using generated monoclonal antibodies and various methods such as radiolabelling, immunoprecipitation pull down assay, Yeast two/three-hybrid assay and two dimensional gel coupled with mass spectrometry analysis. Finally, the identified host factors and their roles in virus replication will be confirmed by RNAi technique and polymerase assay.

Our results from reassortment experiments highlighted one of the ways to overcome a species barrier, but there could still be discrepancy in the pattern of polymerase activity observed in the polymerase reporter assay and the actual virus growth. Moreover, other factors such as temperature, cell type will influence on the polymerase activity and viral replication. To have a better understanding of these factors contributing gene reassortment in the prediction of novel influenza viruses, further studies including optimization of the experimental temperature and the use of different cell types (e.g. respiratory epithelial cells) would be necessary. Together with reassortment studies, it would be worthwhile to further study the viral replication kinetics of infected respiratory epithelial cells where influenza infection occurs. In addition, further investigations using engineered mutations such as site-directed mutagenesis at the important host adaptive residues would also be required to exclude the possibility of poorly transcribing systems and to validate the findings.

The recent emergence of new avian influenza viruses led to a public health concern of the potential for another influenza pandemic outbreak. Current vaccines and anti-influenza drugs are of limited use in those new strains due to high mutation rates of virus and side effects in hosts. Therefore, RNAi therapy has evolved as an alternative antiviral strategy. Using a number of *in vitro* assays, we screened sixteen custom designed siRNAs against NP and polymerase genes of LPAI H5N2 virus. After identifying eight potentially effective siRNAs from our *in vitro* study, it is worthwhile to do further investigations under different experimental conditions including various concentrations of siRNAs and additional doses to determine maximum efficacy and duration of action. It would also be interesting to study the synergistic antiviral effects of two or more siRNAs targeting different polymerase components at the same time. Moreover, we need to examine their specificity and off-target effects. To do so, genome-

wide microarray profiling using human cell lines will be important. Subsequently, it will be essential to explore efficient delivery methods of siRNAs for *in vivo* study. They will provide exciting opportunities for future studies.

Besides the use of siRNAs, another antiviral strategy is to use small-molecule inhibitors of influenza RNA polymerase, some of which have been developed and tested in previous studies. For example, Favipiravir inhibits the RNA dependent RNA polymerase, while ANA-0 and RO-7 specifically inhibits the PA endonuclease activity (Furuta *et al.*, 2013; Jones *et al.*, 2016; Yuan *et al.*, 2016). Hence, it would also be interesting to evaluate these inhibitors with our viruses.

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APPENDIX

1. Complete amino acid sequence alignments of four selected strains based on their PB2 protein sequence alignments. Protein residues that match H1N1/WSN-PB2 exactly are hidden (as ‘.’).

2. Complete amino acid sequence alignments of four selected strains based on their PB1 protein sequence alignments. Protein residues that match H1N1/WSN-PB1 exactly are hidden (as '.').

