MAMMALIAN ADAPTATION MECHANISM OF INFLUENZA A VIRUS REPLICATION MECHINERY

A Thesis

Submitted to the College of Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the

Department of Veterinary Microbiology

Western College of Veterinary Medicine

University of Saskatchewan

Saskatoon

Canada

By

Fangzheng Wang

PERMISSION TO USE

In presenting this thesis/dissertation in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis/dissertation in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis/dissertation work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis/dissertation or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis/dissertation. Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or part should be addressed to:

Head,
Department of Veterinary Microbiology
Western College of Veterinary Medicine
University of Saskatchewan
52 Campus Drive
Saskatoon, Saskatchewan S7N 5B4
Canada

OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9
Canada

ABSTRACT

The incompatibility between avian influenza viruses and host factors within mammalian hosts constitutes the major host restriction on influenza A virus polymerase complex. To overcome restrictions of viral polymerase complex, the virus has evolved diverse adaptive strategies. The PB1 gene segment originated from avian strains has recurrently appeared in 1918, 1957, and 1968 pandemic viruses. Although this recurrent selection of the avian-origin PB1 segment has been correlated with enhanced viral polymerase activity in mammalian cells, the underlying mechanism behinds this phenomenon is still enigmatic. Using 2009 pH1N1 virus which naturally lacks the avian-origin PB1 segment and the canonical mammalian-signature PB2 E627K mutation, we demonstrate that the avian-origin PB1 can markedly enhance functions (both replication and transcription) of pH1N1 polymerase in human cells even in the absence of canonical PB2-associated adaptive mutations (E627K and G590S/Q591R). Mechanistically, acquisition of avian-origin PB1 does not change the interaction between polymerase subunits, or assembly of polymerase complex and viral ribonucleoprotein. In viral replication, acquisition of the avian-origin PB1 specifically facilitates the vRNA synthesis of 2009 pH1N1 polymerase by stimulating the *trans*-activation on cRNA-associated polymerase, thereby leading to the enhanced overall replication and polymerase activity.

We extrapolate our finding to the function of acidic nuclear phosphoprotein 32 family member A (ANP32A), the major host factor underlying host restrictions on viral polymerase complex. We substantiate that the SR polymorphism (G590S/Q591R) is also deployed by viruses to accommodate the species difference in ANP32A, which is like the function of PB2 E627K. Our results also indicate that chicken ANP32A can specifically enhance vRNA synthesis of avian-like (PB2 627E) polymerase by promoting the *trans*-activating effect in replication without altering the genuine vRNP assembly capacity.

Collectively, our results suggest that the *trans*-activation process in vRNA synthesis of un-adapted avian influenza polymerase is a defective step that needs to be restored via mammalian adaptation of viral polymerase complex.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Yan Zhou for recruiting me into her lab three years ago, when the world of molecular virology opened to me. As the saying goes, the strict teacher produces outstanding students. The spiritual strength built under three-year hard training would serve as the beacon guiding me to sail through any mist on the sea of life. For this specialized training and guidance which I would never get from my parents, I show my deepest gratitude to her. I also want to thank Dr. Janet Hill and Dr. Joyce Wilson, who are in my committee, to guide me and help me in the past 3 years.

I would like to acknowledge the help from my lab mates: Hongsu, Guanqun, Magda, Shelby, Amit, Yao, and Dr. Qiang Liu for their help both in the lab and in the life. Hongsu and Guanqun, you two were like the big brothers who taught me the experiment 101 when I was still a freshman in the lab. Especially Guanqun, your support to my lab life is tremendous. I also want to thank Amit as well as Guanqun, who make me feel not alone in the lab in most of the vacations and late nights.

I am very grateful to the funding agencies. The Public Health Agency of Canada (PHAC) funding grant to Dr. Yan Zhou, the Western College of Veterinary Medicine, and Saskatchewan Innovation Scholarship. Without these generous funding agencies, I would never complete this project.

I also want to thank my dear friends who came to Canada to visit me while I could not come back to my hometown. The time we spent together in Banff and Jasper is the most precious memory in the past 3 years which encourages me to accomplish my current project and look forward.

Finally, I want to thank my parents who love and support me thousand miles away. As the only child in the family, I have not come back to home for more than three years, I really feel indebted to you.

Human nature should always be superior to science.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANP32A	Acidic nuclear phosphoprotein 32 family member A
BSA	Bovine Serum Albumin
chANP32A	Chicken acidic nuclear phosphoprotein 32 family member A
cRNA	Complementary RNA
cRNP	Complementary ribonucleoprotein
C-terminal	Carboxyl-terminal
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleotide-5′-triphosphate
DTT	Dithiothreitol
E	Glutamic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
GFP	Green fluorescent protein
HA	Haemagglutinin
293T	Human embryonic kidney 293T cells
Hr	Hour
h.p.i.	hours post-infection
h.p.t.	hours post-transfection
K	Lysine
kDa	Kilodalton
M1	Matrix protein 1
M2	Matrix protein 2
MDCK	Madin-Darby canine kidney
MEM	Minimum Essential Medium
mRNA	Messenger ribonucleic acid
MOI	Multiplicity of infection
N	Asparagine
NA	Neuraminidase

NCR	Non-coding region
NEP	Nuclear export protein
NES	Nuclear export signal
NLS	Nuclear localisation signal
NP	Nucleoprotein
N-terminal	Amino-terminal
NS	Non-structural
NS1	Non-structural protein 1
ON	A/Turkey/Ontario/6213/1966 (H5N1)
ORF	Open reading frame
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming unit
pH1N1	A/Halifax/210/2009 (H1N1)
poly (A)	Polyadenylic acid
poly (U)	Polyuridylic acid
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
S	Serine
SD	Standard deviation
vRNA	Viral ribonucleic acid
vRNP	Viral ribonucleoprotein
WT	Wild-type

CHAPTER 1. LITERATURE REVIEW

1.1 BIOLOGY OF INFLUENZA A VIRUS

1.1.1 Influenza virus taxonomy and nomenclature

Influenza viruses are negative-sense, single stranded RNA virus belonging to the family of *Orthomyxoviridae*. The viruses in *Orthomyxoviridae* family can be further divided into seven different genera: *Alphainfluenzavirus* (influenza A virus), *Betainfluenzavirus* (influenza B virus), *Gammainfluenzavirus* (influenza C virus), *Deltainfluenzavirus* (influenza D virus), Isavirus, quaranjavirus, and thogotovirus [1]. The four genera of influenza viruses are categorized by the antigenic difference in their internal structural proteins, matrix (M) and nucleocapsid (NP) [1]. Based on antigenicity of hemagglutinin (HA) and neuraminidase (NA), influenza A virus could be further divided into different subtypes. Until now, 18 different subtypes of HA and 11 types of NA have been identified [2]. The nomenclature system for influenza virus comprises the host of origin, geographical origin, strain number, and year of isolation [1]. The subtypes of HA and NA should also be described in parentheses for influenza A viruses. For human isolates, the host of origin can be omitted. For instance, A/Halifax/210/2009 (H1N1) indicates the human influenza A virus isolated in Halifax as strain 210 in 2009. This virus can be categorized into H1N1 subtypes based on antigenicity of its HA and NA proteins.

Influenza A virus triggers outbreaks of all influenza pandemics in history. It is also the major pathogen that leads to annual influenza epidemics. Due to recurrent pandemics and annual epidemics, influenza A virus has long been the focus of research. Unlike influenza A virus, the host range of influenza B virus is restricted among human, seal, and ferret. Influenza B virus can only cause occasional epidemics. Influenza C virus is less common compared to influenza A virus and influenza B virus, and it is only responsible for certain minor symptoms in infected patients [3]. The recently identified influenza D virus is believed to primarily infect cattle [4].

1.1.2 Influenza A virion

The virion of influenza A virus is pleomorphic. The spherical or elliptical phenotypes are dominant in laboratory strains, whereas clinical isolates mostly display filamentous phenotypes. The diameter of spherical and elliptical forms usually range from 80-120 nm, but, the diameter of the elongated filamentous structures can reach more than 300 nm and up to 20 μm [5]. The Influenza A virion is enveloped by the host-derived lipid bilayer beneath which the matrix proteins forms the shell lining the inner surface of the lipid envelope [6]. HA, NA, and M2 proteins are anchored to the lipid envelope through their transmembrane domains [7, 8]. The core of the virion is the viral genome packaged in eight viral ribonucleoprotein (vRNP) complexes. Each RNP is composed by individual viral RNA gene segment encapsidated by NP protein and the heterotrimeric polymerase complex consisting of polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), and polymerase acidic protein (PA) [9]. Additionally, non-structural 1(NS1) protein and nuclear export protein (NEP) are also found inside the virion [10, 11]. The schematic diagram of influenza A virion is in (Figure 1.1).

1.1.3 The influenza A virus life cycle

1.1.3.1 Virus attachment

As shown in (Figure 1.2), the life cycle of influenza A virus is started with the recognition and binding of sialic acid (N-acetylneuraminic acid) receptors on cell surface by the integral membrane protein HA. The sialic acid can bind to galactose through the linkage between the carbon-2 of sialic acid hexose and the carbon-3 (SAα2, 3Gal) or carbon-6 (SAα2, 6Gal) of galactose hexose [12, 13]. The distribution of these two types of sialic acid receptors is different among avian species and human, representing one of the major host-species barriers in cross-species transmission of influenza A virus [14, 15]. The avian influenza virus preferentially binds to the α2, 3 linked sialic acid receptor which is dominant in the guts epithelial cells of bird or human lower respiratory tract. Conversely, human strains display stronger affinity to α2, 6 linked sialic acid receptor which is mainly found in the human upper respiratory tract [12, 16]. Due to the low affinity of HA of avian influenza viruses to the sialic receptor present on human upper respiratory tract, the virus usually requires certain adaptive mutations in its HA to accommodate the 2, 6 linked sialic acid receptor. For instance, E190D (glutamic acid to aspartic acid mutation at 190 residue) or G225E (glycine to glutamic acid mutation at 225 residue) in HA can alter the

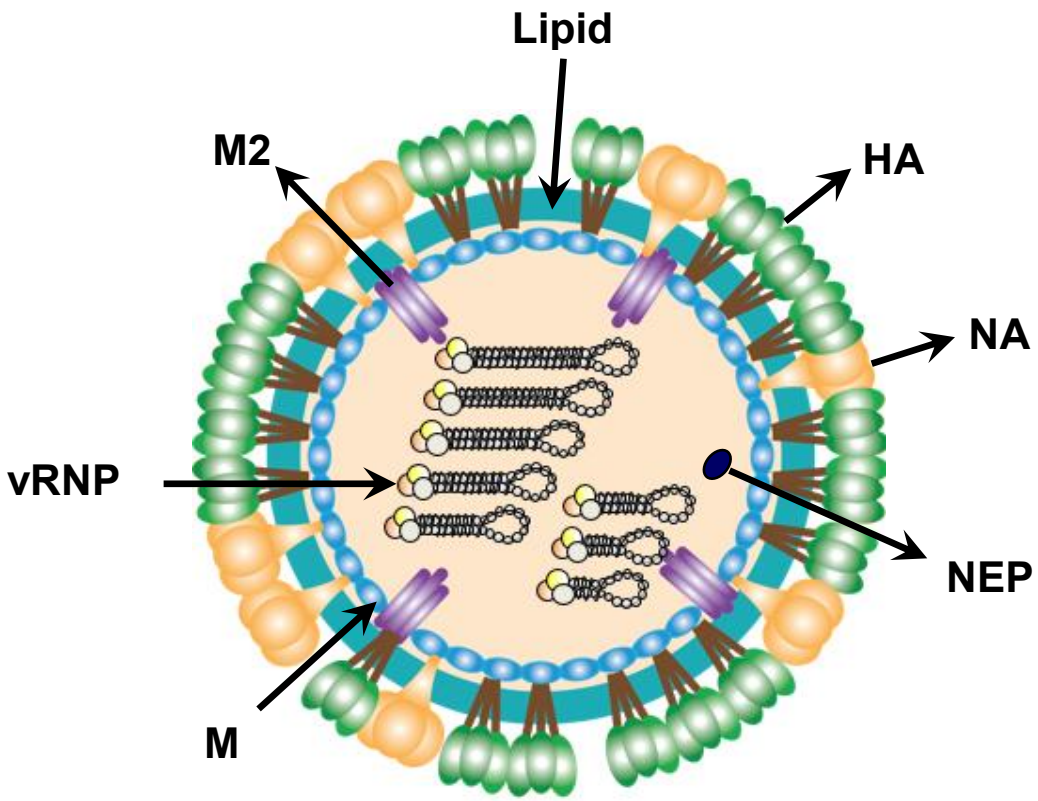
receptor binding specificity of avian influenza viruses, thus rendering avian strains the efficient binding affinity to 2, 6 linked sialic acid receptors present in human cells [17, 18]. Pigs are considered as the 'mixing vessels' of influenza A viruses due to the abundant distribution of both α2, 3 and α2, 6 linked sialic acid receptors in trachea cells [19].

1.1.3.2 Virus entry, fusion, and uncoating

Following the attachment of hemagglutinin to sialic acid receptor, the virus is internalized through clathrin-mediated endocytosis [20]. In addition to virus attachment, hemagglutinin is also critical to virus fusion [21]. The low pH environment in the endosome triggers the conformational changes in HA molecule (HA1+HA2), which allows the fusion peptide in HA2 to attach to the endosome membrane, resulting in the fusion of virus and endosome membranes [21, 22]. The sialic acid receptor binding domain in HA1 mediates virus attachment; the fusion peptide at N-terminus of HA2 mediates virus fusion [21]. Meanwhile, the transmembrane M2 protein serves as the ion channel to conduct H+ ions from endosome into virus, creating the acidic environment within the virion. The acidification of interior environment of virion then leads to the dissociation of vRNPs from matrix proteins [23]. From the fusion pore formed by HA molecules, the vRNPs are released into cytoplasm [24]. In summary, the presence of free vRNPs in the cytoplasm is the results of concerted HA-mediated virus fusion and M2-mediated release of vRNPs.

1.1.3.3 Nuclear import

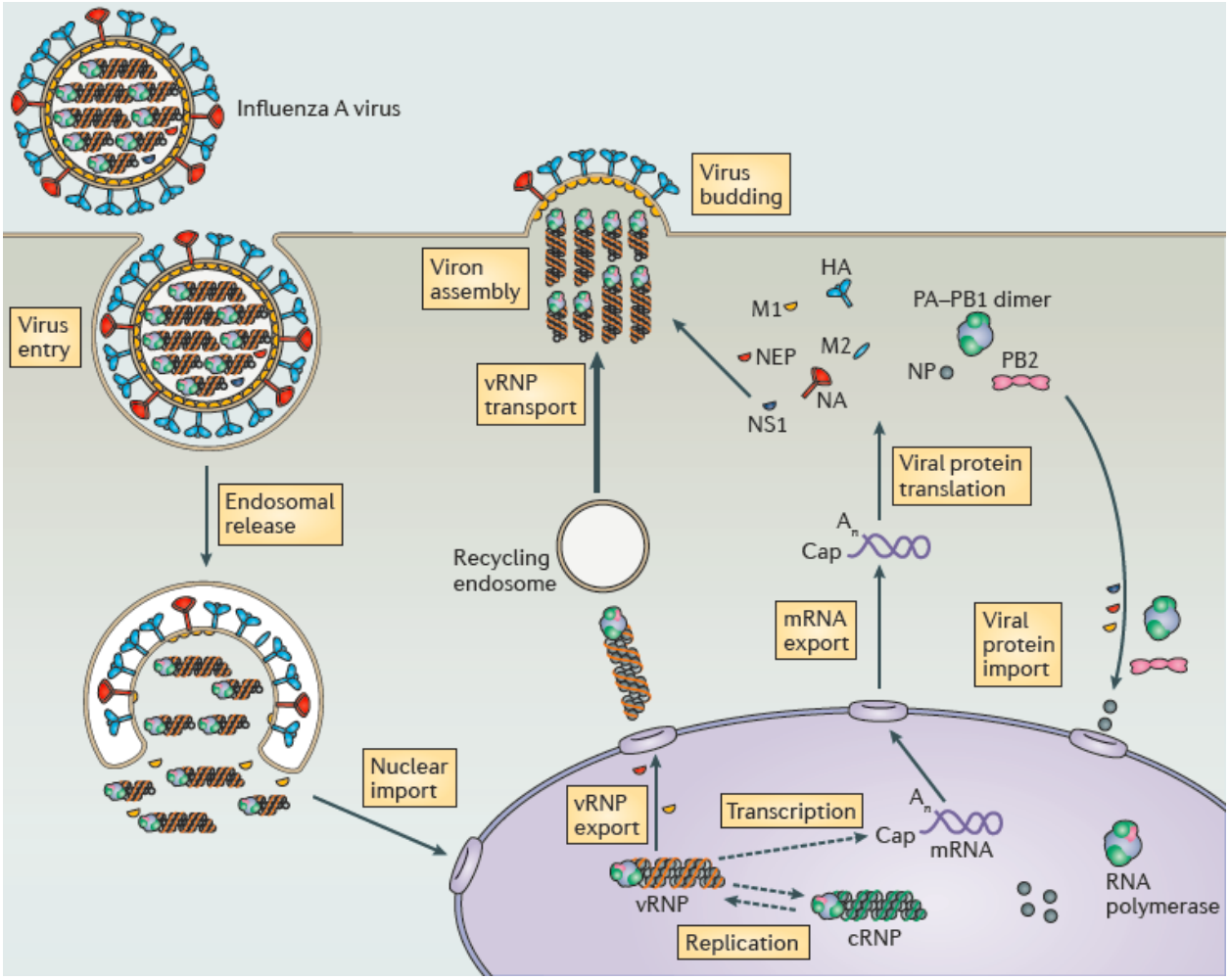
As the RNA synthesis of influenza virus occurs in the nucleus, the free vRNPs in the cytoplasm are trafficked into nucleus using the importin-α / importin-β1 nuclear import pathway [25]. Although all the viral proteins (PB2, PB1, PA, and NP) constituting the vRNPs contain nuclear localization signals (NLSs), the NLS motifs in NP protein are believed to associate with importin-α, and then in turn to associate with importin-β1 to form the ternary importin-α-importin-β1-vRNP complex. This complex subsequently traverses the nuclear pore complex [26], where RAN-GTP binds to importin-β1 leading to the release of importin-α-vRNP in the nucleoplasm [25, 27].



Adapted from Guanqun Liu's original figure with permission.

Figure 1.1 Schematic diagram of influenza A virion.

Influenza A virion with HA, NA, and M2 (three envelope proteins), M1, PB2, PB1, PA, NP (internal proteins), and non-structural protein NEP. The lipid bilayers of the virus is from host cellular membrane.



Reprinted from Nature Reviews. Microbiology, 14(8), 479–493. (te Velthuis et al., 2016) with permission.

Figure 1.2 The life cycle of influenza A virus [9].

The life cycle of influenza virus starts with the attachment of HA molecule to the sialic acid receptors on the cell surface. After virus enters the cytoplasm through endocytosis, vRNPs are released into the cytoplasm and subsequently being transferred into the nucleus for the viral replication and transcription. After translation of viral mRNA, the newly synthesized viral proteins and nascent vRNA would be used for assembly of progeny virions.

1.1.3.4 Virus replication and transcription

Once the vRNPs have been in the nucleus, they are used for the subsequent replication and transcription [28]. The details of RNA synthesis processes will be reviewed in later sections. The viral transcription is a primer-dependent process in which a capped primer generated by cleaving the host pre-mRNA initiates the transcription and leads to the synthesis of 5' capped and 3' polyadenylated viral mRNA [29, 30]. During viral replication, the vRNA is first used as template to synthesize the full-length complementary RNA (cRNA), which in turn is used for the synthesis of progeny vRNA. Unlike viral mRNA, cRNA lacking 5' cap and 3' poly (A) tail is the exact copy of vRNA [31]. Additionally, the synthesis of both vRNA and cRNA belongs to de novo replication which is independent of the primer [31]. The newly synthesized vRNA is encapsidated into vRNP by association with viral polymerase complex and nucleoprotein, waiting for the nuclear export.

1.1.3.5 Nuclear export

Following the viral RNA synthesis and RNPs assembly, the newly synthesized RNPs are exported from nucleus into the cytoplasma for the subsequent genome packaging and virus assembly [32]. The nuclear export of vRNPs is achieved using the Crm1 nuclear export pathway, in which both M1 protein and NEP (nuclear export protein) are involved [33-35]. It has been noticed that the expression of M1 protein occurs in the late stage of infection, suggesting that M1 protein controls nuclear export until RNA synthesis is completed [36]. Similarly, the slow accumulation of NEP caused by utilization of erroneous splicing site is also believed to orchestrate the delayed timing of nuclear export. Optimization of the weak NEP 5' splice site increases the production of NEP, which in turn results in premature vRNP export. [37]. M1 protein alone cannot fully control RNP export, as the nuclear export signals (NESs) motif in M1 protein does not directly link the vRNP to Crm1 protein [38, 39]. Instead, the NES motifs in NEP mediates the direct interaction between NEP and Crm1-RAN-GTP, which in turn associates with M1 protein to form the vRNP-M1-NEP-Crm1-RAN-GTP (daisy chain complex) for nuclear export [33, 38, 40]. Another potential role of NEP in nuclear export is to prevent the nuclear export complex from re-entering the nucleus by masking the NLS in M1 protein [33]. In addition to the Crm1 nuclear export pathway, other mechanisms are also involved in promoting the export of vRNPs. The

infection of influenza A virus triggers apoptosis, in which caspase 3 is activated resulting in increased diffusion size of the nuclear pore, thus promoting nuclear export [41].

The exported vRNPs are accumulated in the perinuclear cytoplasm where the microtubule organising centre (MTOC) is located [42, 43]. Here, vRNPs can bind to recycling endosomes through interaction between PB2 protein and Rab11, which in turn directs transportation of vRNPs to the cell periphery using vesicular transport pathway [44, 45].

1.1.3.6 Genome packaging

To generate the infectious progeny virions, the exact eight viral genomic segments must be incorporated into each virion. Mechanistically, two models were proposed to explain the genome packaging of influenza A virus [46]. Initially, the genome packaging was believed to occur randomly. However, compelling evidences now favour an alternative mechanism, in which only a single copy of each vRNP segment is selectively incorporated into the virion. This selective packaging mechanism is verified by the direct observation of a '1+7' configuration of vRNP packaging using transmission electron microscopy [47, 48]. Based on that observation, one vRNP in the center is circled by the remaining 7 vRNPs during packaging. Further studies on disposition of vRNPs show that the eight vRNPs are interconnected in a 'transition zone' just beneath the matrix later at budding tip of influenza A virion. Within this zone, the intermolecular interactions between vRNP/vRNP mediated by packaging signals on each vRNA segment take place and result in the formation of vRNP supramolecular complex which is essential for the selective packaging of the viral genome [49-51].