	10	20	30	40	50	60	70	80	90	100																							
H1N1/WSN-PB1	MDVNPTLLFLKVP	PAQNAISTTF	PPYSHG	TGTGYTMD	IVNRTHQYSE	RGRTWTNT	ETGAPQLNP	DGGLPEDNE	PSGYAQTDC	VLEAMAFLEESH	P																						
pH1N1/471-PB1	I.....				K.K.....																												
H5N2/F118-PB1					K.K.....																												
H9N2-PB1					K.....																												
	110	120	130	140	150	160	170	180	190	200																							
H1N1/WSN-PB1	GIFETSCLETME	VVQQT	RVVDKLTQGR	QTYDWT	LN	RNPQPA	ATALANTIE	VFRS	NGLTANES	GR	LIDFLKDV	MESMNKE	MEITTH	FQKR	RRV	RD	NMT	KKM	V														
pH1N1/471-PB1	N.....												I.....																				
H5N2/F118-PB1	N.....							DR.....											I														
H9N2-PB1	N.....	I.....											D.....	V.....																			
	210	220	230	240	250	260	270	280	290	300																							
H1N1/WSN-PB1	TQRTIGKRRQRLN	KRSYLIRALT	NTMTKDA	ERGLK	KLKRAIAT	PGMQIRG	FVYFVET	LARSICEK	LEQSG	LPVGGNE	EKKAKL	ANVVR	KMM	TNS	QDTE	ISF																	
pH1N1/471-PB1	K.....	G.....																															
H5N2/F118-PB1	K.....																		L.....														
H9N2-PB1	K.....		I.....																L.....														
	310	320	330	340	350	360	370	380	390	400																							
H1N1/WSN-PB1	TITGDNTKWN	ENQNP	RMFLAMITY	ITRNQPE	WFRN	VL	SIAPIM	FSNKMAR	LCKGY	MFES	KS	MKLRTQ	IPAE	MLASID	LKYFND	STR	KKIEKIR	P	LLIDGT														
pH1N1/471-PB1								I.....	M.....				R.....	I.....			E.....	K.....															
H5N2/F118-PB1																																	
H9N2-PB1																																	
	410	420	430	440	450	460	470	480	490	500																							
H1N1/WSN-PB1	ASLSPGMMGM	GFNMLSTVL	GV	SI	LN	LGG	KRHT	KTTY	WWDGL	QSSDD	FALIV	NAPN	HEGI	QAGVNR	FYRTCK	LLGIN	MSK	KKSY	INRTGT	FTSFF	RYG												
pH1N1/471-PB1															D.....																		
H5N2/F118-PB1															D.....																		
H9N2-PB1																																	
	510	520	530	540	550	560	570	580	590	600																							
H1N1/WSN-PB1	FVANFSMELP	SFGVSGIN	ESADMS	IGVT	VIKNN	MINNDL	GPAT	AQMA	LQ	LF	IKDY	RYTYR	CHRG	DTQIT	RRSF	EIKKL	WEQTH	SKA	GLLV	DGG	P	N	L	Y	N								
pH1N1/471-PB1																																	
H5N2/F118-PB1																																	
H9N2-PB1																																	
	610	620	630	640	650	660	670	680	690	700																							
H1N1/WSN-PB1	IRNLHIPE	VCLKWELM	DE	DYQ	GR	LC	NPLN	PFV	NH	KDIE	SVNN	AV	IMPA	HGPA	KNME	YDA	VAT	THS	WIP	KRNS	ILNT	SQR	GILE	DE	QMY	QKCC	NLF	EK	FF				
pH1N1/471-PB1																																	
H5N2/F118-PB1																																	
H9N2-PB1																																	
	710	720	730	740	750																												
H1N1/WSN-PB1	PSSSYRRPV	GISSMVEA	MYSRAR	IDAR	IDFES	GRIKKEE	FT	EIMKIC	STIEEL	RRQ	K*																						
pH1N1/471-PB1																																	
H5N2/F118-PB1																																	
H9N2-PB1																																	

3. Complete amino acid sequence alignments of four selected strains based on their PA protein sequence alignments. Protein residues that match H1N1/WSN-PA exactly are hidden (as '.').