1.1.3.7 Virus assembly, budding, and release

Virus assembly and budding take place in the lipid rafts, microdomains of plasma membrane with an abundance of cholesterol and sphingolipids [52]. Following translation and post-translational modifications of HA and NA, these viral proteins are directed to associate with lipid rafts via signals in their transmembrane domains [53, 54]. With the accumulation of HA and NA in lipid rafts, the plasma membrane curvature is altered and virus budding is initiated [55]. The M1 protein is recruited to lipid rafts through binding to the cytoplasmic tails of HA and NA

[56]. As M1 protein also interacts with vRNPs, the polymerization of M1 protein then facilitates the incorporation of vRNPs [55]. M2 protein is targeted to the periphery of lipid rafts via interaction with M1 protein, causing the positive membrane curvature and the subsequent membrane scission which results in the release of progeny virions [57-59]. Once virus budding is completed, the HA of the nascent virions still attaches to the sialic acid receptor on the cells. At this point, NA is required to remove the sialic acid presenting on the cells and viral proteins, thus avoiding virus accumulation at the cell surface or aggregation between progeny virions [60, 61].

1.2 THE RNA SYNTHESIS MECHINERY OF INFLUENZA A VIRUS

1.2.1 Influenza A ribonucleoprotein

Each of the eight viral gene segments is encapsidated into viral ribonucleoprotein, which is the basic replication unit of influenza A virus [9]. The negative-sense viral RNA is coated by NP protein, and its 5' and 3' termini are bound by viral polymerase complex consisting of PB2, PB1, PA. Opposite to the polymerase-bound end, the internal vRNA is closed by forming a loop [28, 62]. Structural analyses on reconstituted RNPs or virion-derived RNPs show that the native influenza vRNP displays an antiparallel double helical conformation which is maintained by minor groove defined by connected NP monomer [63, 64]. Each NP monomer is bound to approximately 24 nucleotides [65]. Similarly, the positive-sense cRNA is also encapsidated into complementary ribonucleoprotein (cRNP) which displays similar structure [66] as vRNP. The schematic diagram of vRNA is shown in (Figure 1.3) [28].

1.2.2 The RNA-dependent RNA polymerase

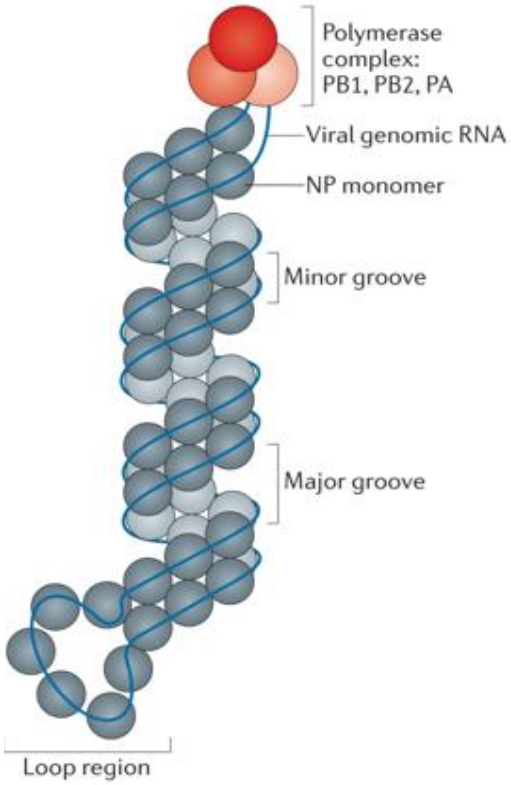
The replication centre of influenza A virus is the 250 kDa heterotrimeric viral polymerase complex composed by polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), and polymerase acidic protein (PA). Both transcription and replication of the negative-sense viral RNA are carried out by the polymerase complex [31]. For the assembly of trimeric polymerase complex, it has been demonstrated that PB1 and PA form the dimer prior to the entrance of nucleus and assembly with PB2 [67]. Recent crystal structure indicates that PB1 binds to PA and PB2 respectively through its N-terminus and C-terminus, forming a U-shaped structure [68].

1.2.3 Viral nucleoprotein

As the major component of RNP, NP protein is critical to the ongoing viral replication and transcription [69]. NP was once considered as the switcher for transition of viral polymerase from transcriptase to replicase [70, 71]. However, recent studies have substantiated that NP represents an elongation factor for viral RNA synthesis, and it is entirely dispensable for viral transcription and replication [72, 73]. The 56 KDa NP protein encoded by gene segment 5 displays a curved, crescent-like shape which can be divided into a head domain, a body domain, and tail-loop [74, 75]. Particularly, this tail loop mediates the oligomerization of NP protein by inserting into the body domain of the neighbouring NP molecule. NP protein with mutations disrupting the tail loop could only exist as monomer [75]. Oligomerization of NP protein is also required for active RNP activity. Mutations in tail loop disrupting the NP oligomerization impair the vRNP activity [76]. Furthermore, although NP protein shows high affinity to viral RNA in a sequence-independent manner [77, 78], the recruit of NP protein to nascent RNP is mediated by NP oligomerization instead of the RNA binding ability [72].

1.2.4 Viral RNA promoter

Prior to the available structure of viral polymerase complex binding to the viral RNA, different models have been proposed for influenza viral RNA promoter based on extensive biochemical studies. Particularly, the proposed vRNA promoter regions among all these models (panhandle model, fork model, and the corkscrew model) both involve the conserved 3’ terminus and 5′ terminus of vRNA noncoding regions. [79-82]. As expected, it has already been confirmed that the viral RNA promoter region is indeed composed by the highly conserved 12 nucleotides at 3′ end and 13 nucleotides at the 5′ end of vRNA.



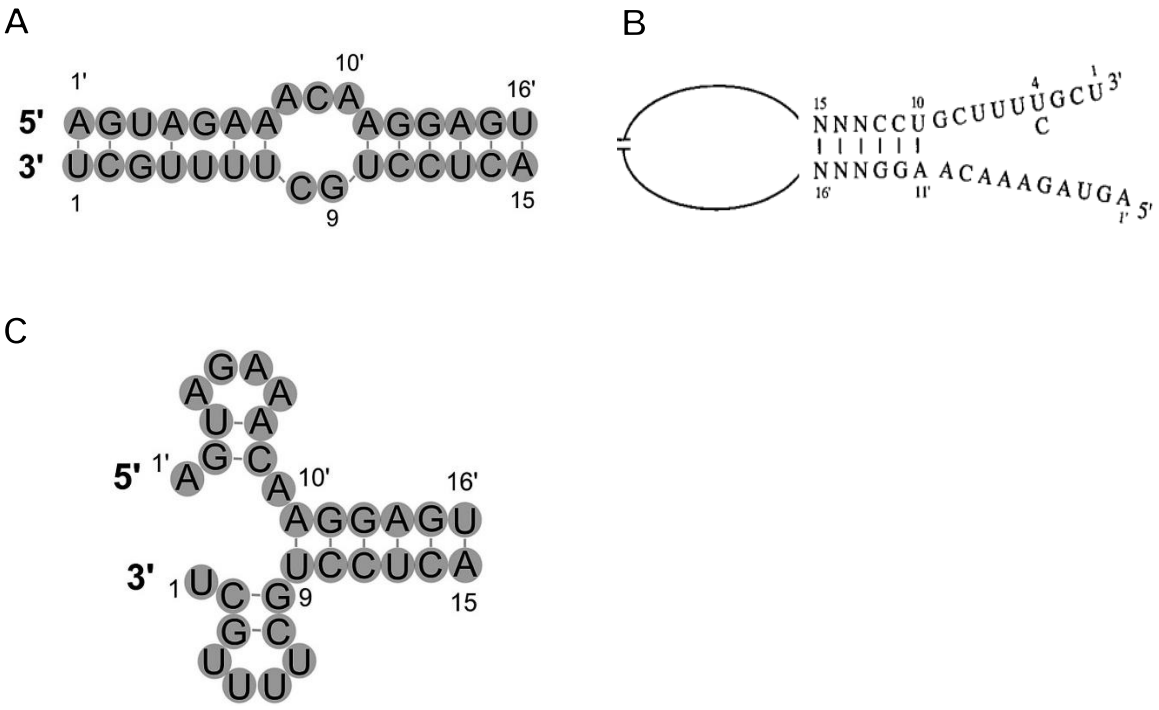
Reprinted from Nature Reviews. Microbiology, 13(1), 28 (Eisfeld et al., 2015) with permission.

Figure 1.3 Schematic diagram of influenza vRNP complex [28].

vRNA is composed of the trimeric polymerase complex (PB2, PB1, and PA), a negative-sense, single-stranded vRNA associated with NP protein. The two termini of vRNA are partially complementary and form the double-stranded structure, where is also the place bound by the trimeric polymerase complex.

Due to the partial and inverted complementarity of the 3’ terminus and 5′ terminus of vRNA, three proposed RNA structures for the annealed ends has been proposed. In the panhandle model, the termini of vRNA forms a non-canonical base-paired duplex (Figure. 1.4 A) [83, 84]. Unlike the panhandle model, the fork model proposed the partially double stranded structure with two ends of vRNA remain as single-stranded. In this way, the polymerase complex binds to the double stranded region and 5’ end of the single-stranded region; the remaining single-stranded 3’ terminus serves as the template for transcription initiation (Figure. 1.4 B) [81, 85] . Later, a ‘corkscrew model’ was proposed. In corkscrew model, the promoter region is divided into a distal six base pair RNA rod, a proximal two stem-loop structures at the ends of vRNA, and an unpaired adenosine at position 10 of 5’ end of vRNA (Figure. 1.4 C) [82, 86]. Both of fork and corkscrew models proposed the distal double-stranded region, even though they have discrepancies concerning to the existence of internal stem-loop at each strand of vRNA. The first available polymerase structure of a bat influenza A virus reveals that 1-10 nucleotides of 5’ end form the intramolecular stem-loop or the “hook” structure which is similar to the prediction of corkscrew model. However, the nucleotides 1-9 of 3’ end remain single-stranded as proposed by the fork model. For the distal promoter element, the nucleotides 10-14 of 5’ end and nucleotides 11-15 form the base-paired duplex [68, 87].

In a similar fashion, the stem-loops structure proposed in the corkscrew model is also required for the cRNA promoter activity [88]. This has been confirmed by the recent available structure of influenza polymerase binding to the 5’ cRNA, in which the 5’ end of cRNA forms the “hook’ and binds to the viral polymerase complex [89] .



Reprinted from Proceedings of the National Academy of Sciences, 111(32), E3335-E3342 (Tomescu et al., 2014), Journal of virology, 69(7), 4012-4019. (Fodor et al., 1995) with permissions

Figure 1.4 The schematic diagrams of different influenza vRNA promoter.

The schematic diagrams of panhandle model (A), fork model (B), and corkscrew (C) model of vRNA promoter [85, 90].

1.3 TRANSCRIPTION

1.3.1 Initiation

Following the nuclear import of incoming vRNPs, the synthesis of viral mRNA occurs. Viral transcription is carried out by vRNP-associated polymerase which is bound to the 3’ and 5’ termini of vRNA [81, 91]. Viral mRNA synthesis requires primer-dependent initiation which involves a 5’ capped primer preferentially derived from host noncoding RNAs (promoter-

associated capped small (cs) RNAs, small nuclear (sn) RNAs, and small nucleolar RNAs (sno) RNAs) by cap-snatching [92, 93]. Cap-snatching is achieved through the cap-binding function of PB2 protein and the endonucleolytic activity of PA protein [94]. Mechanistically, the binding of the 5' end of the vRNA to the PB1 protein induces conformational changes of polymerase complex, which enables the PB2 protein to bind to the 5' cap structure of the host noncoding RNAs. Concomitantly, the endonuclease domain of PA protein cleaves the capped RNA at approximately 8-14 nucleotides from the cap structure. After cleavage, both the 3' ends of capped primer and vRNA are transferred into the polymerase active site for the subsequent transcription initiation. By direction of penultimate C residue (C2) or the G residue at position 3 (G3) in vRNA, a G or C residue is accordingly added to the 3'end of the capped primer, and thus initiates the viral transcription [85].

1.3.2 Elongation and polyadenylation

Elongation of viral mRNA chain proceeds in a template-dependent manner in which the vRNA template is threaded through the active site of polymerase until encountering the poly-uridine stretch located 16 nucleotides before the 5' end of the vRNA. At this stage, the 5' end of the vRNA is still bound to the viral polymerase with the vRNA template being read in a 3' to 5' direction [9]. A 5-7 uridine residues near the 5' end of the vRNA serve as the polyadenylation signal for viral mRNA synthesis, because the polymerase cannot read beyond the uridine-stretch due to steric hindrance caused by the 5'end of the vRNA which is still associated with the transcribing polymerase [30, 81, 95]. This polymerase stuttering leads to the reiterative copying of the poly-U stretch and thus generate the poly (A) tail of viral mRNA. In the meanwhile, the 5' cap is detached from the PB2 protein and is bound by the nuclear cap-binding complex (CBC), leading to the assembly of host mRNP (messenger ribonucleoprotein)-like structure [96].

1.3.3 Splicing

In eukaryotic cells, pre-mRNAs have to undergo splicing, an essential process for gene expression in which the introns of pre-mRNAs are removed and the exons are accurately joined [97]. Influenza A virus can widen its proteomic diversity via the alternative splicing by hijacking

the cellular splicing machinery [98]. Up to date, only the PB2, M, and NS segments are known to encode the viral proteins in spliced mRNA. The PB2 segment can produce PB2 mRNA and PB2-S1 mRNA [99]; M segment respectively produces 4 mRNA transcripts (M1, M2, M3, and M42) [100, 101]; NS segment produces NS1 mRNA, NEP mRNA, and NS3 mRNA [102]. But, unlike the cellular splicing which is highly efficient, the splicing efficiency of viral mRNA has to be relatively lower with the ratio of spliced to unspliced transcripts at about 10% as both of them must be translated into viral proteins [103].

1.4 REPLICATION

Unlike viral transcription, replication is a two-step *de novo* synthesis process which produces full-length copies of viral RNA [9]. The negative-sense vRNA would be first used as template to synthesize the complementary RNA (cRNA), which is the replicative intermediate [104]. Subsequently, the cRNA would be used for the synthesis of progeny vRNA. As vRNA acts as the template for both the synthesis of cRNA and mRNA, the two molecules of which are produced in distinct mechanisms, a switching model of polymerase from transcriptase to replicase has been proposed [104, 105]. In this switching model, the accumulation of NP protein is hypothesized to switch the viral polymerase from transcription status to replication status. However, this model has been challenged by the observation that polymerase alone can perform the cRNA synthesis even in the absence of NP protein [104]. Alternatively, a cRNA stabilization model has been proposed to explain these discrepancies. In the stabilization model, the vRNP-associated polymerase is capable of synthesizing both cRNA and mRNA, but the cRNA is degraded due to the lack of protection from polymerase and NP protein, and thus there is a bias toward viral transcription at early time of viral infection [104].

1.4.1 cRNA synthesis

The first step of viral replication is the synthesis of the replicative intermediate cRNA. It is believed that after the incoming vRNP entered the nucleus, the vRNP-associated polymerase (the resident polymerase) could catalyse the synthesis of cRNA. This idea has been supported by biochemical studies that the virion-derived vRNP can produce the cRNA *in vitro* [66, 106]. Unlike

mRNA which possesses the 5’ cap, vRNA contains a 5’ triphosphate, and thus its replication is initiated in a primer-independent way [107]. The initiation of cRNA synthesis begins with the translocation of 3’ end of the vRNA template into the active site of polymerase. Through terminal initiation on vRNA promoter, a pppApG dinucleotide is formed on U1 and penultimate C2 of the 3’ terminus of the vRNA, and being further used for the subsequent elongation [107]. As cRNA is the full-length copy of the vRNA, the 5’ terminus of the vRNA template bound to the binding pocket needs to be released. The nascent cRNA is bound by a second, newly synthesized polymerase and NP protein for the assembly of complementary ribonucleoprotein (cRNP) [9].

1.4.2 vRNA synthesis

The synthesis of vRNA using the cRNA template is the second step of viral replication. Although vRNA synthesis is also a primer-independent process, its initiation mechanism is different to the *de novo* initiation used in cRNA synthesis. Unlike the terminal initiation of cRNA synthesis, the initiation of vRNA synthesis occurs on the residues U4 and C5 of the 3’ cRNA template (internal initiation). The pppApG dinucleotide is then realigned to residue U1 and C2 of the cRNA 3’ end to allow the subsequent elongation of full-length nascent vRNA [107]. Another difference between the synthesis of cRNA and vRNA is that the synthesis of vRNA requires a second polymerase (*trans*-polymerase) besides the resident polymerase of cRNP [66, 91]. Currently, the function of this additional second polymerase is not fully clear. However, based on different observations, a *trans*-acting model [91] and a *trans*-activating model [66] have been respectively proposed to elaborate the vRNA synthesis process.In the *trans*-acting model, the second polymerase (*trans*-acting polymerase) is believed to perform the synthesis of vRNA [91]. The 3’ terminus of the cRNA template is translocated into the active site of the *trans*-acting polymerase for subsequent internal initiation and elongation. The end of the nascent vRNA is probably bound by a third polymerase for the assembly of vRNP. This *trans*-acting model is supported by the *trans*-complementation assays [91] and the observation of branched arrangement of RNP complexes in cryo-EM [63]. However, a later study reported that the addition of a catalytically inactive *trans*-polymerase to isolated cRNA can still drive the synthesis of vRNA [66]. To resolve the discrepancies among these observations, a *trans*-activating model is proposed. In the *trans*-activating model, the polymerase actually performing the vRNA synthesis is the

cRNA-associated polymerase instead of the second *trans*-polymerase [66]. It is proposed that the second polymerase (*trans*-activating polymerase) induces the conformational change in resident polymerase of cRNP and thus leads to the transfer of 3' end of cRNA to the cRNP-associated polymerase for the further vRNA synthesis. In this model, the 5' end of the nascent vRNA is bound by the *trans*-activating polymerase instead of the third polymerase proposed in *trans*-acting model. The *trans*-activating model is favoured by the fact that the catalytically inactive *trans*-polymerase is still potent in facilitating vRNA synthesis.

1.5 HOST RESTRICTIONS AND ADAPTIVE STRATEGIES OF POLYMERASE

The natural reservoirs of influenza A virus are aquatic birds. However, the virus in the avian species could sporadically jump into other mammalian hosts or even human populations [108]. Most of the cases of human infection with avian influenza virus are usually the dead-end infections which would not lead to further human to human transmission due to species barriers. But, on certain rare occasions such as 1918 influenza pandemic, the virus could acquire adaptive mutations to overcome these species barriers and establish itself as the human-adapted strain which can sustainably transmit among human population [109].

To evolve into a pandemic virus, a strain must develop the countermeasures to jump the species barriers that severely restrict the replication of the virus. Generally, the host restrictions could be categorized into the low receptor binding affinity [17], poor polymerase activity [110], and inability to counteract host innate immune responses [111]. In the following paragraphs, the host restrictions and adaptation on viral polymerase complex will be mechanistically elaborated.

1.5.1 The canonical PB2 E627K mutation and SR polymorphism

PB2 subunit is one of the major determinants of host range, and its role in host adaptation has been intensively studied. The majority of the avian influenza polymerases possess a glutamic acid at 627 residue of their PB2 segment. Intriguingly, a single glutamic acid to lysine mutation at this PB2 627 residue (PB2 E627K) can fully restored the impaired polymerase function of avian influenza viruses in human cells [17]. The increased viral polymerase activity mediated by PB2 E627K is also correlated with the enhanced pathogenicity and transmissibility [112]. Although it

has long been noticed that PB2 E627K is critical to host adaption of viral polymerase complex, the exact molecular mechanism is still unclear yet.

Initially, the function of PB2 E627K and the defect of avian influenza polymerase were implicated in different body temperatures of avian and mammalian species. The avian-like polymerase complex (PB2 627E) is sensitive to the relatively cold environment of human upper respiratory tract where the temperature is lower (33 ℃) compared to the environment within avian species (40 ℃) [113, 114]. Alternatively, the defective vRNP assembly was speculated as the major defect of avian-like polymerase complex, as evidenced by the observation that acquisition of PB2 E627K mutation increased the interaction between NP and PB2, and thus facilitates the vRNP formation in mammalian cells [115]. However, this view was challenged by another study showing that the enhanced vRNP assembly actually was the result of increased RNA synthesis by polymerase with PB2 627K instead of the facilitated vRNP assembly capacity [116]. Intriguingly, the restriction on polymerase containing PB2 627E is diminished when the vRNA template is shortened to 37 nucleotides, indicating that the restriction might be the results of low processivity of the polymerase. Moreover, introducing mutations in vRNA promoter region which disrupt intrastrand base pairing between nucleotides proposed to interact in the corkscrew model while stabilizing the panhandle structure could enhance the transcription and replication of 627E-polymerase to the comparable level as 627K-polymerase (Figure. 1.4 A), suggesting that the restrictions might occur in the promoter binding by viral polymerase [82, 117].

Nevertheless, certain human isolates do not possess the canonical PB2 E627K mutation [118, 119]. The best example is the 2009 pandemic H1N1 virus which still retains the avian-signature (PB2 627E). Interestingly, studies have found that this most recent pandemic virus has deployed an alternative adaptive strategy to compensate the absence of PB2 E627K. The SR polymorphism (PB2 590S, 591R) presented in 2009 pH1N1 virus partially restores the impaired function caused by the lack of PB2 E627K mutation [120]. Mechanistically, SR polymorphism restores the positively charged surface of PB2 627 domain, which has been hypothetically implicated to alter the interaction with unknown host factors [120, 121].

Indeed, in addition to the viral polymerase, or vRNP itself, numerous recent studies have demonstrated that the defective function of avian influenza polymerase is caused by the incompatibility between avian-like polymerase complex and host factors within mammalian cells

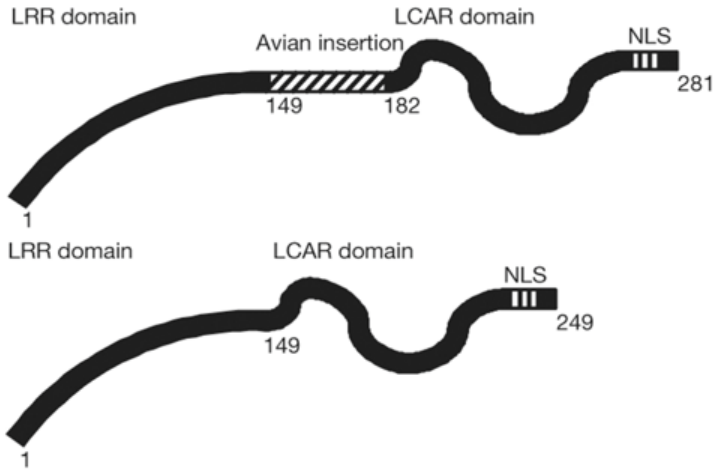
[115, 122]. Therefore, the recurrent selection of PB2 E627K among human isolates is probably to accommodate the viral polymerase complex to the different cellular host factors in human cells. The differential use of importin-α isoforms by avian-like or mammalian-like polymerases has been proved as one of the mechanisms underlying the host adaptation of the virus. The activity of mammalian-like polymerase with PB2 627K requires importin-α1 and importin-α7, while the activity of avian-like polymerase (PB2 627E) depends on importin-α3 [123-125]. However, considering the high identity of importin-α3 in avian and mammalian cells, it is still enigmatic why avian and human influenza viruses display different preferences to importin-α isoforms.

1.5.2 ANP32A is a host factor underlying restrictions on influenza virus polymerase

Acidic nuclear phosphoprotein 32 family member A (ANP32A) consists of an N-terminal leucine-rich repeat domain (LRR) and C-terminal low-complexity acidic region (LCAR) mainly comprised of glutamic or aspartic acid. As a multifunctional protein, ANP32A is involved in the regulation of chromatin, cell death, phosphatase activity, and intracellular transport [126]. Proteomic studies have reported the interaction between ANP32A and influenza viral polymerase complex [127, 128]. Later study showed that this interaction is achieved by ANP32A with the full heterotrimeric polymerase complex instead of individual polymerase subunit or binary subcomplex [129]. But, both of these studies have not investigated the species differences in ANP32A and its potential role in host adaptation of viral polymerase complex.

By using the chicken genome radiation hybrid panel, ANP32A has recently been identified as one of the positive host factors that underlies the host restrictions on influenza virus polymerase complex [130]. Overexpression of chicken ANP32A (chANP32A), but not human ANP32A protein, could restored the poor activity of avian influenza virus polymerase complex in mammalian cells. Avian and mammalian ANP32A display high similarity except that avian ANP32A harbours an additional 33 amino acid insertion (176-208) in the LCAR domain (Figure 1.5). This avian insertion is comprised of 27 amino acid repeat of residue 149-175 and an extra of 6 amino acids. Intriguingly, later study identified a hydrophobic SUMO interaction motif (SIM)-like sequence within this unique 6 amino acid-long region, which seems to be critical to the polymerase-elevating effect of avian ANP32A [131].

Functionally, the defect of avian influenza polymerase in mammalian cells has been demonstrated in the step of vRNA synthesis. It was suspected that the avian-like polymerase (PB2 627E) cannot provide bona fide cRNA templates for further vRNA synthesis in human cells [132]. Intriguingly, ANP32A was also shown as an essential factor to the vRNA synthesis [129]. Therefore, the observed poor polymerase activity of avian influenza polymerase in human cells could be the consequences of impairment in vRNA synthesis. Moreover, compared to human ANP32A, avian ANP32A displays much stronger interaction with viral RdRp despite the identity of PB2 627 residue [131]. This observation implies that the function of PB2 627K is independent of the species-specific interaction.



Reprinted from Nature, 529(7584), 101. (Long. et al., 2016) with permission.

Figure 1.5 Schematic diagram of human ANP32A and chicken ANP32A [130].

The schematic diagram of chicken ANP32A with 33 amino acid insertion relative to the human homologue. The avian-insertion in chANP32A it critical to its polymerase activity enhancing effect.

1.5.3 Other critical adaptive mutations in PB2 segment

PB2 subunit is the major determinant of influenza A virus host range. Multiple other adaptive mutations other than PB2 E627K have been identified in the PB2 segment. The mutation

of aspartic acid to asparagine at 701 residue (D701N) is also a frequently observed adaptive mutation in the PB2 segment among human isolates [133]. The PB2 D701N, which is similar to other adaptive mutation in the polymerase genes, can enhance viral polymerase activity in human cells. Particularly, the D701N mutation can compensate the function of PB2 E627K and increase viral replication and transmissibility [112]. Mechanistically, the function of PB2 D701N has been implicated in increased binding affinity of PB2 subunit to importin which in turn leads to the more efficient assembly of trimeric polymerase complex [134]. This finding is in agreement with the role of importin in underlying host restrictions on viral polymerase [123-125]. Moreover, the PB2 T271A has been demonstrated as an important adaptive mutation to the triple reassortant swine influenza virus and 2009 pH1N1 virus. PB2 T271A can cooperatively promote viral replication [135] with SR polymorphism. In a word, numerous potential adaptive mutations in PB2 have been identified from isolates in different species. Nevertheless, the fundamental function of these mutations is the same which is to enhance the viral polymerase activity and thereby allowing better viral replication within hosts.

1.5.4 Adaptive mutations in PB1 subunit

The PB1 subunit is the enzymatic core of viral polymerase complex [9]. Therefore, unlike the PB2 subunit which is versatile and susceptible to adaptive mutations, PB1 is relatively conserved during virus evolution. This conservation of the PB1 subunit has been suspected to maintain the authentic enzymatic function of viral polymerase complex, although no evidence to support this theory yet [136]. The PB1 segment originated from avian strains participated in the emergence of 1957 and 1968 pandemic viruses [137], which raises the speculation that avian-origin PB1 might contribute to the pandemic formation. Indeed, a study showed that the avian-derived PB1 segment facilitates viral replication and transmissibility of 1968 H3N2 pandemic virus. However, the enhancing effect mediated by avian-origin PB1 observed in this study was quite marginal and could only be detected by a highly sensitive competitive fitness experiment [138].

A couple of mutations have been identified by comparing the PB1 segments originated from avian or human strains. The 473V and 598P present in the PB1 segment of a H5N1 virus can increase the viral polymerase activity of the attenuated human isolate. Intriguingly, the human-

derived PB1 segment of 2009 pH1N1 virus also retains these two signatures of avian-origin PB1 segment. Substitutions of these residues with leucine which is amino acid present in most of the human-derived PB1 segments decreased the polymerase activity as well as the viral replication of 2009 pH1N1 virus [139].

Alignment of PB1 segments of 1918, 1957, and 1968 pandemic viruses lead to the identification of a conserved asparagine to serine mutation at 375 residue (PB1 N375S) [140]. Most of the mammalian isolates including samples from swine or equine possess the serine in this residue, while the majority of avian isolates preferentially carry the asparagine. This N375S is located in in the cRNA binding domain [68]. But, the molecular mechanism of the recurrent selection of this N375S mutation by human isolates is still unclear.

1.5.5 Adaptive mutations in PA subunit

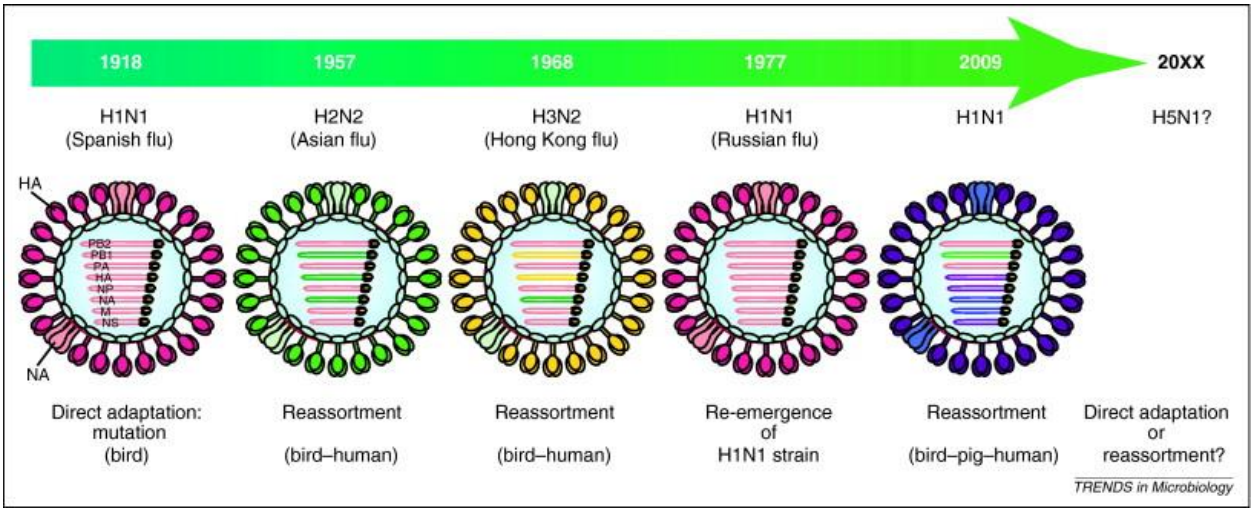
PA subunit is responsible for the endonucleolytic activity of the viral polymerase complex [9]. Multiple adaptive mutations have been identified in PA segment, indicating that PA also plays a critical role in overcoming the host restrictions on viral polymerase complex [120, 141-143]. A switch of lysine to arginine in 356 residue (PA K356R) can increase viral replication and pathogenicity of avian H9N2 virus in mice, demonstrating that adaptive mutation in the PA segment could contribute to the emergence of a pandemic strain. Different studies on the PA segment of 2009 pandemic H1N1 virus lead to the identification of a series of adaptive mutations which are critical to the active viral polymerase of this recent pandemic virus. T85I, G186S, L336M, and T552S in pH1N1-PA segment both have been identified as residues which can increase polymerase activity and promote viral replication in mammalian hosts [142, 144]. Coherently, introducing the PA segment of 1918 or 2009 pandemic virus into the un-adapted avian-like polymerase can fully restored the restricted polymerase activity in human cells [120]. Probably, it is the adaptive mutations carried by human-adapted PA segments rescue the defect of avian-like polymerase and thus promotes the viral polymerase activity.

1.6. Pandemic viruses and genetic traits of their polymerase

Influenza pandemic represents one of the most serious threats to public health. The best example is 1918 pandemic H1N1 virus outbreak 100 years ago which killed approximately 20 to 50 million people worldwide. In the last century, there are five times of influenza pandemic outbreaks as shown in (Figure 1.6): 1918 Spanish flu (H1N1), 1957 Asian flu (H2N2), 1968 Hong Kong flu (H3N2), 1977 (Russian flu (H1N1), and 2009 pandemic flu (H1N1) [145]. As mammalian adaptation of viral polymerase is an essential step in evolution towards the pandemic strain, analysis on their polymerase genes can shed light on the conserved features used by the virus and thus promote the understanding of adaptive mechanism used by the virus.

Reassortment of the gene segments from avian, swine, or human strains frequently occurs during the emergence of pandemic influenza viruses, as observed in all the past pandemics except 1918 and the potential recurrent strain (1977 Russian H1N1 virus) [145]. The reassortment process provides opportunities for the virus to achieve the optimal compatibility between polymerase subunits, and thereby enhancing the viral replication or transcription abilities [120, 146]. However, the deeper reason for the enhanced activity of reassortant polymerase complex might be the accumulative effect of multiple adaptive mutations carried within the entire gene segment [144]. Since the emergence of 1918 pandemic virus, all the other following pandemic viruses could be regarded as the descendants of 1918 virus [147]. Intriguingly, these viruses still retain the PB1 segment derived from avian strains during reassortment, as evidenced by 1957 and 1968 pandemic viruses both possess the avian-origin PB1 segment [138]. Although there is speculation that avian-origin PB1 might contribute to the pandemic formation, no mechanism has been revealed for this hypothesis.

The presence of PB2 627K is another trait of these pandemic polymerases. Except the most recent 2009 pandemic virus, all the other past pandemic viruses contain the PB2 627K compared to the un-adapted avian strain. Even the 2009 pH1N1 virus lacks the PB2 627K mutation, it adopts SR polymorphism, an alternative adaptive strategies, to maintain the active functions of the polymerase [120]. Therefore, it is highly possible that the next pandemic strain would keep these canonical adaptations to overcome the host restrictions on its polymerase complex.



Reprinted from Trends in microbiology, 20 (1), 11-20. (Watanabe et al., 2012) with permission.

Figure 1.6 Timeline of past influenza pandemic viruses [145].

The pandemic viruses emerged in the 20th century. Among these viruses, the most recent pH1N1 virus contain avian-derived PB2, PA, and a human-derived PB1. For the rest of pandemic viruses, their PB1 segments are originated from avian strains.

CHAPTER 2. OBJECTIVE, HYPOTHESIS AND AIMS

The main **objective** of this project is to identify the adaptation pathway which could be deployed by influenza A virus (especially the most recent 2009 pH1N1 virus) to achieve mammalian adaptation. Additionally, through the study on the fundamental molecular mechanism of influenza A viral replication machinery, we try to reveal the molecular mechanism by which the viral polymerase complex becomes adapted to human.

We **hypothesized** that acquisition of PB1 segment from avian strains by 2009 pH1N1 virus can render viral polymerase the fully-adapted status in human cells.

Aim 1: To investigate the viral polymerase activities of 2009 pH1N1 virus subsequent to the acquisition of avian-origin PB1 segment.

Aim 2: To investigate the molecular mechanism by which avian-origin PB1 confers pandemic polymerase replication advantages in mammalian cells.

Aim 3: To study the potential mechanism by which in the cellular factor ANP32A confers enhanced replication of avian-like viral RNA polymerase in mammalian cells.

CHAPTER 3. AVIAN-ORIGIN PB1 GENE CONFERS SELECTIVE ADVANTAGES TO 2009 PANDEMIC H1N1 VIRUS RNA TRANSCRIPTION AND REPLICATION

ABSTRACT

The constant crosstalk between the large avian reservoir of influenza A viruses (IAV) and its mammalian hosts drives viral evolution and facilitates host switching. Direct adaption of an avian strain to human or reassortment of avian-origin gene segments with that of human strains led to the emergence of the 1918, 1957, and 1968 pandemic viruses. The avian PB1 segment, which encodes the catalytic subunit of IAV RNA-dependent RNA polymerase (RdRp), is frequently subjected to reassortment events and is present in the 1957 and 1968 pandemic viruses. However, the biological consequence and molecular basis of such gene exchange remain less well understood. Using the 2009 pandemic H1N1 polymerase naturally lacking an avian-origin PB1 subunit, we demonstrate that the acquisition of an avian PB1 markedly facilitates viral RNA synthesis. This enhancement is also effective in the absence of PB2 adaptive mutations, which are key determinants of host switching. Mechanistically, the avian-origin PB1 does not appear to affect polymerase assembly but imparts the reassorted pandemic polymerase augmented viral primary transcription and replication. Moreover, compared to the parental pandemic polymerase, the reassorted polymerase displays comparable cRNA stabilizing activity but is specifically enhanced in progeny vRNA synthesis from cRNA in a *trans*-activating manner. Overall, our results provide the first insight into the mechanism by which avian-origin PB1 facilitates viral RNA synthesis by the 2009 pandemic virus polymerase.

3.1 INTRODUCTION

Influenza A virus (IAV) has a broad host range in which the avian species constitute the largest natural reservoir. The spillover of avian strains to mammalian hosts leads to occasional fatal cases and more frequently, the exchange of gene segments with mammalian counterparts through reassortment [148]. The reassortment event not only expands viral diversity but facilitate host switching and the emergence of novel viruses with pandemic potential. Except for the 1918 pandemic virus whose genesis is under debate [136, 149-151], all the other three major pandemic viruses (i.e. 1957, 1968, and 2009) arose indisputably as a consequence of genetic reassortment [118, 137, 152]. These viral strains contain genome segments that are directly contributed by avian

viruses or can be traced back to avian origins. Notably, the reassortment process is subjected to constraints, among which the incompatibility of polymerase subunits may lead to a loss of viral fitness [153]. The IAV polymerase is composed of three subunits which act in concert to catalyze viral RNA synthesis. The PB1 subunit is the catalytic core of the polymerase complex, while the PB2 and PA subunits are well characterized for their roles in the cap-snatching process of viral mRNA synthesis[154]. Due to stronger functional constraints, PB1 exhibits lower evolutionary rate and limited evolutionary divergence between host-specific lineages [155]. In contrast, PB2 and PA coevolve and encompass the majority of host adaptive mutations, such as the well-known host range determinant at the residue 627 of PB2 [155, 156].

Given the relatively conserved nature of PB1, the contribution of PB1 mutations 66 to host adaption is less well understood. On the other hand, it has long been observed that human seasonal and pandemic IAV strains, such as that of 1957 and 1968, frequently contain reassorted PB1 segments originated from avian viruses [137, 157]. While these reassortment events partially corroborate the genetic compatibility of avian PB1 with human PB2 and PA, the biological consequence and molecular basis of such frequent emergence of avian-origin PB1 remain unclear. In mammalian cells, introducing avian PB1 of H7N1 or H2N2 into the background of mammalian H1N1 and H3N2 polymerases led to increased or comparable activity [146]. An avian H5N1 PB1 also rescued the attenuated polymerase activity and partially restored viral replication of a PB2-627E containing mammalian H1N1 virus [139]. Moreover, the reassortment between a human H3N2 and an avian H5N1 virus led to the generation of an avian PB1-containing reassortant with increased virulence *in vivo*, though the reassorted polymerase activity remained unchanged *in vitro* [158]. Swapping the PB1 segment of a human seasonal H2N2 virus with that of the 1968 pandemic strain, which is of avian-origin, increased viral polymerase activity and facilitated replication and transmission of the reassortant virus in guinea pigs [138].

The 2009 pandemic H1N1 is a “quadruple reassortant” virus resulted from the reassortment between the “triple reassortant” North American swine H1N2 viruses and the Eurasian “avian-like” swine H1N1 viruses. It contains the PB2 and PA gene segments of avian-origin, and interestingly the PB1 segment originated from human seasonal H3N2 viruses, unlike that of the previous pandemic strains. In a ferret model, the 2009 pandemic virus exhibited increased viral replication and pathogenicity compared to a seasonal H1N1 strain [159], whereas it showed mild to moderate

virulence in mice compared to the reconstructed 1918 pandemic H1N1 and a highly pathogenic H5N1 virus [159]. Due to the cocirculation of 2009 pandemic viruses with avian strains, it is of significant interest to monitor the potential reassortment events that may further confer the 2009 pandemic H1N1 gene segments of avian-origin. Interestingly, upon the reassortment between a 2009 pandemic N1H1 and an avian H9N2, reassortant viruses containing the avian PB1 were mostly more virulent than that containing the pandemic PB1 [160]. However, the underlying mechanism remains unknown.

Here we further address the biological significance of the acquisition of avian PB1 by the 2009 pandemic H1N1 virus polymerase. We found that the avian-origin PB1 significantly enhances pandemic polymerase activity, and such enhancement is independent of the PB2 adaptive mutations. Mechanistically, the avian-origin PB1 does not affect polymerase assembly but imparts the reassorted pandemic polymerase augmented viral primary transcription and replication. Moreover, the reassorted polymerase displays comparable cRNA stabilizing activity to the parental polymerase but is specifically enhanced in vRNA synthesis from cRNA in a *trans*-activating manner. Overall, our results provide the first insight into the mechanism by which avian-origin PB1 facilitates viral RNA synthesis by the 2009 pandemic virus polymerase.

3.2 MATERIALS AND METHODS

3.2.1 Cells

Madin-Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco). Human embryonic kidney 293T (293T) cells and chicken embryonic fibroblast (DF-1) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% FBS. MDCK cells and 293T cells were maintained at 37 °C; DF-1 cells were grown at 39 °C.

3.2.2 Plasmids

Polymerase proteins and NP protein expressing plasmids were constructed by cloning coding sequences of PB2, PB1, PA, and NP segments from pH1N1, A/Turkey/Ontario/6213/1966 (H5N1) (ON H5N1) [161], A/chicken/British Columbia/CN-6/2004 (H7N3) (BC H7N3), and

A/British Columbia/2015 (H7N9) (BC H7N9) [162] into pcDNA vector, generating plasmids (pcDNA-pH1N1-PB2, pcDNA-pH1N1-PB1, pcDNA-pH1N1-PA, pcDNA-pH1N1-NP), (pcDNA-ON H5N1-PB2, pcDNA-ON H5N1-PB1, pcDNA-ON H5N1-PA, pcDNA-ON H5N1-NP), pcDNA-BC H7N9-PB1, pcDNA-BC H7N3-PB1. The pcDNA-ON H5N1-PB1 (PB1-F2 KO) construct was created by introducing stop codons at 12^{th} and 58^{th} residues of PB1-F2 ORF to disrupt the expression of PB1-F2 protein. For the mutant pH1N1-PB2 constructs, 627K, 590G and 591Q were respectively introduced into pcDNA-pH1N1-PB2 to create the pcDNA-pH1N1-PB2 (627K), pcDNA-pH1N1-PB2 (590G/591Q), pcDNA-pH1N1-PB2 (590G/591Q, 627K). For the construction of catalytically inactive PB1a of either pH1N1 virus or ON H5N1 virus, the double mutations (D445A and D446A) were introduced into the WT pcDNA-pH1N1-PB1 and pcDNA-ON H5N1-PB1. The replication defective mutation PA E410A was introduced into pcDNA-pH1N1-PA to create the replication defective construct pcDNA-pH1N1-PA (E410A). For vRNA expressing plasmids, pHH21-pH1N1-vNP and pHH21-pH1N1-vNA, the full-length vRNA of segment 5 and 6 of pH1N1 virus were respectively introduced into the pHH21 vector. The pPOLI-NP-Luc constructs with species-specific polymerase I promoter and pTK-RLuc used in minireplicon luciferase report assay were described elsewhere [120, 163]. Chicken ANP32A gene (Gallus gallus, XP_413932.3) was synthesized and constructed into pcDNA vector by Genscript.

3.2.3 Viruses

2009 pandemic H1N1 virus and the recombinant pH1N1-$PB1_{Avi}$ virus was generated by using eight-plasmid reverse genetic system [164]. Viruses were propagated in MDCK cells. Virus titer was titrated by plaque assay. The origin of PB1 segment was verified by sequencing. Recombinant virus pH1N1-$PB1_{Avi}$ refers to the reassortant pH1N1 virus with PB1 segment from avian strain ON H5N1 virus.

3.2.4 Viral growth kinetics

For the single-cycle growth curve, confluent monolayers of A549 cells were infected by respective viruses at an MOI of 5. After 1h of adsorption at 37 °C, cells were washed with DMEM and cultured in viral growth medium (DMEM containing 0.2% Bovine serum albumin and 1 µg/ml

of TPCK-Trypsin). Supernatants were collected at 2, 4, 6, 8, 10 hours post infection and were stored at -80 °C. Viral titer of each time point was titrated in MDCK cells by plaque assay.