	10	20	30	40	50	60	70	80	90	100
H1N1/WSN-PA	MEDFVRQCFNPMIVELAEKAMKEYGEDLKIETNKFAAICTHLEVCFMYSDFHFI	DEQGESIVVELGDPNALLKHRFEIIEGRDRTIAWTIVINSICNTTGA								
pH1N1/471-PA			P.....	R.....I.....S.....				IM.....V.....	V.....	
H5N2/F118-PA			P.....	R.....I.....S.....				M.....V.....	V.....	
H9N2-PA			P.....	R.....MI.....S.....				M.....V.....	V.....	
	110	120	130	140	150	160	170	180	190	200
H1N1/WSN-PA	EKPKFLPDLYDYKKNRFIEIGVTRREVIHYYLEKANKIKSEKTHIHIFSFTGEEMATKADYTLDEESRARIKTRLFTIRQEMASRG	LWDSFRQSERGEET								
pH1N1/471-PA	E.....							S.....		
H5N2/F118-PA	ED.....									
H9N2-PA	E.....									
	210	220	230	240	250	260	270	280	290	300
H1N1/WSN-PA	IEERFETITGMRKLADQSLPPNFSSLENFRAYVDGFEPNGYIEGKLSQMSKEVNA	RIEPPFLKSTPRPLRLPDGPPCSQSRKFLMLDAIKLSIEDPSHEGE								
pH1N1/471-PA			C.....	K.....	RT.....	L.....H.....				
H5N2/F118-PA	R.....N.....		C.....		S.....T.....					
H9N2-PA	R.....		C.....		T.....					
	310	320	330	340	350	360	370	380	390	400
H1N1/WSN-PA	GIPLYDAIKCMRTFFGNKEPNVVKPHEKGINPNYLLSWKQVLAEIQDIENE	EKIPTKNNMKTSQKWLGENMAPEKVD	DDCKDVGD	LKQYDS	DEPEL					
pH1N1/471-PA	K.....	I.....	MA.....		R.....				P.....	
H5N2/F118-PA	K.....D.....	I.....	A.....		K.....		E.....	S.....R.....	S.....	
H9N2-PA	K.....	I.....D.....L.....A.....	G.....	K.....			E.....	S.....R.....	S.....	
	410	420	430	440	450	460	470	480	490	500
H1N1/WSN-PA	RSLASWIQNEFNKACELTDSSWIELDEIGEDAAPIEHIASMRNRYFTA	EVSHCRATEYIMKGVYINTALLNASCAAMDDFQLIPMISKCRTEGRRKTNL								
pH1N1/471-PA	V.....		V.....							
H5N2/F118-PA	S.....		V.....							
H9N2-PA	S.....		V.....					N.....		
	510	520	530	540	550	560	570	580	590	600
H1N1/WSN-PA	YGFIIKGRSHLRNDTDVVNFVSM	EFSLTDPRLPHKWEKVCVLEVGDM	LLRSAIGHVSRPMFLYVRTNGTSKIKMKWGME	MRCLLQSLQQIESMIEAES						
pH1N1/471-PA			I.....	T.....Q.....						
H5N2/F118-PA			I.....	T.....Q.....						
H9N2-PA			I.....	T.....Q.....						
	610	620	630	640	650	660	670	680	690	700
H1N1/WSN-PA	SVKEKDMTKEFFENKSETW	VPVGESEPKGVEEGSIGKVCRTLLAKSVFN	SLYASPOLEGFSAESRKLL	LIVQALRDNLEPGTFDLGGLYE	AIIECLINDPWV					
pH1N1/471-PA		I.....R.....								
H5N2/F118-PA	D.....	I.....S.....								
H9N2-PA	V.....	I.....M.....								
	710									
H1N1/WSN-PA	LLNASWFNSFLTHALR*									
pH1N1/471-PA	K*									
H5N2/F118-PA	K*									
H9N2-PA	K*									

4. Pairwise comparison of each polymerase gene segment and its associated protein(s) among four different influenza A virus strains (H1N1/WSN, pH1N1/471, H5N2/F118 and H9N2). Percentage nucleotide identity is indicated in the bottom left half of the table (highlighted in blue) while protein sequence identity is in the top right half of each table (highlighted in yellow).

PB2	H1N1-WSN	pH1N1-471	H5N2-F118	H9N2
H1N1-WSN		94.6	95.7	95.5
pH1N1-471	85.7		97.0	97.2
H5N2-F118	82.8	83.7		98.3
H9N2	83.2	85.0	87.8	

PB1	H1N1-WSN	pH1N1-471	H5N2-F118	H9N2
H1N1-WSN		95.6	97.1	97.4
pH1N1-471	82.0		96.4	96.3
H5N2-F118	83.5	86.5		98.3
H9N2	84.0	86.1	91.7	

PA	H1N1-WSN	pH1N1-471	H5N2-F118	H9N2
H1N1-WSN		95.7	95.5	95.4
pH1N1-471	84.3		96.7	96.5
H5N2-F118	86.3	89.0		98.2
H9N2	85.8	90.1	93.2	

5. Phylogenetic trees for each polymerase gene of selected four different influenza A virus strains (H1N1/WSN, pH1N1/471, H5N2/F118 and H9N2).

6. Brief summary of the additional proteins encoded by the larger influenza genome segments.

Genome Segment	Additional proteins	Predicted M.W (kDa)	Mechanism	Functions
PB1	PB1-F2	10.54	Ribosomal frameshift	Pro-apoptotic protein with membrane-disrupting properties; contributes to viral pathogenicity; possible virulence factor
	PB1-N40	82.31	Alternative initiations	Interacts with PB2 and the polymerase complex during infection
PA	PA-X	29.00	Ribosomal frameshift	Modulates cellular gene expression; host cell shutoff and limitation of viral pathogenesis
	PA-N155	62.00	Alternative initiations	Required for efficient virus replication by a process that appears to be independent of virus gene transcription
	PA-N182	60.00		

7. Coomassie blue staining of purified His-tagged proteins after refolding: PA-pH1N1-vc007 ~57 kDa (lane 1), PB2-pH1N1-vc007 ~60 kDa (lane 2) and PB1-pH1N1-vc008 ~55 kDa (lane 3) used as antigens for mice immunization. Lane M represents molecular weight markers. Target proteins are indicated by arrows between 50 kDa and 75 kDa markers.