3.2.5 Minireplicon luciferase reporter assay

The luciferase reporter assay was performed as previously described [120]. 293T cells or DF-1 cells were transfected with plasmids expressing indicated PB2, PB1, PA, and NP proteins (50 ng each) together with pPOLI-vNP-Luc expressing firefly luciferase (50 ng) and *Renilla* luciferase expressing plasmid pTK-RLuc (50 ng) for internal control. After 24 hours incubation at 37 °C (293T cells) or 39 °C (DF-1 cells), cells were lysed for the dual-luciferase reporter assay (Promega).

3.2.6 Split luciferase complementation assay (SLCA)

N-terminus (1-229) and C-terminus (230-311) of *Renilla* luciferase gene were respectively fused to different termini of full-length PB1 or PA segment of pH1N1 virus or ON H5N1 virus via protein linker (GGGSGGGS) as described elsewhere [165], generating plasmids LN-PB1 $_{(pH1N1)}$, PA $_{(pH1N1)}$-LC, and LN-PB1 $_{(ON\ H5N1)}$. For selection of functional SLCA constructs, 293T cells were co-transfected with 50 ng of different PB1 SLCA constructs and PA SLCA constructs. In conformations of heterotrimeric polymerase complex or vRNP, 50 ng of pcDNA-pH1N1-PB2or with the additional 50 ng of pHH21-pH1N1-vNA and pcDNA-NP were co-transfected with SLCA constructs. The relative *Renilla* luciferase activity were measured at 24 h.p.t. by luciferase assay (Promega).

3.2.7 vRNP reconstitution assay and primer extension analysis

293T cells in 6-well plates were transiently transfected with expression plasmids for three polymerase subunits (500 ng each), NP protein (500 ng), and vRNA-expressing plasmid (500ng) pHH21-vNA expressing the full-length NA segment vRNA template. Forty-eight hours post-transfection (h.p.t), cells were harvested for extraction of total RNA by Trizol. The levels of vRNA, cRNA, mRNA, and 5S rRNA were analyzed by primer extension as previously described [104,

166]. Briefly, the 5’ end of primers for probing the vRNA and 5S rRNA are labeled with Tide Fluor 6 which have the spectral propriety similar to IRDye 700, and the 5’ end of primer for probing cRNA and mRNA are labeled with Tide Fluor 8 which have the spectral propriety similar to IRDye 800. The expected sizes of the products are 61 nt (vRNA), 75 nt (cRNA), 100 nt (5S rRNA), and 85 to 88 nt depending on the length of the capped primer (mRNA). Products of primer extension were analysed by 15% PAGE containing 7M urea in TBE buffer and were detected by infrared imaging system (Odyssey, LI-COR).

3.2.8 NP-cascade vRNP reconstitution assay

293T cells were co-transfected with 100 ng of plasmids expressing PB2, PB1, and PA protein along with pHH21-vNP expressing full length segment 5 RNA. Cells were lysed at 48 h.p.t for detection of NP protein by western blotting.

3.2.9 Strand-specific real-time RT-PCR

The relative quantification of vRNA and cRNA accumulated in cRNA stabilization assay were measured by strand-specific real-time RT-PCR as previously described [167]. Briefly, 350 ng of total RNA was reverse transcribed using hot-start reverse transcription at 60 °C for 1 hour. The 20 μl reaction system containing 4.5 μl RNA (350 ng), 6.5 μl saturated trehalose, 1 μl tagged RT primers (10 pmol), 4 μl First-strand buffer (5 ×, Invitrogen), 1 μl DTT (0.1M), 1 μl dNTP (10mM), 1 μl RNaseOUT (Invitrogen), and 1 μl SuperScript III reverse transcriptase (200U/μl ,Invitrogen). Real-time PCR was performed in 20 μl reaction system with 2 μl of cDNA, 1 × Power SYBR Green PCR master mix (Applied Biosystems), and 500 nM primers by StepOnePlus real-time PCR system (Applied Biosystems).

3.2.10 cRNA stabilization assay

As described elsewhere [104], 293T cells in 6-well plate were transiently transfected with plasmid expressing three polymerase subunits (250 ng) and NP protein (1000 ng) using TransIT-LT1. 24 h.p.t, cells were infected by indicated viruses at an MOI of 10 in presence of

cycloheximide (100 µg/ml). 1 or 6 hours post-infection, cells were collected for total RNA extraction by Trizol. vRNA or cRNA were quantified by strand-specific real-time RT-PCR.

3.2.11 Immunoblotting

Viral PB1 and NP proteins were probed with homemade rabbit antibody, actin was probed with mouse anti-actin antibody (Cell Signaling). Standard immunoblotting protocol was performed as previously described [163], and the nitrocellulose membranes were visualized with infrared imaging system (Odyssey, LI-COR).

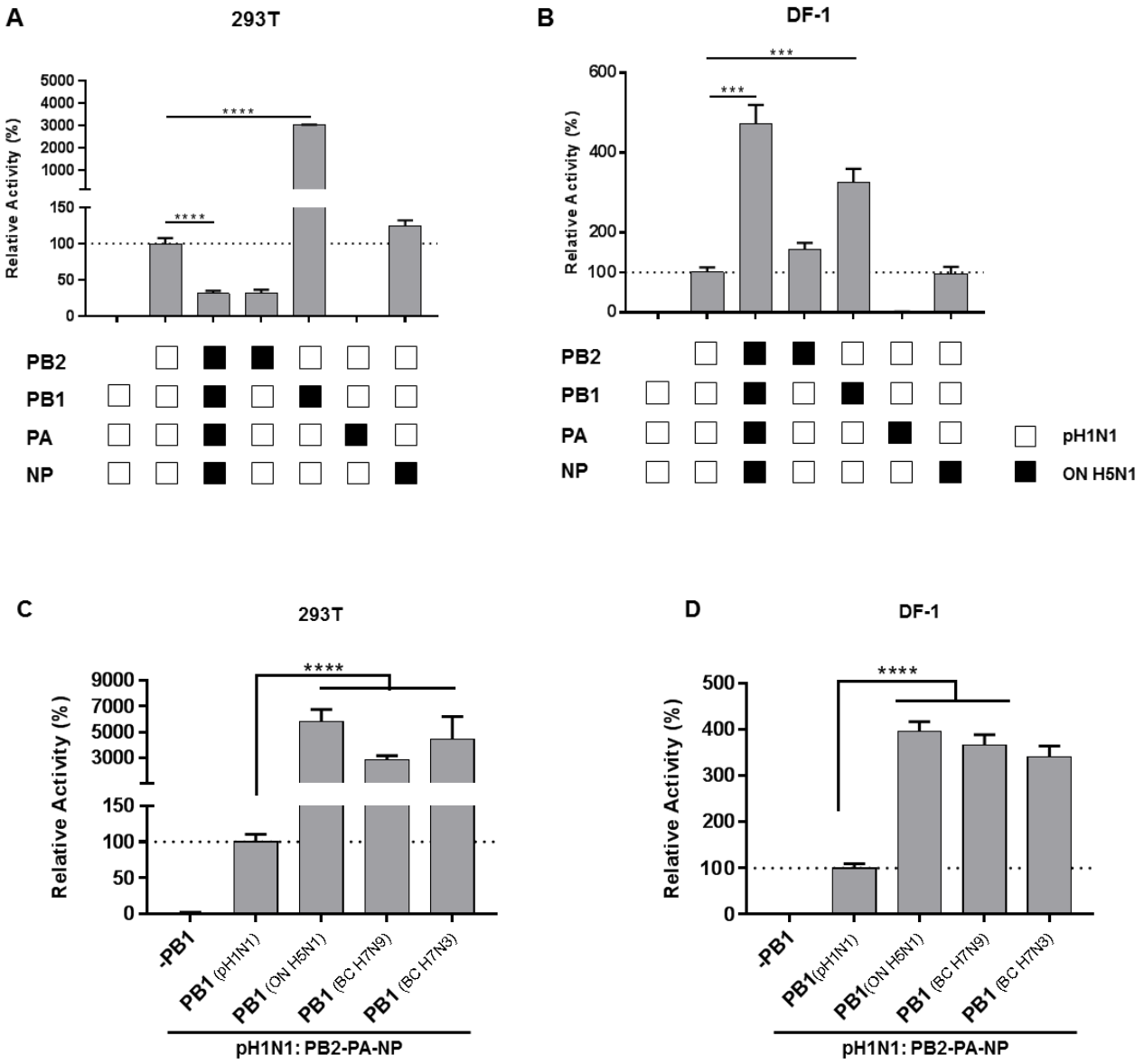
3.2.12 Statistical analysis

The statistical analyses were performed in GraphPad Prism 7 (GraphPad Software, Inc., USA) using Student's t test, one-way ANOVA, or two-way ANOVA. Significant differences are denoted by the asterisks as follow: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$; $P < 0.0001$.

3.3 RESULTS

3.3.1 Avian-origin PB1 segment enhances viral polymerase activity of 2009 pH1N1 virus.

To evaluate the effect of avian-origin PB1 segment on 2009 pH1N1 viral polymerase activity, we replaced the pandemic PB1 subunit, or its PB2, PA, and NP segments one at a time with the respective avian-origin segments derived from A/Turkey/Ontario/6213/1966 (H5N1) (ON H5N1). Polymerase activity of the reconstituted vRNP in either human 293T cells or avian DF-1 cells were measured by minireplicon assay [120]. As expected, while avian polymerase functioned well in avian cells (Figure. 3.1B), the human pH1N1 vRNP exhibited higher level of polymerase activity than the avian vRNP in 293T cells (Figure. 3.1A). Exchange of individual segment resulted in various vRNP activity compared to that of WT pH1N1, exchanging the PB1 segment of pH1N1 virus with avian PB1 segment markedly enhanced the polymerase activity in 293T cells (Figure. 3.1A). This elevating effect induced by avian PB1 was also observed in DF-1 cells even though it was at a lesser degree (Figure. 3.1B).


A
293T
Relative Activity (%)
5000
4000
3000
2000
1000
150
100
50
0
****
****
PB2
PB1
PA
NP
B
DF-1
Relative Activity (%)
600
400
200
100
0
***
***
PB2
PB1
PA
NP
pH1N1
ON H5N1
C
293T
Relative Activity (%)
9000
7000
5000
3000
150
100
50
0
****
-PB1
PB1 (pH1N1)
PB1 (ON H5N1)
PB1 (BC H7N9)
PB1 (BC H7N3)
pH1N1: PB2-PA-NP
D
DF-1
Relative Activity (%)
500
400
300
200
100
0
****
-PB1
PB1(pH1N1)
PB1(ON H5N1)
PB1 (BC H7N9)
PB1 (BC H7N3)
pH1N1: PB2-PA-NP

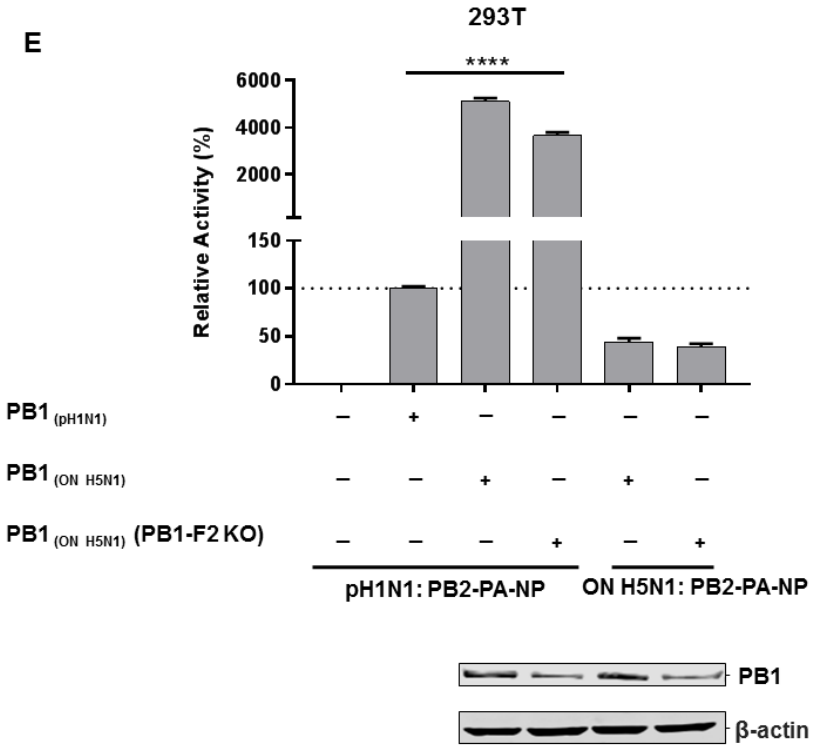


Figure 3.1 Avian-origin PB1 enhances polymerase activity of pH1N1 virus.

(A and B) The polymerase activity of reassortant vRNPs. HEK-293T (A) or DF-1 cells (B) were co-transfected with plasmids expressing the indicated PB2, PB1, PA and NP proteins of pH1N1 virus and avian H5N1 virus, along with a species-specific polymerase I-driven vRNA-luciferase reporter plasmid and a *Renilla* luciferase expressing plasmid. (C and D) The polymerase activity of reassortant pH1N1 polymerases with PB1 segments from different avian strains. Relative polymerase activities of reassortant pH1N1 vRNPs with PB1 segment from ON H5N1 virus, BC H7N3 virus, or BC H7N9 virus were measured in 293T (C) or DF-1 cells (D). (E) PB1-F2 expressed by avian-origin PB1 segment does not affect the enhanced polymerase activity. The activity of ON H5N1 polymerase or the reassortant pH1N1 polymerase with mutant ON H5N1 PB1 (PB1-F2 KO) was measured in 293T cells. In above experiments, omission of PB1 segment (-PB1) was used as the negative control. Firefly luciferase activities were normalized to the *Renilla* luciferase activities. The polymerase activity of 2009 pH1N1 virus was set as 100%. Error bars represent the standard deviation of three biological replicates (±SD); the pattern of results are consistent in at least three independent experiments.

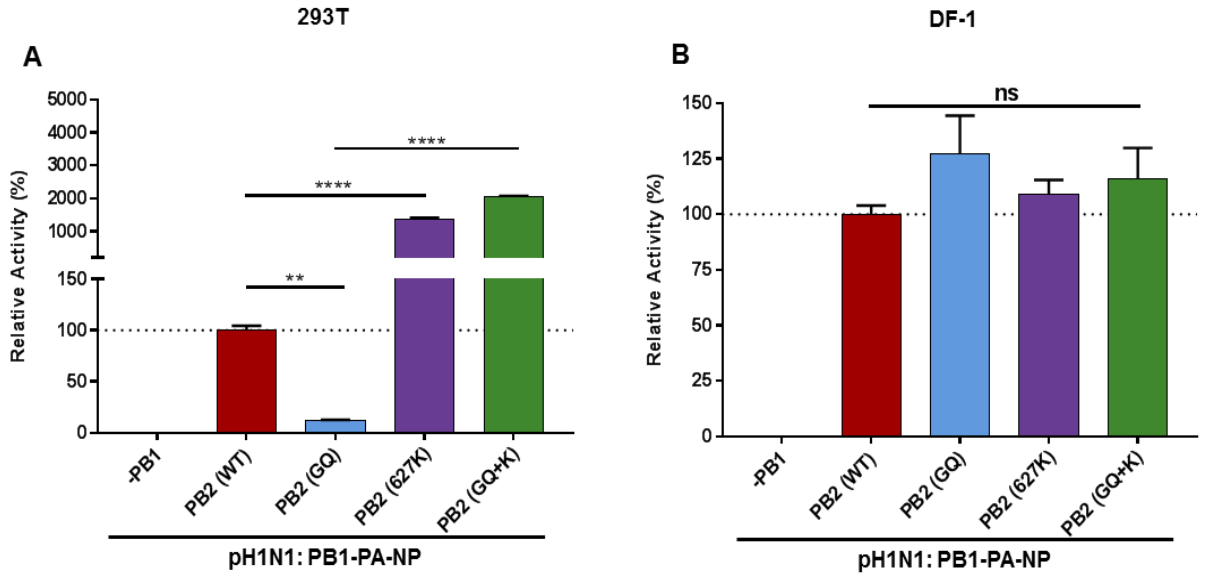
To address whether the enhancement in viral polymerase activity mediated by PB1 derived from ON H5N1 virus was a strain-specific effect or not, reassortant pH1N1 vRNPs with PB1 segment originated from different avian strains were reconstituted in both human and avian cells. In addition to the aforementioned low pathogenic avian influenza virus (LPAIV) ON H5N1, PB1 segment from LPAI (A/chicken/British Columbia/CN-6/2004) (BC H7N3) or highly pathogenic avian influenza (HPAI) (A/British Columbia/2015 (H7N9) (BC H7N9) both markedly boosted the polymerase activity of 2009 pH1N1 virus in human cells (Figure. 3.1C). These avian-origin PB1 segments also enhanced the activity of 2009 pH1N1 polymerase in avian cells; however, to a lesser extent (Figure. 3.1D). PB1-F2 protein encoded by PB1 segment has been shown to play a role in regulating viral polymerase activity in a strain-specific manner [168]. While 2009 pH1N1 virus does not express PB1-F2 due to the presence of three stop codons in the PB1-F2 ORF, most of avian-origin PB1 segments encode PB1-F2 protein. To examine whether the enhanced viral polymerase activity was attributable to the PB1-F2 protein encoded by avian PB1 gene or not, we inserted two stop codons into ON H5N1 PB1 segment to abolish the expression of PB1-F2 protein without disrupting the expression of PB1 protein. Abrogating PB1-F2 expression slightly decreased the PB1 protein expression; however, neither the activity of WT ON H5N1 vRNP nor the enhanced activity of reassortant pH1N1 vRNP with ON H5N1-PB1 was affected (Figure. 3.1E).

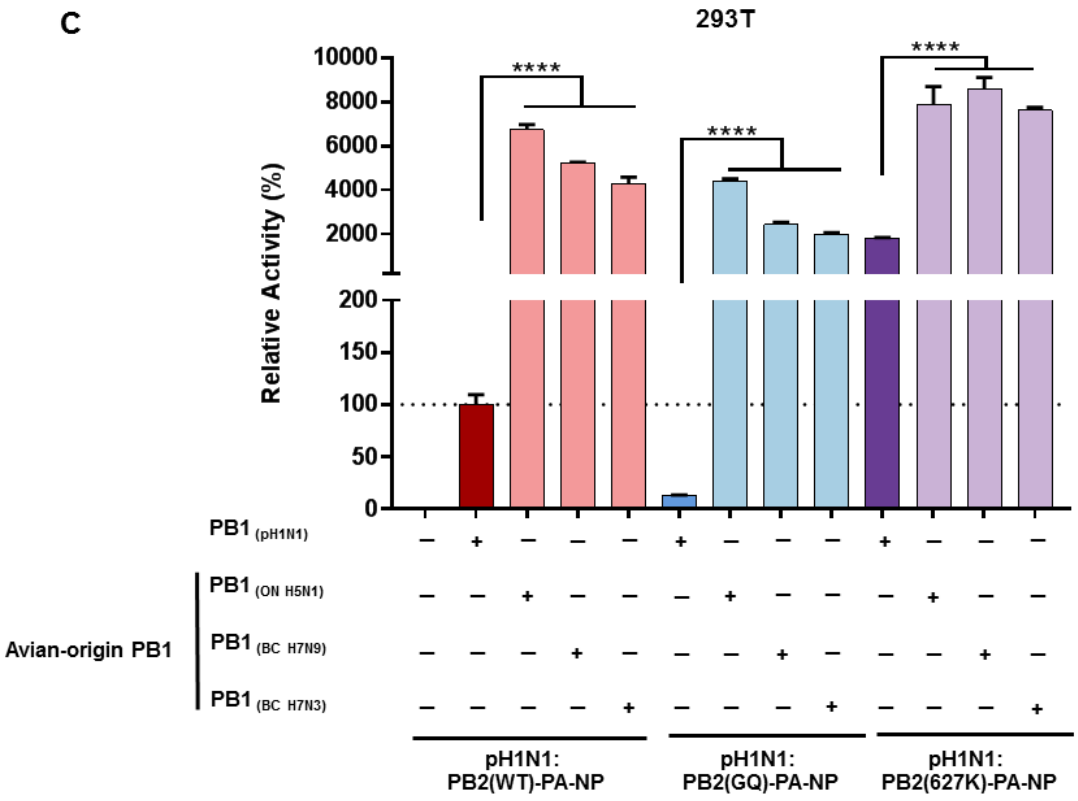
3.3.2 Avian-origin PB1 segment assists pH1N1 polymerase to overcome host restriction conferred by PB2.

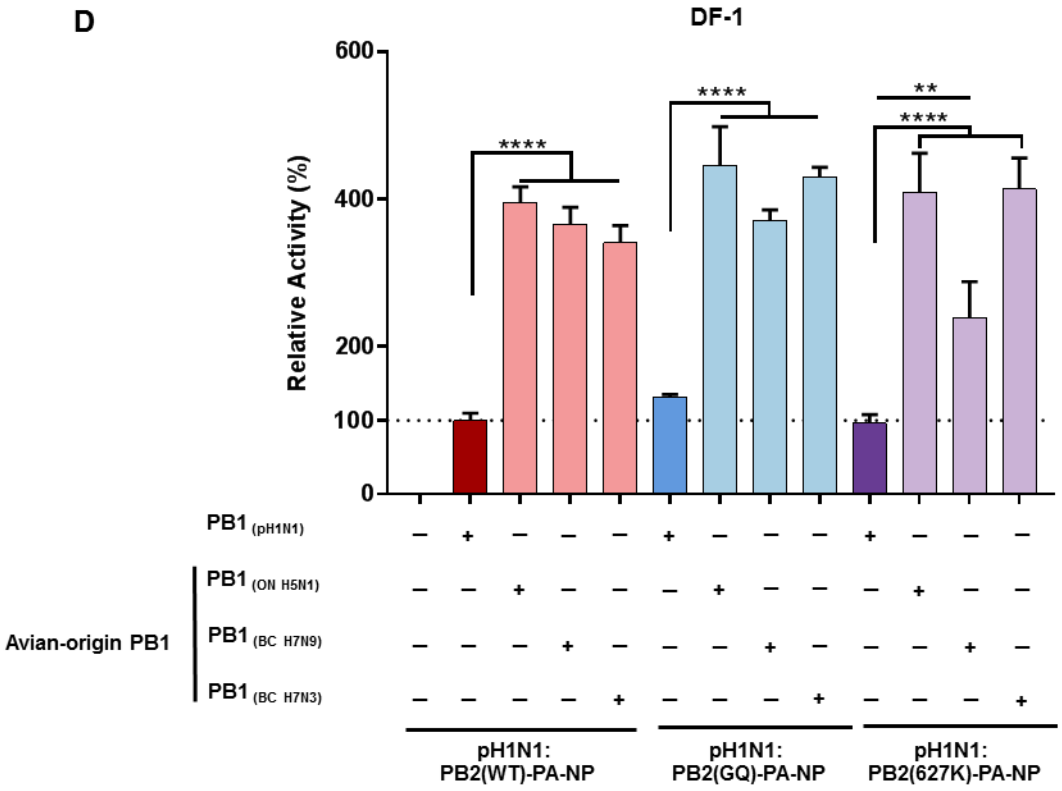
We next investigated whether acquisition of avian-origin PB1 could enhance pH1N1 polymerase activity in a reassortant situation where PB2 carries avian-like signatures. The 2009 pH1N1 polymerase possesses 627E avian-like signature in PB2; however, it employs SR polymorphism (serine at position 590 and arginine at position 591) as an alternative mammalian adaptive strategy [120]. First, we confirmed the functions of these previously characterized PB2-associated mammalian adaptive mutations in minireplicon assay. Indeed, in human cells, in the background of pH1N1 vRNP, while replacing SR polymorphism with GQ sequence (S590G, R591Q) in PB2 significantly decreased the polymerase activity, a single mutation of E627K in PB2 dramatically enhanced polymerase activity despite the presence of SR polymorphism (Figure.