8. Locations of binding sites of monoclonal antibodies against the PA and PB2 proteins (N: N-terminal domain, C: C-terminal domain, L: Linker region and NLS: NLS region).

9. Showing the epitopes (yellow) of mAbs-PA and mAb-PB2 on the 3D structures of PA protein (magenta) and PB2 protein (cyan) respectively. PA-N: PA N-terminal domain, PA-C: PA C-terminal domain, PB2-N: PB2 N-terminal domain, PB2-C: PB2 C-terminal domain. 3D structures of PA and PB2 proteins are adapted from NCBI PDB database.

10. Nucleotide sequence homology of the siRNA constructs with their respective influenza viral genes.

siRNA constructs		Sequence homology of the siRNAs with their respective viral genes
siRNAs against H1N1 strain	siNP-H1N1	1 nt mismatch to H5N2 NP sequence
	siPA-H1N1	match to H5N2 PA sequence
siRNAs against H5N2 strain	siNP-1	2 nt mismatch to H1N1 NP sequence
	siNP-2	3 nt mismatch to H1N1 NP sequence
	siNP-3	4 nt mismatch to H1N1 NP sequence
	siNP-4	1 nt mismatch to H1N1 NP sequence
	siPA-1	3 nt mismatch to H1N1 PA sequence
	siPA-2	2 nt mismatch to H1N1 PA sequence
	siPA-3	6 nt mismatch to H1N1 PA sequence
	siPA-4	6 nt mismatch to H1N1 PA sequence
	siPB1-1	2 nt mismatch to H1N1 PB1 sequence
	siPB1-2	3 nt mismatch to H1N1 PB1 sequence
	siPB1-3	4 nt mismatch to H1N1 PB1 sequence
	siPB1-4	4 nt mismatch to H1N1 PB1 sequence
	siPB2-1	3 nt mismatch to H1N1 PB2 sequence
	siPB2-2	3 nt mismatch to H1N1 PB2 sequence
	siPB2-3	6 nt mismatch to H1N1 PB2 sequence
	siPB2-4	3 nt mismatch to H1N1 PB2 sequence

11. Locations of mutations (green) with their respective amino acid positions from the introductory section are shown on the 3D structures of PA protein (magenta), PB1 protein (red) and PB2 protein (grey). 3D structures of PA, PB1 and PB2 proteins are adapted from NCBI PDB database.

PUBLICATIONS AND POSTERS

Papers:

Myaing, M.Z., Jumat, M.R., Yeo, D.S.Y., Gan, J., Ayi, T.C., Ng, C.G., Wong, P.S., Tobias, Cornvik, Tan, B.H., Sugrue, R.J. (2016) Truncated forms of the PA protein containing only the C-terminal domains are associated with the RNP complex within H1N1 influenza virus particles. (Submitted to Journal of General Virology)

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Biruhalem, T., Chen, Hui., Myaing, M.Z., Tan, B.H., Sebastian M.S., Sugrue, R.J. (2015) Integration of host gene expression from human and low pathogenic avian influenza A virus infections with interactome and small interfering RNA (siRNA) screen highlights new plausible host targets. (Submitted to BMC Genomics)

Sutejo, R., Yeo, D.S.Y., Myaing, M.Z., Hui, C, Xia, J., Ko, D., Cheung, P.C., Tan, B.H., Sugrue, R.J. (2012) Activation of type I and III interferon signalling pathways occurs in lung epithelial cells infected with low pathogenic avian influenza viruses, PLoS One. 7(3): e33732.

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Posters:

“Characterization of Monoclonal Antibodies against PA Subunit of 2009 Pandemic H1N1 Influenza Virus and Epitope Mapping” at 12th NUS-Nagasaki Joint Symposium, NUS, Singapore (2015).

“*In Vitro* Evaluation of siRNAs against Low Pathogenic Avian Influenza H5N2 Virus” at 34th Annual Meeting of the American Society for Virology at London, Ontario, Canada (2015).