3.2A). In comparison, all these PB2 variant polymerases displayed comparable level of activities in avian cells (Figure 3.2B).

To explore whether the SR polymorphism is the prerequisite of the enhanced viral polymerase activity mediated by avian-origin PB1, we then reconstituted reassortant vRNPs using pH1N1 PB2 variants (different identities in 590, 591, and 627 residues) PA, NP, and avian-origin PB1. Avian-origin PB1 massively enhanced pH1N1 polymerase activity not only when PB2 possessed SR polymorphism, but also when PB2 possessed GQ sequence. Even in the background of fully adapted pandemic PB2 carrying 627K, acquisition of the avian-origin PB1 segment still enhanced its activity by more than 4-fold. The enhancing effect was consistently observed by PB1 derived from different avian strains (Figure. 3.2C). In contrast, these enhancements were less profound in avian cells (Figure. 3.2D). In addition to the luciferase assay, we also scrutinized the effect of avian-origin PB1 on the functions of pH1N1 polymerase by primer extension assay (27). As seen in Figure 3.2E, pH1N1 polymerases with avian-origin PB1 (lane 4 and 5) produced more vRNA, cRNA, and mRNA than WT pH1N1 polymerase did (lane 1 and 2). In agreement with the above observation, avian-origin PB1 could overcome the viral RNA synthesis restricted by PB2 lacking SR polymorphism or PB2 lacking 627K (lane 5 vs 1 and 2). Overall, these data indicated that acquisition of avian-origin PB1 by pH1N1 vRNP could fully overcome the restrictions rendered by the absence of critical PB2-associated mammalian adaptive mutations.




C
293T
Relative Activity (%)
10000
8000
6000
4000
2000
200
150
100
50
0
****
****
****
PB1 (pH1N1)
Avian-origin PB1
PB1 (ON H5N1)
PB1 (BC H7N9)
PB1 (BC H7N3)
pH1N1: PB2(WT)-PA-NP
pH1N1: PB2(GQ)-PA-NP
pH1N1: PB2(627K)-PA-NP


D
DF-1
Relative Activity (%)
600
400
200
100
0
****
****
**
****
PB1 (pH1N1)
PB1 (ON H5N1)
PB1 (BC H7N9)
PB1 (BC H7N3)
Avian-origin PB1
pH1N1: PB2(WT)-PA-NP
pH1N1: PB2(GQ)-PA-NP
pH1N1: PB2(627K)-PA-NP

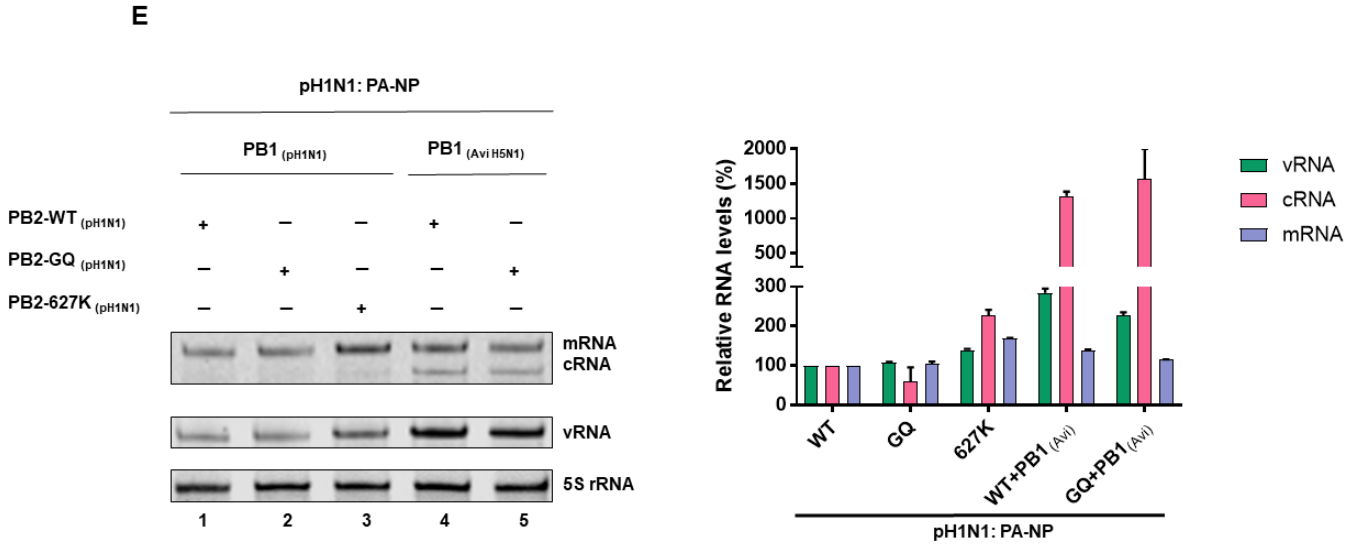

E
pH1N1: PA-NP
PB1 (pH1N1)
PB1 (Avi H5N1)
PB2-WT (pH1N1)
PB2-GQ (pH1N1)
PB2-627K (pH1N1)
mRNA
cRNA
vRNA
5S rRNA
1
2
3
4
5
Relative RNA levels (%)
2000
1500
1000
500
300
200
100
0
WT
GQ
627K
WT+PB1 (Avi)
GQ+PB1 (Avi)
pH1N1: PA-NP
vRNA
cRNA
mRNA

Figure 3.2 Avian-origin PB1 compensates the function of canonical PB2-associated adaptive mutation in pH1N1 polymerase.

(A and B) The effect of PB2 E627K and SR polymorphism on viral polymerase activity. Polymerase activities of WT pH1N1 polymerase and variant pH1N1 polymerases bearing the indicated mutations were respectively measured in 293T cells (A) or DF-1 cells (B). (C and D) Avian-origin PB1 renders optimal polymerase activity to pH1N1 polymerase. Reassortant polymerases with different avian-origin PB1 and pH1N1 PB2-assoicated mutations were measured in 293T cells (C) or DF-1 cells (D). Firefly luciferase activities were normalized to the *Renilla* luciferase activities. The polymerase activity of 2009 pH1N1 virus was set as 100%. Error bars represent the standard deviation of three biological replicates (±SD); the pattern of results are consistent in at least three independent experiments. (E) The levels of three species of viral RNAs produced by reassortant pH1N1 polymerases carrying PB2 mutations with or without ON H5N1-PB1 (avian-origin PB1) were assessed by primer extension assay. 5S rRNA was served as loading control. The corresponding relative signal intensity normalized to WT pH1N1 polymerase (100%) was analysed using Imagine J software. Error bars represent the standard deviation of two biological replicates (±SD).

3.3.3 Avian-origin PB1 does not facilitate the assembly of viral polymerase complex or vRNP.

The vRNP assembly capacity has been demonstrated as the factor which affects the viral polymerase activity. To evaluate the effect of the avian-origin PB1 on the assembly of viral polymerase complex or vRNP, we established a split luciferase complementation assay (SLCA) as described elsewhere [165]. As PB1 and PA form the dimer prior to the assembly of trimeric polymerase complex with PB2 [67], each half of the *Renilla* luciferase gene was respectively tagged to PB1 and PA segments derived from pH1N1. The diagram of different tested combinations of PB1-PA SLCA constructs were shown in Figure. 3.3A. After co-transfection in 293T cells, the *Renilla* luciferase activity at 24 h.p.t were measured by luciferase activity assay. The N-terminally luciferase-tagged PB1 (LN-PB1) and C-terminally luciferase-tagged PA (PA-LC) displayed the highest *Renilla* luciferase activity, whereas other combinations merely showed background signals (Figure. 3.3B). Therefore, the LN-PB1 and PA-LC constructs were used for the rest of experiments, and the luciferase activity generated by these SLCA constructs was measured as the proxy for polymerase complex or vRNP assembly capacity.

Co-transfection of PB1 and PA in SLCA assay revealed that the relative *Renilla* luciferase activity (normalized to WT pH1N1 constructs) generated by pH1N1-PA and avian origin-PB1 combination was not significantly enhanced compared to that of PA with PB1 derived from pH1N1 (Figure. 3.3C) indicating that the PB1-PA interaction is not altered followed by acquisition of avian-origin PB1. We next included a plasmid expressing pH1N1 PB2 in SLCA assay to assess the polymerase assembly capacity when all three polymerase subunits are present. Figure. 3.3D showed that avian-origin PB1 moderately increased the luciferase reading but statistically not significant. Next, we assessed the vRNP assembly capacity by including NP and viral RNA in the system. As shown in Fig. 3E, the avian-origin PB1 did not lead to marked enhancement on *Renilla* luciferase activity. Collectively, these data suggest that avian-origin PB1 neither strengthens the PB1-PA interaction nor facilitates the assembly of trimeric polymerase complex or vRNP.

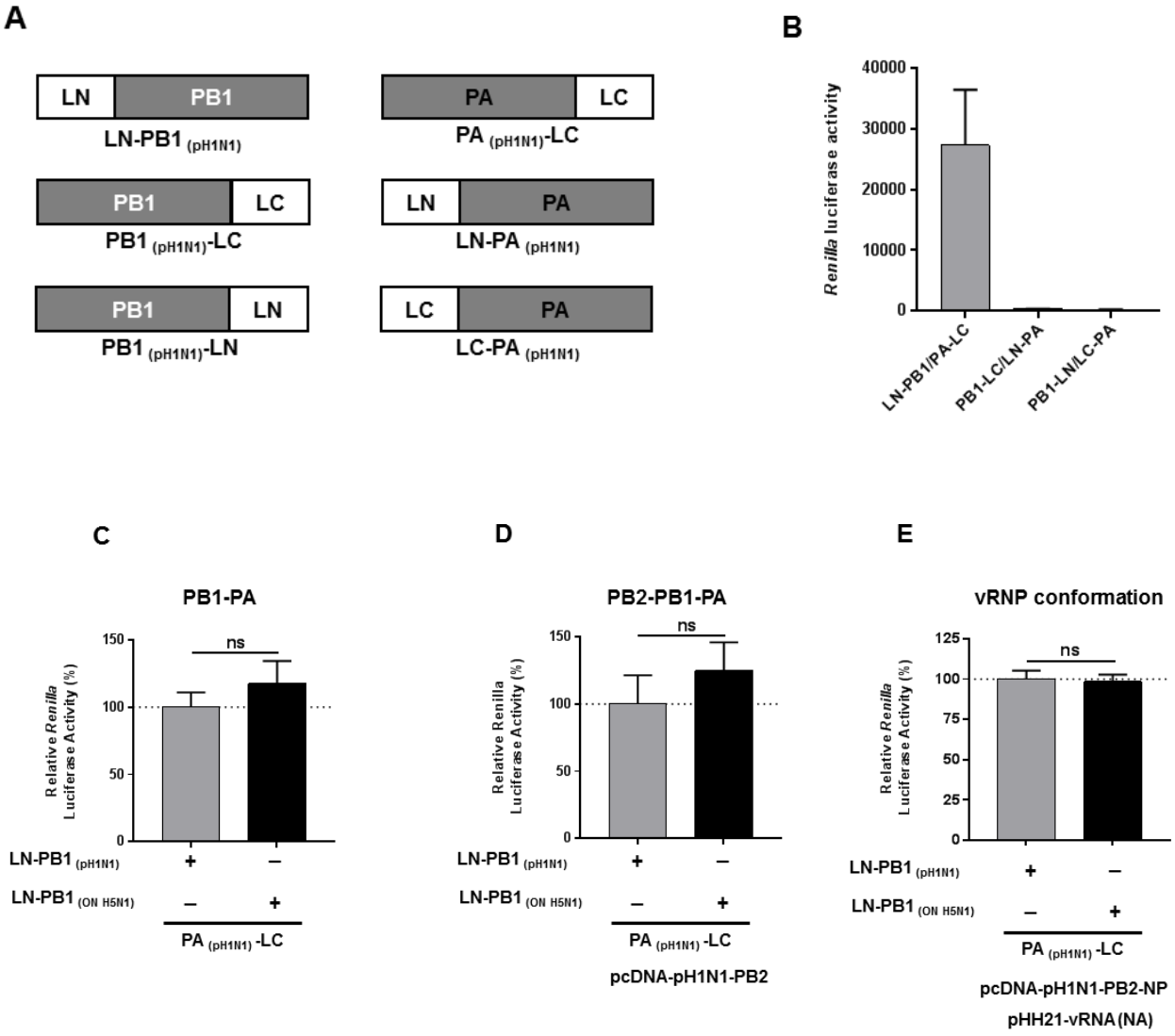

A
LN
PB1
LN-PB1 (pH1N1)
PA
LC
PA (pH1N1)-LC
PB1
LC
PB1 (pH1N1)-LC
LN
PA
LN-PA (pH1N1)
PB1
LN
PB1 (pH1N1)-LN
LC
PA
LC-PA (pH1N1)
B
Renilla luciferase activity
40000
30000
20000
10000
0
LN-PB1/PA-LC
PB1-LC/LN-PA
PB1-LN/LC-PA
C
PB1-PA
Relative Renilla Luciferase Activity (%)
150
100
50
0
ns
LN-PB1 (pH1N1)
+
–
LN-PB1 (ON H5N1)
–
+
PA (pH1N1) -LC
D
PB2-PB1-PA
Relative Renilla Luciferase Activity (%)
150
100
50
0
ns
LN-PB1 (pH1N1)
+
–
LN-PB1 (ON H5N1)
–
+
PA (pH1N1) -LC
pcDNA-pH1N1-PB2
E
vRNP conformation
Relative Renilla Luciferase Activity (%)
125
100
75
50
25
0
ns
LN-PB1 (pH1N1)
+
–
LN-PB1 (ON H5N1)
–
+
PA (pH1N1) -LC
pcDNA-pH1N1-PB2-NP
pHH21-vRNA (NA)

Figure 3.3 The polymerase subunit interaction or assembly of polymerase complex and vRNP is not altered by avian-origin PB1.

(A) Schematic diagram of PB1 and PA constructs used for split-luciferase complementation assay. (B) Selection of functional SLCA constructs. *Renilla* luciferase activities were measured after co-expression of indicated SLCA constructs in 293T cells. (C) Avian-origin PB1 does not enhance PB1-PA interaction. 293T cells were transfected by PA $_{(pH1N1)}$-LC along with the SLCA constructs fused to PB1 derived from either pH1N1 or ON H5N1. The *Renilla* luciferase were measured. (D) Avian-origin PB1 does not promote assembly of trimeric polymerase complex. *Renilla* luciferase activities were measured after co-transfection of the indicated combination of SLCA constructs along with a plasmid expressing pH1N1 PB2 protein in 293T cells. (E) Avian-origin PB1 does not change assembly of vRNP. 293T cells were co-transfected with the indicated combination of SLCA constructs together with the Pol II driven plasmids expressing pH1N1 PB2 and NP protein, and a Pol I driven plasmid generating a full-length vRNA of NA segment. In above experiments, *Renilla* luciferase activities were measured at 24 hr post transfection by luciferase assay. The *Renilla* luciferase activity generated by LN-PB1 $_{(pH1N1)}$ and PA $_{(pH1N1)}$-LC in each setting was set as 100%. Error bars represent the standard deviation of three biological replicates (±SD); the pattern of results are consistent in at least three independent experiments.

3.3.4 Acquisition of avian-origin PB1 segment enhances RNA transcription of pH1N1 polymerase.

Given that PB1 functions as the catalytic core of the viral polymerase, we next hypothesized that avian PB1 might directly affect the synthesis of viral RNA species. Depending on the mode of viral polymerase, it can act as a transcriptase to catalyze viral mRNA synthesis from viral genomic RNA (vRNA), or a replicase to synthesize more vRNA via the replicative intermediate complementary RNA (cRNA). While the minireplicon assay results as shown in Figure. 3.1 and 3.2 directly reflected viral mRNA levels, it remained unclear which arm of the viral polymerase activity was enhanced as a result of an avian PB1 acquisition, since an enhanced replication activity (i.e. more vRNA) could also lead to more viral mRNA production. To specifically assess whether avian-origin PB1 enhances viral transcriptase activity, we established a modified minireplicon system in which the Pol II-driven NP plasmid was omitted (Figure. 3.4A). Since ongoing viral replication requires concurrent NP encapsidation of newly synthesized vRNA/cRNA, such NP-free condition would largely diminish viral replication and the production of full-length progeny vRNA. In contrast, NP is not required for viral primary transcription; full-length viral mRNA is able to be synthesized from input vRNA followed by translation into viral proteins. Taking advantage of this property, we particularly supplied to the NP-free system a vRNA-expressing plasmid encoding the viral NP segment. We rationalized that if the viral polymerase conducted sufficient levels of primary transcription, the resulting NP mRNA would be translated into NP proteins to resume ongoing viral replication. This would convert the NP-free condition back to the standard minireplicon condition, leading to the accumulation of large amounts of NP. Therefore, the expression levels of NP from the NP-free minireplicon system would be an indicator of the primary transcription activity of the tested polymerase. Using this system, we showed that viral polymerases of avian origins (i.e. H5N1 and H7N3) were highly restricted at the level of primary transcription (Figure.3.4B, lanes 2, 3, and 8). Removal of the SR polymorphism within the PB2 subunit of pH1N1 polymerase also diminished primary transcription (Fig. 3.3.4B, lanes 6 and 7). Strikingly, under both the SR and GQ backgrounds, introducing an avian-origin PB1 enhanced or restored viral primary transcription (Fig. 3.3.4B, lanes 4, 5, 9, and 10), echoing the polymerase enhancing effect observed in Fig. 3.3.2C.

Collectively, these results demonstrated that avian-origin PB1 enhances the transcription activity of the pH1N1 polymerase.

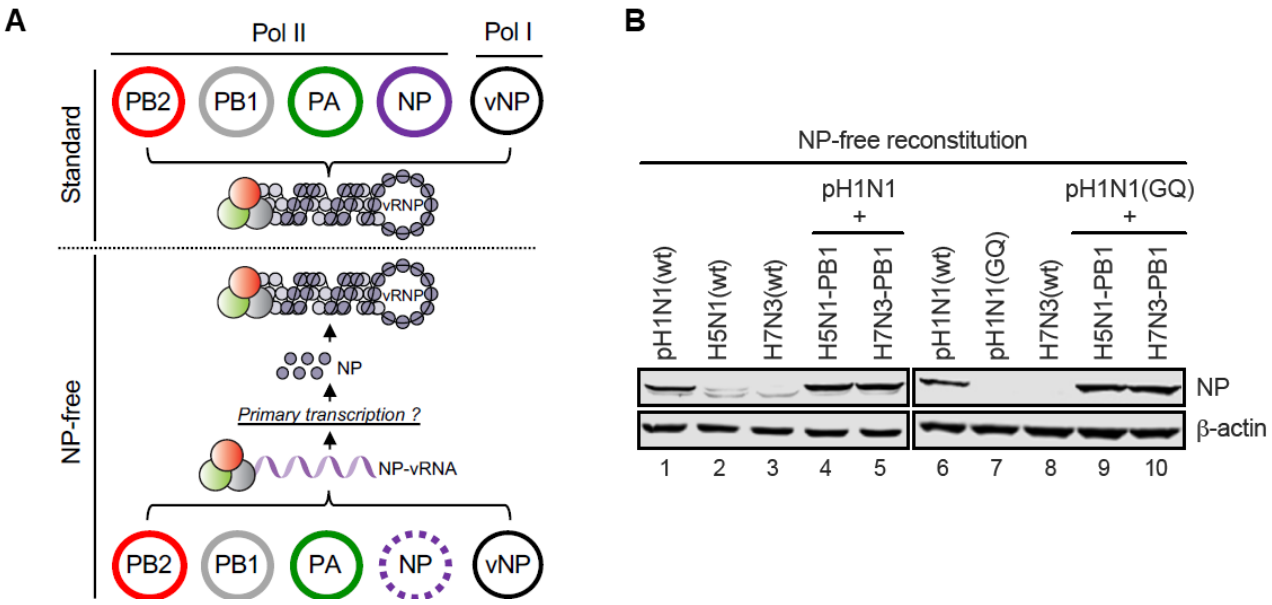


Figure 3.4 Avian-origin PB1 enhances transcription of pH1N1 polymerase in human cells.

(A) Schematic diagram of the NP-free minireplicon system. Cells were co-transfected with plasmids expressing polymerases subunits PB2, PB1 and PA along with pHH21- vNP generating a full-length segment 5 vRNA. (B) NP protein levels generated by different combination of RdRp in the NP-free system were assessed by western blotting 24 h.p.t.

3.3.5 Avian-origin PB1 facilitates synthesis of progeny vRNA.

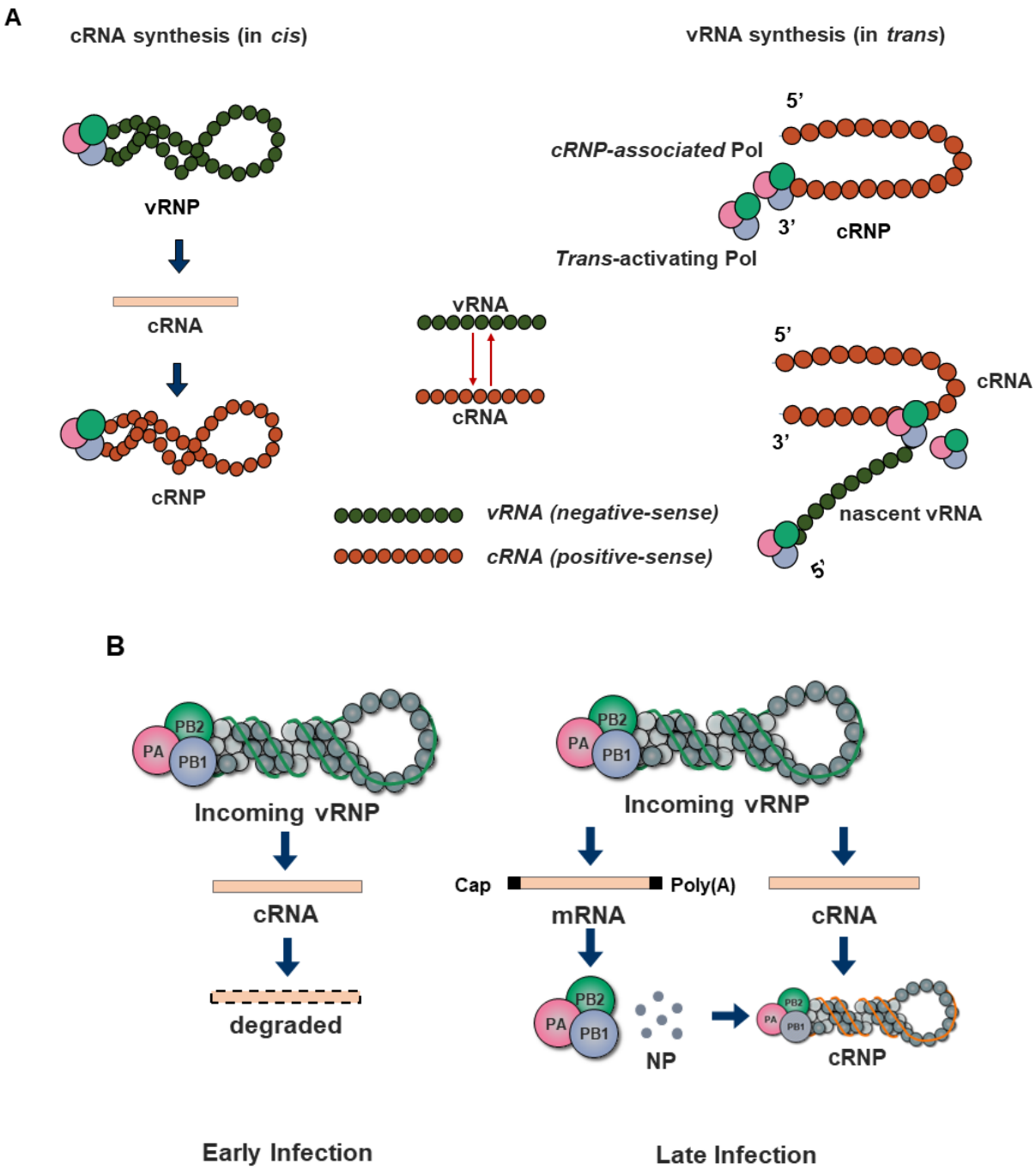
Influenza virus replication is a two-step process. The first step is the synthesis of cRNA from the incoming vRNP in *cis*. The second step is the synthesis of progeny vRNA using the cRNA as template in *trans* [154] (Figure. 3.5.A). Based on the current cRNA stabilization model [104], the newly synthesized cRNA from incoming vRNP requires protection from polymerase complex and NP protein (Figure. 3.5.B).

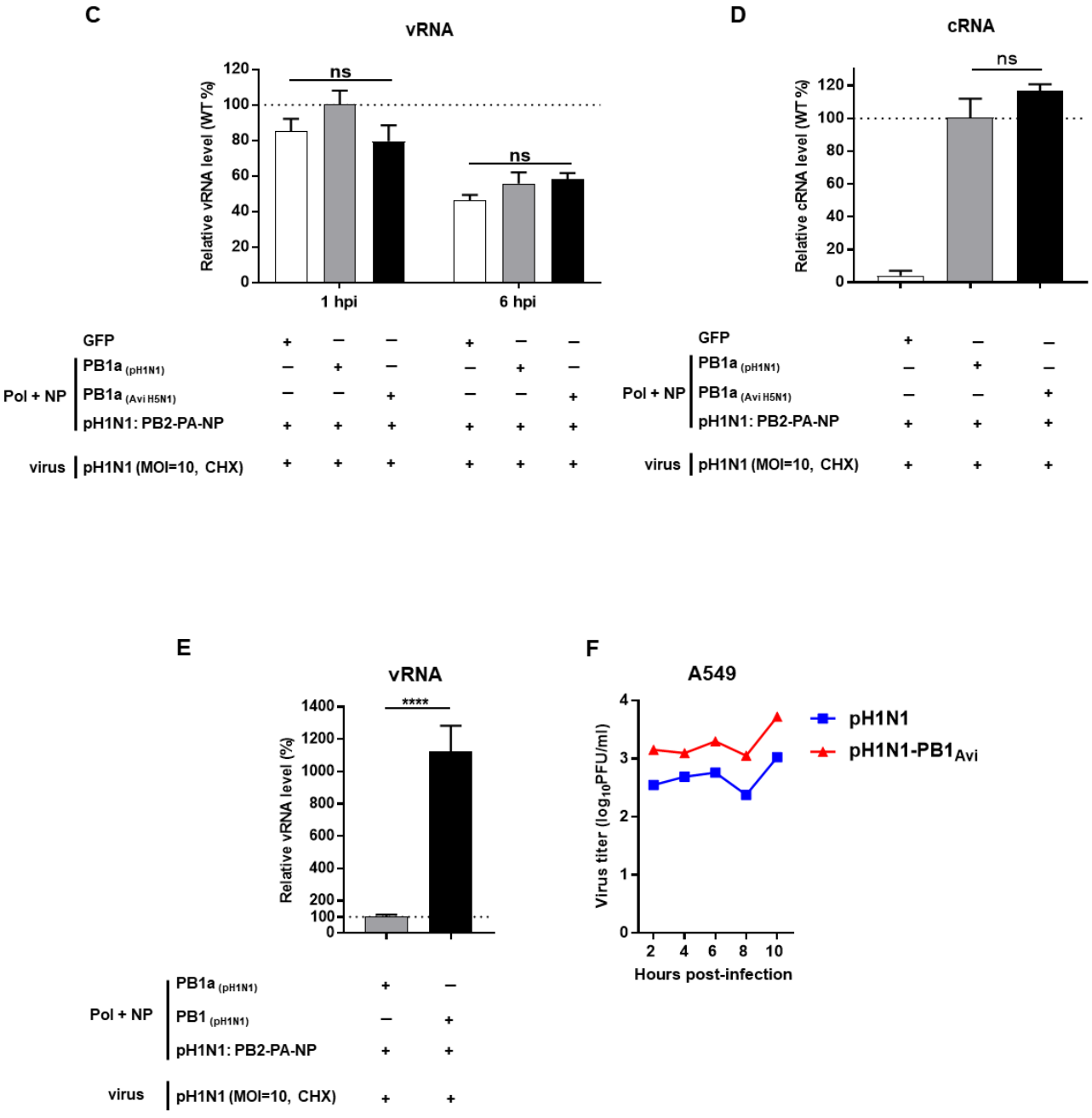
We next asked whether the acquisition of an avian-origin PB1 would facilitate the replication of pH1N1 polymerase. Given that cRNA stability is a prerequisite for ongoing viral replication, we first explored whether the reassorted polymerase containing an avian-origin PB1 has a stronger ability to stabilize nascent cRNA and to prevent it from degradation. We used a cRNA stabilization assay in which 293T cells were pre-expressed with viral polymerase complex and NP before infected with the pH1N1 virus in the presence of protein synthesis inhibitor cycloheximide (CHX) [132]. Under this condition, nascent cRNA synthesized from the incoming vRNPs would be assembled into cRNP containing the pre-expressed viral protein components to avoid degradation. Moreover, the pre-expressed polymerase complex contained a catalytically inactive PB1 subunit (PB1a) to abrogate cRNP replication, which eliminated interference from accumulative cRNA synthesis. Consistent with previous reports, cRNA stabilizing effect could only be detected in cells pre-expressing an intact viral polymerase but not that with the PB1 subunit replaced by GFP (Figure 3.5D). Pre-expression of polymerase complexes containing either pH1N1 or H5N1 PB1a led to similar levels of cRNA accumulation as determined by a strand-specific qPCR, indicating comparable cRNA stabilization abilities. It is also noted that input vRNA levels remained consistent across conditions at 1-hour post-infection (h.p.i.) and 6 h.p.i (Figure 3.5 C), ruling out the possibility of any difference in viral infection. The results also showed that the vRNA level at 6 h.p.i. did not increase compared to that at 1 h.p.i. (Figure 3.5C), indicating no progeny vRNA is produced when the pre-expressed polymerase is catalytically inactive. Furthermore, a much higher level of vRNA synthesis was observed when we exchanged the catalytically inactive polymerase with an active one (Figure. 3.5E). Given that the nascent cRNA associated catalytically inactive polymerase incapacitates the synthesis of progeny vRNA, it suggests that the second step of viral RNA replication (cRNA

to vRNA) relies on the resident polymerase on cRNP, which is in agreement with the *trans*-activating model that the polymerase does not act as a replicase in *trans* [66].

We subsequently sought to assess the effect of avian-origin PB1 on cRNA synthesis ability. cRNA is synthesized by vRNP-associated polymerase in *cis*. To provide the vRNP-associated polymerase containing avian-origin PB1, we resuced the recombinant pH1N1 virus with its PB1 segment replaced by avian ON H5N1 PB1 (the virus is referred as pH1N1-PB1Avi). This recombinant virus showed a one-log increase in replication during a single-cycle infection in A549 cells (Figure. 3.5F). In 293T cells pre-expressed with catalytically inactive PB1a of pH1N1, the recombinant virus synthesized similar levels of cRNA as the parental pH1N1, demonstrating that the avian PB1 as contained in the incoming vRNPs conferred no advantage to cRNA synthesis (Figure. 3.5G). These results together with the enhanced specifically increases the nascent vRNA synthesis.

To verify whether acquisition of avian-origin PB1 could indeed provide advantages to vRNA synthesis step, we used the homologous combinations of pre-expressed polymerases (cRNP-associated polymerase) and viruses (incoming vRNP) in the cRNA stabilization assay. As expected, in cells infected with pH1N1-PB1$_{Avi}$ virus, the pre-expressed avian-origin PB1 containing polymerase produced more vRNA and cRNA compared to the WT combinations did (Figure. 3.5H). Cumulatively, our results indicate that avian-origin PB1 specifically enhances the vRNA synthesis from cRNA template, and thereby facilitating the overall replication ability of pH1N1 polymerase in human cells.


A
cRNA synthesis (in *cis*)
vRNA synthesis (in *trans*)
vRNP
cRNA
cRNP
vRNA
cRNA
5'
cRNP-associated Pol
3'
cRNP
Trans-activating Pol
5'
cRNA
3'
nascent vRNA
5'
vRNA (negative-sense)
cRNA (positive-sense)
B
PB2
PA
PB1
Incoming vRNP
cRNA
degraded
Early Infection
PB2
PA
PB1
Incoming vRNP
Cap
Poly(A)
mRNA
cRNA
PB2
PA
PB1
NP
PB2
PA
PB1
cRNP
Late Infection


C
vRNA
Relative vRNA level (WT %)
ns
ns
1 hpi
6 hpi
GFP
Pol + NP
PB1a (pH1N1)
PB1a (Avi H5N1)
pH1N1: PB2-PA-NP
virus
pH1N1 (MOI=10, CHX)
D
cRNA
Relative cRNA level (WT %)
ns
E
vRNA
Relative vRNA level (%)
****
PB1a (pH1N1)
PB1 (pH1N1)
F
A549
Virus titer (log10PFU/ml)
Hours post-infection
pH1N1
pH1N1-PB1Avi

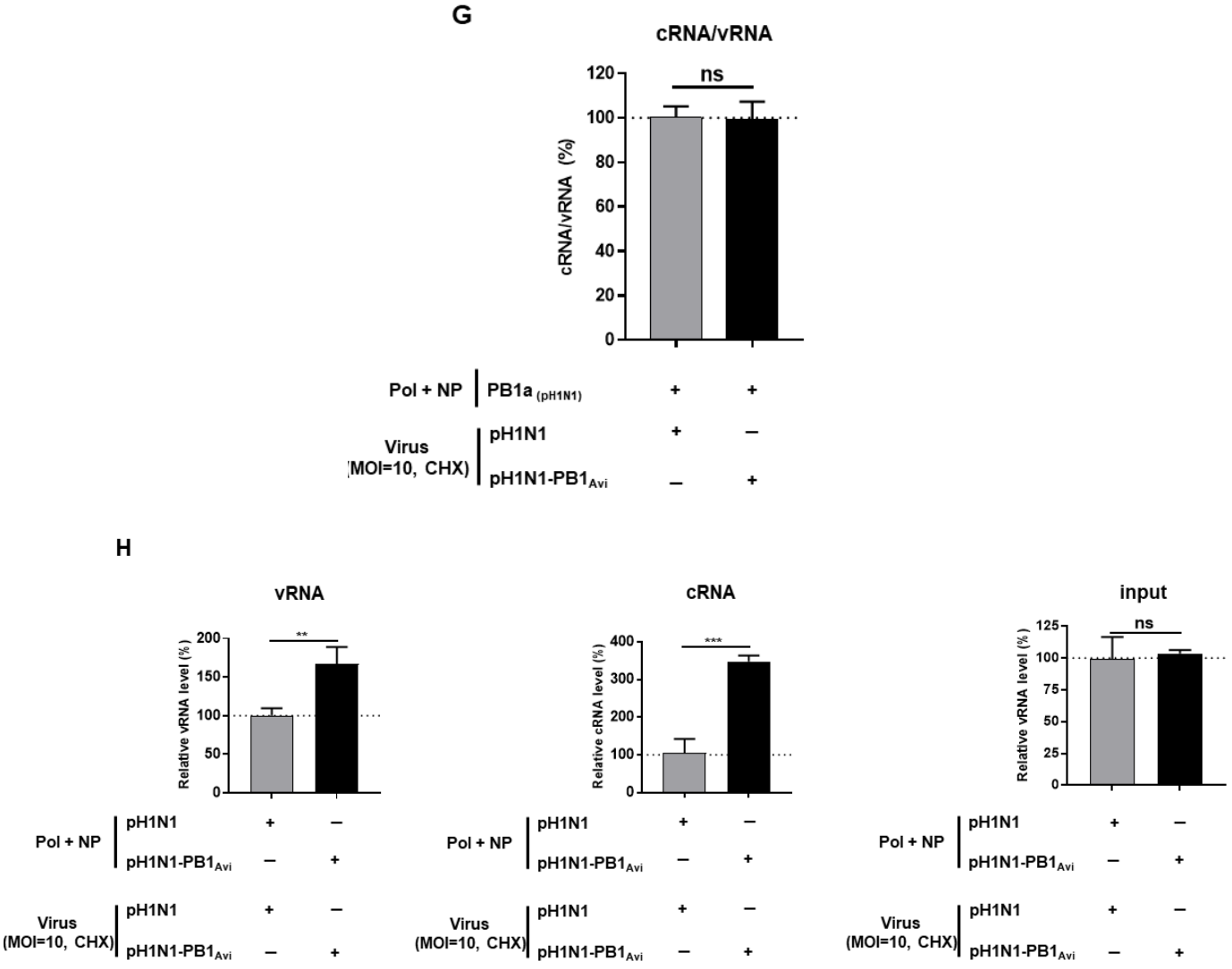

G
cRNA/vRNA
ns
cRNA/vRNA (%)
120
100
80
60
40
20
0
Pol + NP
PB1a (pH1N1)
+
+
Virus (MOI=10, CHX)
pH1N1
+
–
pH1N1-PB1Avi
–
+
H
vRNA
**
Relative vRNA level (%)
200
150
100
50
0
cRNA
***
Relative cRNA level (%)
400
300
200
100
0
input
ns
Relative vRNA level (%)
125
100
75
50
25
0
Pol + NP
pH1N1
pH1N1-PB1Avi
Virus (MOI=10, CHX)

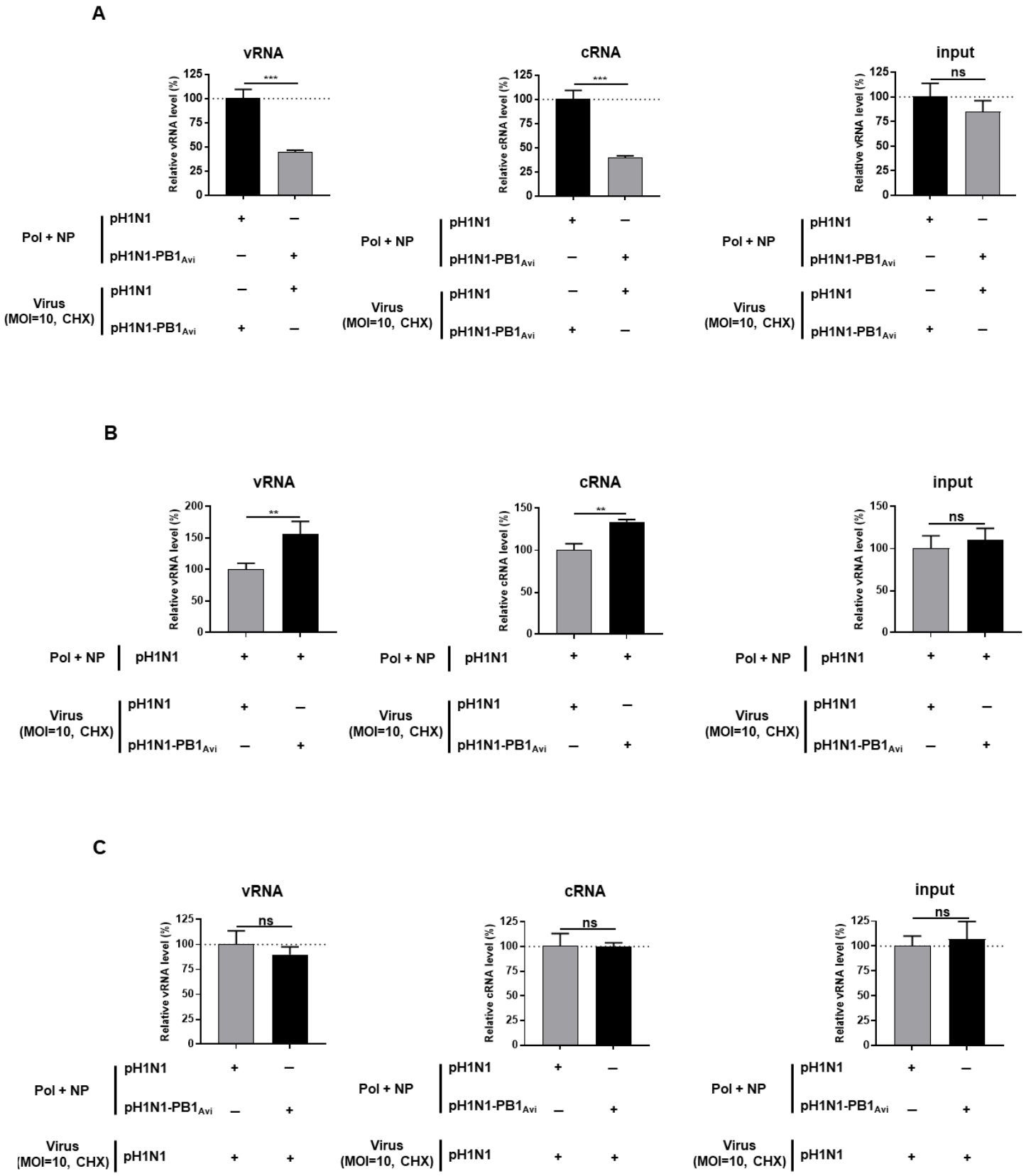
Figure 3.5 Avian-origin PB1 facilitates vRNA synthesis from cRNA template.

(A) Replication mechanism of influenza A virus. The schematic diagrams of *trans*-activation model of vRNA synthesis and cRNA synthesis in *cis*. (B) The schematic diagram of cRNA stabilization model for influenza viral infection. (C) Progeny vRNA synthesis is disrupted by the pre-expressed catalytically inactive polymerase. 293T cells were co-transfected with plasmids expressing indicated polymerase subunits and NP protein followed by infection of pH1N1 virus at an MOI of 10 in presences of CHX. vRNA levels at 1 h.p.i and 6 h.p.i were measured respectively by strand-specific real-time RT-PCR. The vRNA level in cells expressing catalytically inactive pH1N1 polymerase at 1 h.p.i was set as 100%. (D) avian-origin PB1 does not change cRNA stabilization ability. Same as conditions in (C), the cRNA level was measured at 6 h.p.i. The cRNA level in cells expressing catalytically inactive pH1N1 polymerase was set as 100%. (E) The resident polymerase on cRNP carries out the vRNA synthesis. After expression of either catalytically inactive polymerase or active polymerase, cells were infected with pH1N1 virus at an MOI of 10 in presences of CHX. The vRNA levels at 6 h.p.i were measured. vRNA level in cells expressing inactive pH1N1 polymerase was set as 100%. (F) Growth kinetics of pH1N1 virus and pH1N1-$PB1_{Avi}$ virus in A549 cells. A549 cells were infected with the respective virus at an MOI of 5 and incubated at 37 ℃. Supernatants were collected at indicated time points, and virus titers were determined by plaque assay in MDCK cells. (G) avian-origin PB1 does not facilitate cRNA synthesis from vRNA template. After pre-expression of catalytically inactive pH1N1 polymerase, cells were infected with indicated viruses at an MOI of 10 in the presence of CHX. The vRNA and cRNA levels in cells infected with pH1N1 virus were measured at 6 h.p.i. The ratio of cRNA and vRNA in cells infected with pH1N1 virus was set as 100%. (H) avian-origin PB1 facilitate vRNA synthesis from cRNA template. 293T cells were transfected with plasmids expressing indicated polymerase subunits and NP protein and subsequently infected with pH1N1 virus or pH1N1-$PB1_{Avi}$ virus at an MOI of 10 in the presence of CHX. The vRNA and cRNA levels in cells expressing pH1N1 polymerase and infected with pH1N1 virus was set as 100%. vRNA levels in both settings at 1 h.p.i. was served as control of input vRNA. The levels of vRNA and cRNA in all aforementioned cRNA stabilization assays were measured using strand-specific real-time PCR. Error bars represent the standard deviation of three biological replicates (±SD); the pattern of results is consistent in at least three independent experiments.

3.3.6 Avian-origin PB1 enhances activation of the *trans*-activating polymerase.

The enhanced vRNA synthesis could be the result from adapted cRNP-associated polymerase, or the stronger activating effect exerted by the adapted *trans*-polymerase. To address this question, we first assessed vRNA and cRNA levels in the heterologous scenarios in which cells pre-expressed WT pH1N1 polymerase were infected by pH1N1-PB1$_{Avi}$ virus, or vice versa. In these two conditions, the pre-expressed polymerase would be the one that biochemically performs the vRNA synthesis and be subsequently incorporated into cRNP and the nascent vRNP. Intriguingly, the WT pH1N1 polymerase in cells infected by pH1N1-PB1$_{Avi}$ virus produced significantly higher amounts of vRNA and cRNA than avian-origin PB1 containing polymerase did in WT pH1N1 infected cells (Figure. 3.6A). Considering the only fully-adapted polymerase in the enhanced replication setting is the one carried by the incoming vRNP (pH1N1-PB1$_{Avi}$), these results imply that the incoming vRNP-associated polymerases could also serve as the *trans*-activating polymerase to cooperatively assist the cRNP-associated polymerase to perform the vRNA synthesis. These data also suggest that the vRNP-associated polymerase is be the major determinants of the final consequence of viral replication

To corroborate whether the vRNP-associated polymerase could determine the final consequence of viral replication or not, we pre-expressed the WT pH1N1 polymerase (cRNP-associated polymerase) and followed by infection of either pH1N1 virus or pH1N1-PB1$_{Avi}$ virus. As expected, the same cRNP-associated polymerase exhibited increased RNA synthesis ability when a fully-adapted *trans*-activating polymerase carried by incoming vRNP was provided (Figure. 3.6B). To obtain direct evidences that the enhanced vRNA synthesis is not due to adaptation in cRNP-associated polymerases, cells were pre-expressed with NP protein along with either WT pH1N1 polymerase or the avian-origin PB1 containing polymerase followed by infection of same virus (pH1N1/ pH1N1-PB1$_{Avi}$) in the presence of cycloheximide. Coherently, these two different polymerases displayed similar replication ability when a same *trans*-activating polymerase (incoming vRNP-associated polymerase) was provided (Figure. 3.6C and D). Collectively, these data indicate that the acquisition of avian-origin PB1 renders fully-adapted status to the *trans*-activating polymerase, and thereby facilitating the viral replication.


A
vRNA
cRNA
input
Relative vRNA level (%)
Relative cRNA level (%)
***
ns
Pol + NP
pH1N1
pH1N1-PB1Avi
Virus (MOI=10, CHX)
B
**
C

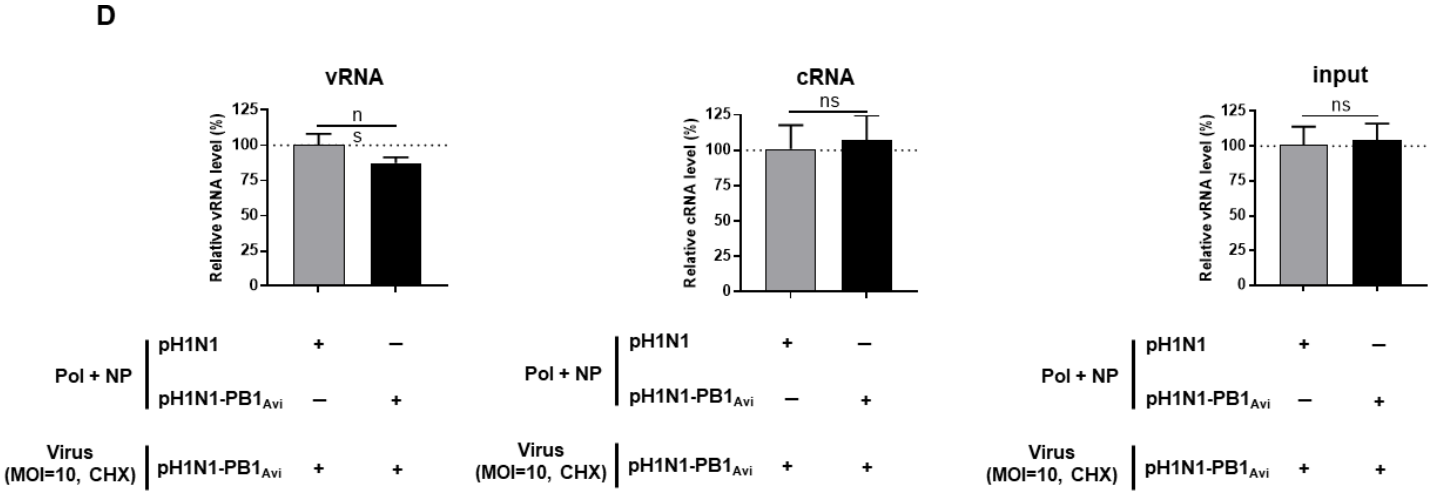


Figure 3.6 Avian-origin PB1 promotes vRNA synthesis in a trans-activating manner.

(A) avian-origin PB1 enhances the *trans*-activating effect. 293T cells were co-transfected with plasmids expressing indicated polymerase subunits and NP protein followed by infection of either pH1N1 virus or pH1N1-PB1$_{Avi}$ virus. The relative vRNA and cRNA levels at 6 h.p.i was measured. The vRNA and cRNA levels in cells expressing pH1N1 polymerase and infected with pH1N1-PB1$_{Avi}$ virus were set as 100%. (B) The enhanced vRNA synthesis is mainly attributed to the vRNP-associated polymerase with avian-origin PB1. After pre-expression of WT pH1N1 polymerase and NP protein, cells were infected by either pH1N1 virus or pH1N1-PB1 $_{Avi}$ virus. The relative vRNA and cRNA levels at 6 h.p.i were measured. The vRNA and cRNA levels in cells infected with pH1N1 virus were set as 100%. (C and D) The cRNP-associated polymerase was not the reason for the enhanced vRNA synthesis mediated by avian-origin PB1. After pre-expression of NP protein with either WT pH1N1 polymerase or pH1N1 polymerase carrying avian-origin PB1, 293T cells were infected with pH1N1 virus (C) or pH1N1-PB1$_{Avi}$ virus (D). The relative vRNA and cRNA levels at 6 h.p.i were measured. The vRNA and cRNA levels in cells pre-expressing WT pH1N1 polymerase were set as 100%. All the virus infection was performed at an MOI of 10 in the presence of CHX. The levels of vRNA and cRNA were measured using strand-specific real-time PCR. The vRNA levels at 1 h.p.i. were served as control of input. Error bars represent the standard deviation of three biological replicates (±SD); the pattern of results are consistent in at least three independent experiments.

3.4 Discussion

The recurrent presence of avian-origin PB1 in past pandemic viruses has long been observed. Avian-origin PB1 appears to be important to the active viral polymerase activity in human cells, even though the underlying mechanism has never been revealed. In this study, using 2009 pH1N1 virus naturally lacking avian-origin PB1 segment as the platform, we examined the role of avian-origin PB1 in polymerase adaptation. Results of vRNP reconstitution assays suggested that avian-origin PB1 can enhance viral RNAs synthesis of pH1N1 polymerase, or even the avianized pH1N1 polymerase lacking critical PB2-associated adaptive mutations (SR polymorphism and 627K) (Figure. 3.2 and 3.4). Furthermore, acquisition of avian-origin PB1 by pH1N1 virus did not alter PB1-PA interaction or assembly of polymerase complex and vRNP, indicating the advantage provided by avian-origin PB1 is not result from the enhanced vRNP assembly capacity. (Figure. 3.3). Using cRNA stabilization assay, we demonstrated that avian-origin PB1 did not change the cRNA stabilization or synthesis abilities (Figure. 3.5). Instead, the resident polymerase on the incoming vRNP with avian-origin PB1 could serve as the more powerful *trans*-activating polymerase to cooperatively facilitate the vRNA synthesis performed by cRNP-associated polymerase (Figure. 3.6). These results revealed a possible mechanism by which avian-origin PB1 enhances viral polymerase activity and contributes to a potential pandemic virus formation in reassortment event between avian and human strains.

Our results substantiate that acquisition of avian-origin PB1 segment by pH1N1 virus can result in enhanced polymerase functions in human cells. Strikingly, the stark polymerase activity enhancing effect was not diminished by removal of SR polymorphism in PB2 segment, suggesting that avian-origin PB1, together with pandemic PA, rescues the major defect of avian-like polymerases in human cells (Figure. 3.1 and 3.2). In agreement with these findings, other studies also showed that introducing avian-origin PB1 segment into the A/WSN/33 virus remarkably enhanced the viral polymerase activity in human cells [169]. The virulence of 2009 pH1N1 virus is quite mild compared to earlier pandemic viruses such as 1918 pH1N1 virus [170]. Additionally, introducing the missing PB2 E627K mutation into 2009 pH1N1 virus cannot enhance the viral replication, pathogenicity, and transmissibility, suggesting that the mild pathogenicity probably is due to other missing virulence markers [171]. Interestingly, a recent study using competitive fitness experiments showed that avian-origin PB1 promoted the replication and transmission of

1968 pandemic H3N2 virus [138]. Therefore, it would be tempting to evaluate the effect of avian-origin PB1 on virulence and transmissibility of 2009 pH1N1 virus in animal models.

Using the split luciferase complementation assay, we quantified the dynamic interactions of PB1-PA in different conformations (Figure. 3.3). Our results indicate that avian-origin PB1 did not alter PB1-PA interaction or assembly of the heterotrimeric polymerase complex and vRNP. These results match to the previous finding that the defective assembly of vRNP is not responsible for the restricted functions of avian polymerase in human cells [116, 172].

Assessment of viral RNAs synthesis provides insights into the mechanism by which avian-origin PB1 enhances polymerase activity of pH1N1 virus. Primer extension results demonstrated that the vRNA, cRNA, and mRNA levels produced by polymerases containing avian-origin PB1 were elevated compared to WT pH1N1 polymerase. However, due to the interdependency of viral replication and transcription, this result cannot rule out the possibility that the increased mRNA level was simply the consequence of more vRNA template involved in the transcription. Through a modified vRNP reconstitution system, we showed that the basal transcription ability of pH1N1 polymerase was elevated after acquisition of avian-origin PB1. This notion was further verified by results acquired from regular vRNP reconstitution system with replication defective mutation which directly controlled the level of vRNA template used for mRNA synthesis (Figure. 3.4).

Avian-origin PB1 did not change the cRNA stabilization effect of polymerase (Figure. 3.5B), recapitulating the previous finding that the stabilization of nascent cRNA is probably just a biophysical process which is independent of the enzymatic function of polymerase [104]. Our observation that the pre-expressed catalytically inactive polymerase blocked the vRNA synthesis in cRNA stabilization assay favours the *trans*-activating model for vRNA synthesis, which allows us to further decipher the molecular mechanism of how avian-origin PB1 enhances viral replication (Figure. 3.5E). We showed that avian-origin PB1 specifically enhanced vRNA but not cRNA synthesis (Figure. 3.5), which was same to the previously reported function of PB2 E627K [132]. A proposed reason for the defective vRNA synthesis of avian influenza polymerase is the inability of providing bona fide cRNA template [132]. However, in our experiments, cRNA was detected by stand-specific real-time PCR instead of primer extension assay. Therefore, it is unlikely that the cRNA template is defective interfering molecules which cannot be used as template for the subsequent vRNA synthesis.

Based on *trans*-activating model for vRNA synthesis, we next asked whether avian-origin PB1 provided the advantages to the cRNP-associated polymerase or another *trans*-activating polymerase. In the heterologous settings of pre-expressed polymerases and incoming viruses (Figure. 3.6A), we noticed the enhanced viral replication performed by cRNP-associated polymerase when the incoming vRNP possessing the polymerase containing avian-origin PB1. These results suggested a stronger *trans*-activating effect from incoming vRNP-associated polymerase with avian-origin PB1. We also analysed the replication performed by the same cRNP-associated polymerases when different incoming vRNP-associated polymerases were provided (Figure. 3.6B). Coherently, the pH1N1-$PB1_{Avi}$ virus seemed to provide a stronger *trans*-activating polymerase, and thereby increasing the vRNA synthesis performed by the same cRNP-associated polymerase. Conversely, when the incoming vRNPs were the same (either pH1N1 virus or pH1N1-$PB1_{Avi}$ virus), the different cRNP-associated polymerases displayed the comparable replication capacity (Figure. 3.6C and D). These results implied that the incoming vRNP-associated polymerase could serve as the *trans*-activating polymerase, which would exert stronger *trans*-activating effect on the cRNP-associated polymerase to promoted vRNA synthesis once it acquired the avian-origin PB1. Intriguingly, a similar trend was observed in a study on PB2 E627K mutation [132]. Thus, it is plausible that the proposed model in here for avian-origin PB1 could also be applied to PB2 E627K or other mutations.

Our proposed model involved the interaction between different polymerase complexes. Indeed, structural studies showed that oligomerization of polymerase complexes is critical to the viral replication and transcription [173, 174]. As all the polymerase subunits are involved in this oligomerization process, it is tempting to speculate that PB2 E627K or certain amino acids on avian-origin PB1 might facilitate the oligomerization process and thus enhance the *trans*-activating effect. If this is the case, the role of ANP32A protein in host restrictions on polymerase could be hypothetically linked to this polymerase-polymerase interaction. However, further studies are required to verify these speculations.

In summary, we demonstrate the acquisition of avian-origin PB1 segment in reassortment event between avian and human influenza viruses could be a powerful adaptive strategy to overcome host restrictions on viral polymerase in human cells. We proposed that the polymerase

activity enhancing effect of avian-origin PB1 is because of the stronger *trans*-activating effect in vRNA synthesis as well as the enhanced transcription ability.

TRANSITION BETWEEN CHAPTER 3 AND CHAPTER 4

In above chapter, we substantiate that acquisition of avian-origin PB1 segment can lead to further mammalian adaptation of 2009 pH1N1 polymerase, even without the canonical PB2-associated adaptive mutations (SR polymorphism and 627K). Mechanistically, we substantiate that acquisition of avian-origin PB1 enhances both viral replication and transcription. In the replication step, acquisition of avian-origin PB1 specially enhances the vRNA synthesis in a *trans*-activating manner. These results imply the potential reason that avian-derived PB1 segments are recurrently selected by past pandemic viruses.

A recent study reported that the species difference in ANP32A (Acidic nuclear phosphoprotein 32 family member A) underlies the host restriction on influenza A virus polymerase complex [130]. Particularly, ANP32A has been demonstrated as an essential host factor specifically to the vRNA synthesis of influenza A virus [129]. Chicken ANP32A can boost the avian-like polymerase lacking the critical PB2-associated adaptive mutations, which is similar to the polymerase activity enhancing effect of avian-origin PB1 that we observed in pH1N1 background. Therefore, we are interested in interrogating whether the mechanistic model we proposed in chapter 3 could also be applied to explain the function of ANP32A in polymerase adaptation.

CHAPTER 4. CHICKEN ANP32A SPECIFICALLY ENHANCES VRNA SYNTHESIS IN A TRANS-ACTIVATING MANNER

ABSTRACT

The incompatibility between influenza viral polymerase complex and host factors within mammalian cells defines one of the major species barriers that restrict the viral replication in human. Diverse adaptive strategies have been deployed by the virus to overcome the restriction on its polymerase complex. The preferential selection of the canonical adaptive mutation PB2 E627K has recently been correlated to ANP32A, the major host factor underlying host restrictions on viral polymerase complex. Here, we report that the SR polymorphism in PB2 segment is deployed by virus to accommodate the species difference in ANP32A. Our results indicate that chANP32A can specifically enhance vRNA synthesis of avian-like (PB2 627E) polymerase by promoting the *trans*-activating effect in replication without altering the genuine vRNA assembly capacity.

4.1 Introduction

The host restriction on heterotrimeric polymerase complex of influenza A virus is one of the species barriers that block the virus from establishing itself among human population [156, 175]. It has long been observed that the avian influenza polymerase displays heavily restricted functions in human cells prior to the acquisition of adaptive mutations in its polymerase genes. Among the three viral polymerase subunits, PB2 is the major determinant of virus host range [9]. Multiple canonical adaptive mutations in PB2 segment have been identified and characterized [110, 120]. The PB2 E627K is recurrently selected by human-adapted strains and it is linked with increased viral replication, transmissibility, and pathogenicity [112, 176]. Alternatively, certain human isolates use SR polymorphism (serine at 590 residue, arginine at position 591 residue) in PB2 segment as an alternative adaptive strategy to compensate the naturally missed PB2 E627K mutation [120]. Although different theories have been proposed to explain the function of these canonical adaptive mutations [115, 130, 177], the exact molecular mechanism is not clear.

Acidic nuclear phosphoprotein 32 family member A (ANP32A) has recently been identified as one of primary host factors underlying host restrictions on viral polymerase complex [130, 131, 178]. ANP32A from avian species partially restores the impaired function of avian-like polymerase (PB2 627E) in human cells, where human ANP32A does not have this enhancing effect due to the lack of 33 amino acids avian-insertion compared to its avian counterparts [130]. Intriguingly, a SUMO-interaction motif (SIM)-like sequence found within this avian-insertion contributes to the enhancing effect mediated by chicken ANP32A [131]. But, a more recent study reported that the avian splicing isoforms naturally lacking this SIM-like sequence can still enhance viral polymerase activity, suggesting the SIM-like sequence is entirely dispensable [178]. Functionally, the polymerase activity enhancing effect mediated by chANP32A has been implicated in the enhanced vRNP assembly, recapitulating a previous finding that the defect of avian-like polymerase containing PB2 627E might be the impaired vRNP assembly [115]. However, both of these studies did not obviate interferes from the replicating RNA which was involved in the vRNP assembly [116]. Therefore, the exact molecular mechanism by which ANP32A defines the restriction on viral polymerase complex remains unknown.

Here, we dissect the effect of chANP32A on different steps of viral replication process as well as its implication in the selection of adaptive mutations at different residues. Our results

demonstrate that the selection of SR polymorphism in PB2 segment by the virus is also to accommodate the species difference in ANP32A protein. We also report that chANP32A specifically enhances vRNA synthesis, but not cRNA synthesis, without altering the genuine vRNA assembly capacity. Mechanistically, our data suggests that chANP32A can promote vRNA synthesis by strengthening the *trans*-activating effect.

4.2 MATERIALS AND METHODS

4.2.1 Cells

Madin-Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco). Human embryonic kidney 293T (293T) cells and chicken embryonic fibroblast (DF-1) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% FBS. MDCK cells and 293T cells were maintained at 37 °C; DF-1 cells were grown at 39 °C.

4.2.2 Plasmids

Polymerase proteins and NP protein expressing plasmids were constructed by cloning coding sequences of PB2, PB1, PA, and NP segments from pH1N1, A/Turkey/Ontario/6213/1966 (H5N1) (ON H5N1) (37), A/chicken/British Columbia/CN-6/2004 (H7N3) (BC H7N3) into pcDNA vector, generating plasmids (pcDNA-pH1N1-PB2, pcDNA-pH1N1-PB1, pcDNA-pH1N1-PA, pcDNA-pH1N1-NP), (pcDNA-BC H7N3-PB2, pcDNA-BC H7N3-PB1, pcDNA-BC H7N3-PA, pcDNA-BC H7N3-NP), pcDNA-ON H5N1-PB1. For the mutant pH1N1-PB2 constructs, 627K, 627S, 627L, 627I, 590G and 591Q were respectively introduced into pcDNA-pH1N1-PB2 to create the pcDNA-pH1N1-PB2 (627K/S/L/I), pcDNA-pH1N1-PB2 (590G/591Q), pcDNA-pH1N1-PB2 (590G/591Q, 627K/S/L/I). For the construction of catalytically inactive PB1a of pH1N1 virus, the double mutations (D445A and D446A) were introduced into the WT pcDNA-pH1N1-PB1. For vRNA expressing plasmids, pHH21-pH1N1-vNP and pHH21-pH1N1-vNA, the full-length vRNA of segment 6 of pH1N1 virus were respectively introduced into the pHH21 vector. The pPOLI-NP-Luc constructs with species-specific polymerase I promoter and pTK-RLuc used in minireplicon luciferase report assay were described elsewhere (10, 30).

Chicken ANP32A gene (Gallus gallus, XP_413932.3) was synthesized and constructed into pcDNA vector by Genscript.

4.2.3 Viruses

2009 pandemic H1N1 virus, the mutant pH1N1-PB2 (GQ) virus without SR polymorphism in PB2 segment, and the recombinant pH1N1-PB2 (GQ)-PB1 (Avi) virus with PB1 segment derived from an avian ON H5N1 virus were respectively generated by using eight-plasmid reverse genetic system (39). Viruses were propagated in MDCK cells. Virus titer was titrated by plaque assay. The origin of PB1 segment and the GQ mutations in PB2 590/591 residues were verified by sequencing.

4.2.4 Viral growth kinetics

For the single-cycle growth curve, confluent monolayers of MDCK cells were infected by respective viruses at an MOI of 0.001. After 1h of adsorption at 37 °C, cells were washed with MEM and cultured in viral growth medium (MEM containing 0.2% Bovine serum albumin and 1 μg/ml of TPCK-Trypsin). Supernatants were collected at 12, 24, 36, 48, 60, and 72 hours post infection and were stored at -80 °C. Viral titer of each time point was titrated in MDCK cells by plaque assay.

4.2.5 Split luciferase complementation assay (SLCA).

N-terminus (1-229) and C-terminus (230-311) of *Renilla* luciferase gene were respectively fused to different termini of full-length PB1 or PA segment of pH1N1 virus via protein linker (GGGSGGGS) as described elsewhere [165], generating plasmids LN-PB1 $_{(pH1N1)}$ and PA $_{(pH1N1)}$-LC. For selection of functional SLCA constructs, 293T cells were co-transfected with 50 ng of different PB1 SLCA constructs and PA SLCA constructs. In conformations of heterotrimeric polymerase complex or vRNP, 50 ng of pcDNA-pH1N1-PB2or with the additional 50 ng of pHH21-pH1N1-vNA and pcDNA-NP were co-transfected with SLCA constructs. The relative *Renilla* luciferase activity were measured at 24 h.p.t. by luciferase assay (Promega).

4.2.6 vRNP reconstitution assay and primer extension analysis.

293T cells in 6-well plates were transiently transfected with expression plasmids for three polymerase subunits (500 ng each), NP protein (500 ng), and vRNA-expressing plasmid (500ng) pHH21-vNA expressing the full-length NA segment vRNA template. Forty-eight hours post-transfection (h.p.t), cells were harvested for extraction of total RNA by Trizol. The levels of vRNA, cRNA, mRNA, and 5S rRNA were analyzed by primer extension as previously described [104, 166]. Products of primer extension were analysed by 15% PAGE containing 7M urea in TBE buffer and were detected by infrared imaging system (Odyssey, LI-COR).

4.2.7 Strand-specific real-time RT-PCR.

The relative quantification of vRNA and cRNA accumulated in cRNA stabilization assay were measured by strand-specific real-time RT-PCR as previously described [167]. Briefly, 350 ng of total RNA was reverse transcribed using hot-start reverse transcription at 60 °C for 1 hour. The 20 μl reaction system containing 4.5 μl RNA (350 ng), 6.5 μl saturated trehalose, 1 μl tagged RT primers (10 pmol), 4 μl First-strand buffer (5 ×, Invitrogen), 1 μl DTT (0.1M), 1 μl dNTP (10mM), 1 μl RNaseOUT (Invitrogen), and 1 μl SuperScript III reverse transcriptase (200U/μl ,Invitrogen). Real-time PCR was performed in 20 μl reaction system with 2 μl of cDNA, 1 × Power SYBR Green PCR master mix (Applied Biosystems), and 500 nM primers by StepOnePlus real-time PCR system (Applied Biosystems).

4.2.8 cRNA stabilization assay.

As described elsewhere [104], 293T cells in 6-well plate were transiently transfected with plasmid expressing three polymerase subunits (250 ng) and NP protein (1000 ng) using TransIT-LT1. 24 h.p.t, cells were infected by indicated viruses at an MOI of 10 in presence of cycloheximide (100 μg/ml). 1 or 6 hours post-infection, cells were collected for total RNA extraction by Trizol. vRNA or cRNA were quantified by strand-specific real-time RT-PCR.

4.2.9 Statistical analysis

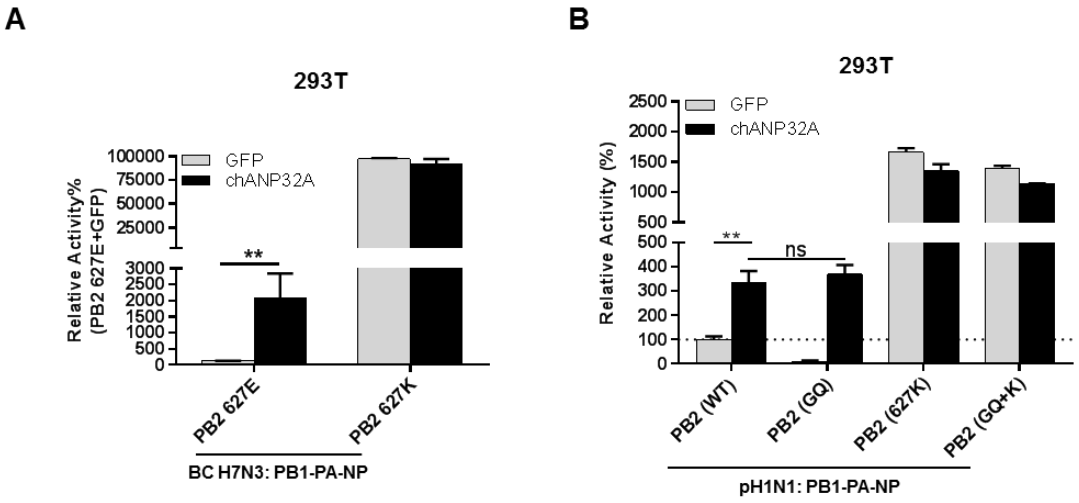
The statistical analyses were performed in GraphPad Prism 7(GraphPad Software, Inc., USA) using Student's t test, one-way ANOVA, or two-way ANOVA. Significant differences are denoted by the asterisks as follow: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$; $P < 0.0001$.

4.3 RESULTS

4.3.1 The SR polymorphism in PB2 segment is to accommodate the species difference in ANP32A.

To substantiate the reported enhancing effect of chANP32A on avian-like polymerase (PB2 627E) in human cells, we respectively measured the activity of either an H7N3 avian polymerase (A/chicken/British Columbia/CN-6/2004) or its cognate PB2 E627K mutant in presence of chANP32A. As previously reported, overexpression of chANP32A in 293T cells significantly increased the activity of PB2 627E polymerase (>200 folds) but not PB2 627K polymerase, and a single PB2 E627K mutation fully restored the impaired functions of avian polymerase in human cells (Figure. 4.1A). The most recent 2009 pandemic virus naturally lacks PB2 E627K mutation [118]. Alternatively, pH1N1 virus uses SR polymorphism (PB2 590S/591R) as the compensating adaptive strategy to maintain its active polymerase activity within human cells, as evidenced by removal of SR polymorphism severely impaired the activity of pH1N1 (WT) polymerase (Figure. 4.1B). Therefore, we thought to evaluate the effect of chANP32A on a series of mutant pH1N1 polymerases carrying different adaptive mutations at PB2 590, 591, and 627 residues. In a similar fashion, chANP32A elevated polymerase activities of all mutants lacking PB2 E627K, regardless the presence of SR polymorphism. Once the polymerase acquired the PB2 E627K mutation, its functions reached the optimal level which cannot be further enhanced by chANP32A. Intriguingly, chANP32A increased the activity of pH1N1 (WT) polymerase and avianized pH1N1 (GQ) polymerase to a similar level, implying that the mutations at PB2 590/591 residues might also to accommodate the species difference in ANP32A (Figure. 4.1B). In order to corroborate this finding, we differentiated the effect of chANP32A on PB2 590/591 and 627 residues by substituting the original glutamic acid (E) at 627 residue into serine, leucine, or isoleucine. Unlike PB2 E627K, the moderate enhancing effect mediated by these uncharged amino acids substitutions did not mask the adaptive effect of SR polymorphism. In these scenarios, removal of SR polymorphism can still decrease polymerase activities (Figure. 4.1C; bar 9, 13, 17 vs 11, 15, 19),

meanwhile, chANP32A cannot further enhance their polymerase activity (9, 13, 17 vs 10, 14, 18). Thus, we have the window to test the effect of chANP32A on PB2 590/591 residues alone without interfere from PB2 627 residue. As expected, chANP32A fully restored the impaired activities caused by lack of SR polymorphism (Figure. 4.1C), indicating that SR polymorphism could assist the viral polymerase to accommodate the species differences in ANP32A.



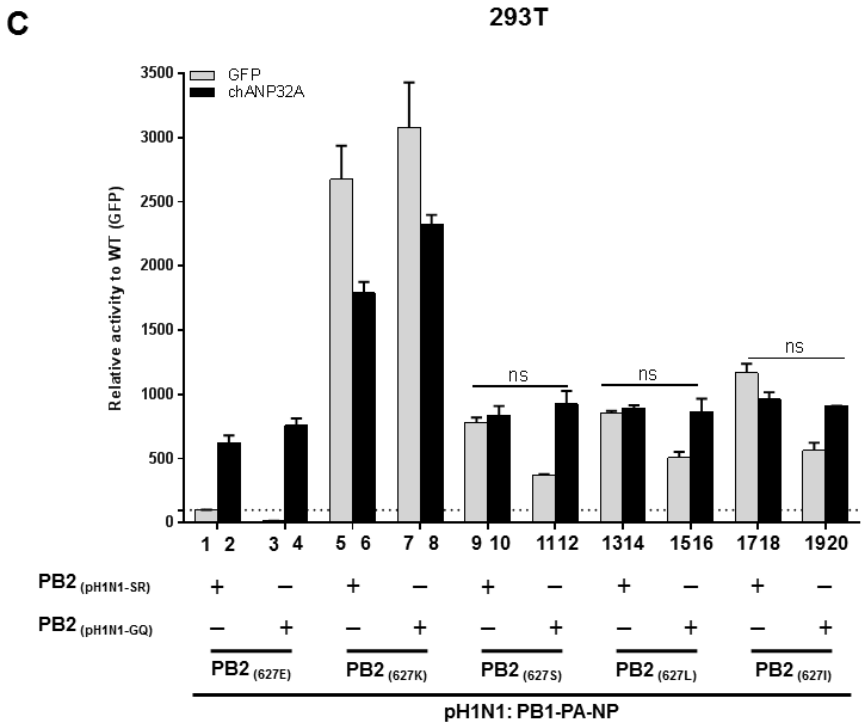
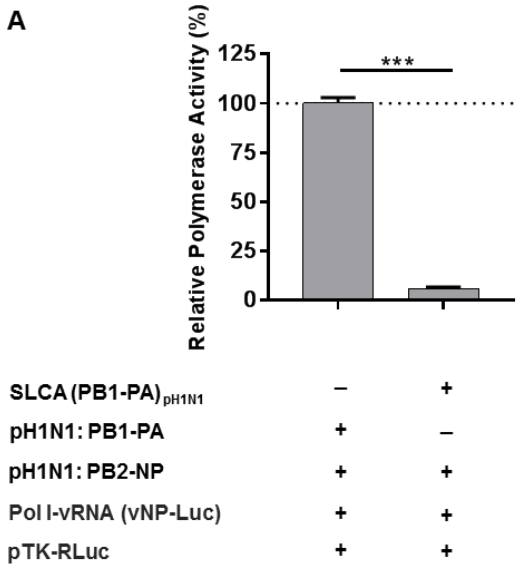


Figure 4.1 Adaptation of SR polymorphism is to accommodate to the species differences in ANP32A

(A) The effect of chANP32A on activities of avian polymerase or human-adapted polymerase in 293T cells. Polymerase activities of BC H7N3 (PB2 627E) polymerase or BC H7N3 (PB2 627K) polymerase were measured after co-expression of chANP32A (50 ng) or equal amount of pcDNA-GFP (negative control) in 293T cells. (B) The effect of chANP32A on activities of WT or mutant pH1N1 polymerases in 293T cells. Polymerase activities of pH1N1 (WT) polymerase or its PB2 mutants with indicated PB2 mutations were measured in presence of chANP32A or GFP in 293T cells by minireplicon assay (C) The function of SR polymorphism is to accommodate the species difference in ANP32A protein. E627S, E627L, and E627I were respectively introduced into 627 residue of pH1N1-PB2 segment with or without SR polymorphism. Plasmids expressing chANP32A or GFP were co-expressed with the reconstituted variant pH1N1 polymerases possessing indicated mutations in PB2 segment. Polymerase activities were measured by minireplicon assay. In above experiments, firefly luciferase activities were normalized to the *Renilla* luciferase activities. The polymerase activity of 2009 pH1N1 (WT) with GFP was set as 100%. Error bars represent the standard error of the mean of three biological replicates (±SD); the pattern of results are consistent in at least three independent experiments.

4.3.2 chANP32A does not alter the genuine assembly capacity of avian-style vRNP

The restricted function of avian-like polymerase was once attributed to the defective vRNP assembly [115]. Coherently, a recent study reported that chANP32A could partially restore this defect and thus elevate the activity of avian influenza polymerase [178]. However, the interfere from the replicating viral RNA participating in the vRNP assembly, which can be easily influenced by chANP32A or adaptive mutations in PB2 segment, was not obviated in these studies. Therefore, we designed the split luciferase complementation assay to measure the effect of chANP32A on the genuine assembly capacity of different vRNPs. As previously reported [115, 165], the *Renilla* luciferase activity generated by the association of N-terminally tagged *Renilla* luciferase PB1 and C-terminally tagged *Renilla* luciferase PA can be used as proxy to monitor the vRNP assembly capacity (Figure. 4.2B). The PB1-PA constructs with a tag on their interacting domains incapacitated the RNA replication, as evidenced by the background reading observed in the minireplicon assay (Figure. 4.2A). Co-expressing of chANP32A with PB2 (WT, GQ, or 627K), NP, viral RNA, and SLCA constructs (PB1-PA) did not increase *Renilla* luciferase readings (Figure. 4.2 C-E), suggesting that the authentic vRNA assembly capacity is not altered by chANP32A. Therefore, the polymerase activity enhancing effect of chANP32A is not due to the enhanced vRNP assembly capacity.



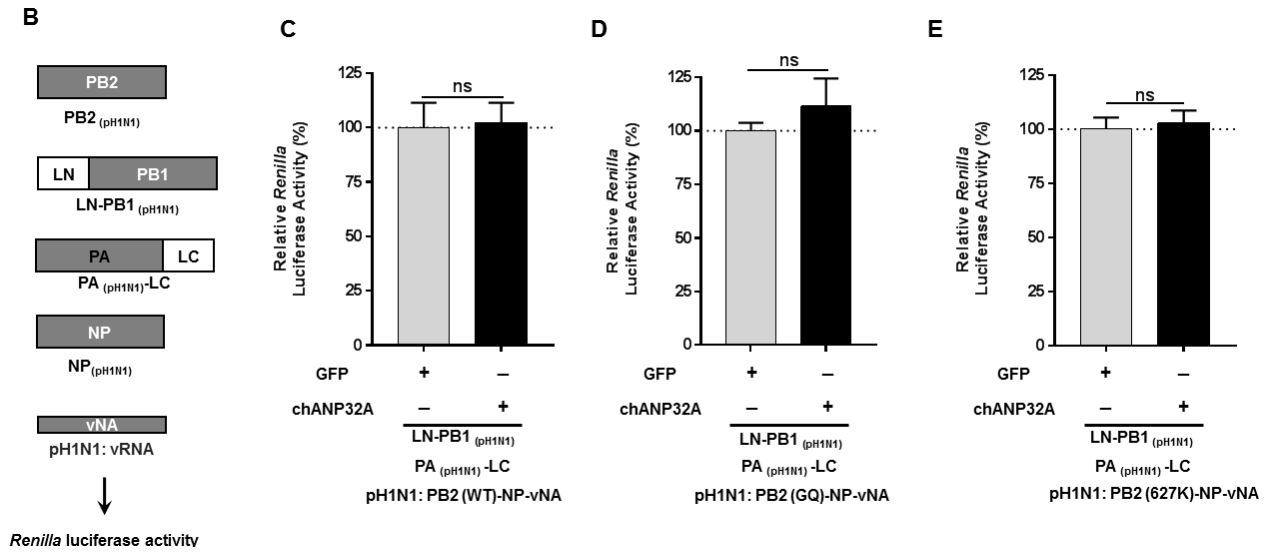


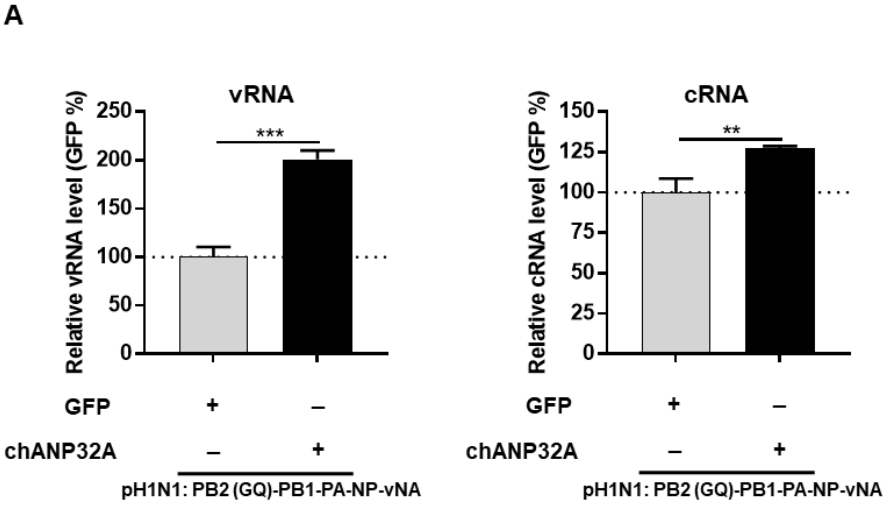
Figure 4.2 chANP32A does not enhance the genuine vRNP assembly capacity

(A) The tagged SLCA constructs (PB1-PA) disrupt the replication by viral polymerase. Polymerase activities of reconstituted pH1N1 (WT) vRNP with either pcDNA-PB1 and pcDNA-PA, or tagged SLCA (PB1-PA) constructs were measured by minireplicon assay. The activity of pH1N1 (WT) reconstituted by normal PB1-PA without any tag on their interacting domains was set as 100%. (B) The schematic diagram of SLCA in vRNP conformations. 293T cells were co-transfected each of the indicated vRNP component (50ng). The relative *Renilla* luciferase activity was measured at 24 h.p.t by luciferase assay. (C-E) chANP32A did not alter the genuine vRNP assembly capacity. Relative *Renilla* luciferase activities in 293T cells were measured at 24 hours after co-transfection of indicated pcDNA-pH1N1-PB2, pcDNA-pH1N1-NP, SLCA constructs (PB1-PA), pHH21-pH1N1-vNA expressing full-length vRNA of NA segment along with chANP32A or GFP. The *Renilla* luciferase produced by SLCA constructs with GFP was set as 100%. Error bars represent the standard error of the mean of three biological replicates (±SD); the pattern of results are consistent in at least three independent experiments.

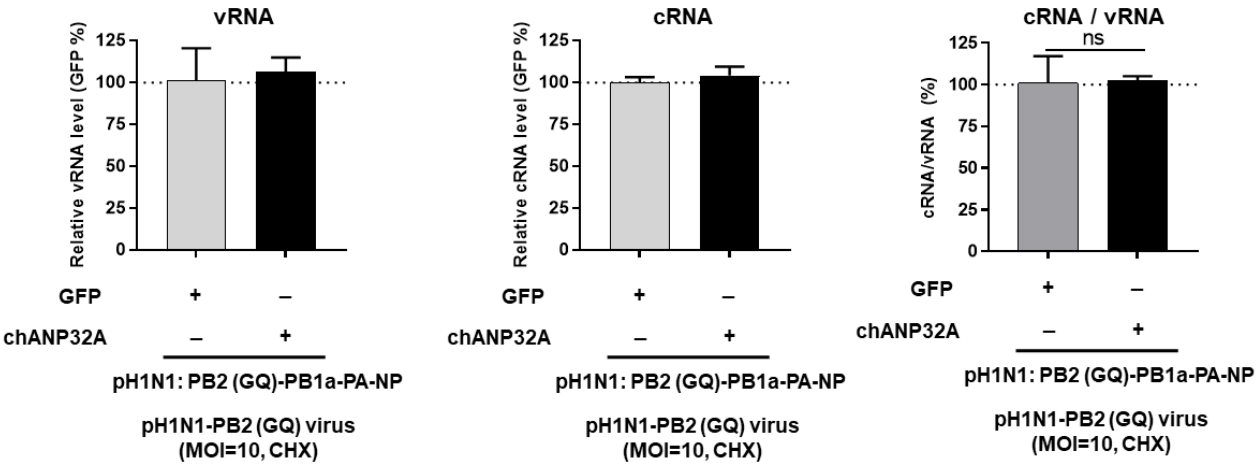
4.3.3 chANP32A specifically enhances vRNA synthesis of avian-like polymerase

Functionally, the defect of avian-like polymerase has been implicated in replication instead of transcription [132], which suggests that chANP32A could restored impaired viral replication of

avian-like polymerase. To investigate the effect of chANP32A on viral replication, the avianized pH1N1 (GQ) polymerase lacking SR polymorphism was reconstituted with chANP32A or GFP in 293T cells. As shown in Figure. 4.3A, chANP32A significantly increased the accumulation levels of vRNA and cRNA, demonstrating that chANP32A indeed facilitates replication of avian-like polymerase in human. To dissect which step of viral replication was promoted by chANP32A, we respectively analysed the effect of chANP32A on cRNA synthesis and vRNA synthesis using cRNA stabilization assay as previously described [104]. The pH1N1 PB2 (GQ), PB1a (catalytically inactive) [140], PA, NP together with either GFP or chANP32A were pre-expressed prior to the infection of avianized pH1N1 (GQ) virus in presence of cycloheximide. The catalytically inactive polymerase disrupts the interdependency of vRNA and cRNA synthesis, which allows us to measure cRNA synthesis alone without interfere from newly synthesized vRNA. Overexpression of chANP32A did not significantly change the cRNA/vRNA ratio, demonstrating that the cRNA synthesis, the first step of viral replication, is not promoted by chANP32A (Figure. 4.3B). Next, we exchanged the pre-expressed inactive polymerase into an active polymerase in the same experiment setting to restore the ongoing viral replication. chANP32A significantly increased the vRNA level and cRNA level, indicating that chANP32A can specifically enhance synthesis of nascent vRNA (Figure. 4.3C).



B


vRNA
Relative vRNA level (GFP %)
125
100
75
50
25
0
GFP + –
chANP32A – +
pH1N1: PB2 (GQ)-PB1a-PA-NP
pH1N1-PB2 (GQ) virus
(MOI=10, CHX)
cRNA
Relative cRNA level (GFP %)
125
100
75
50
25
0
GFP + –
chANP32A – +
pH1N1: PB2 (GQ)-PB1a-PA-NP
pH1N1-PB2 (GQ) virus
(MOI=10, CHX)
cRNA / vRNA
ns
cRNA/vRNA (%)
125
100
75
50
25
0
GFP + –
chANP32A – +
pH1N1: PB2 (GQ)-PB1a-PA-NP
pH1N1-PB2 (GQ) virus
(MOI=10, CHX)

C

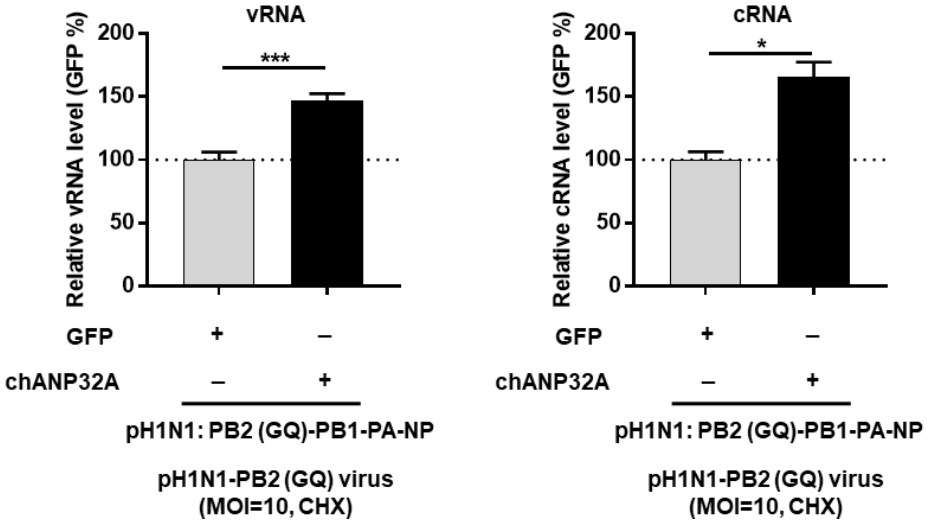

vRNA
***
Relative vRNA level (GFP %)
200
150
100
50
0
GFP + –
chANP32A – +
pH1N1: PB2 (GQ)-PB1-PA-NP
pH1N1-PB2 (GQ) virus
(MOI=10, CHX)
cRNA
*
Relative cRNA level (GFP %)
200
150
100
50
0
GFP + –
chANP32A – +
pH1N1: PB2 (GQ)-PB1-PA-NP
pH1N1-PB2 (GQ) virus
(MOI=10, CHX)

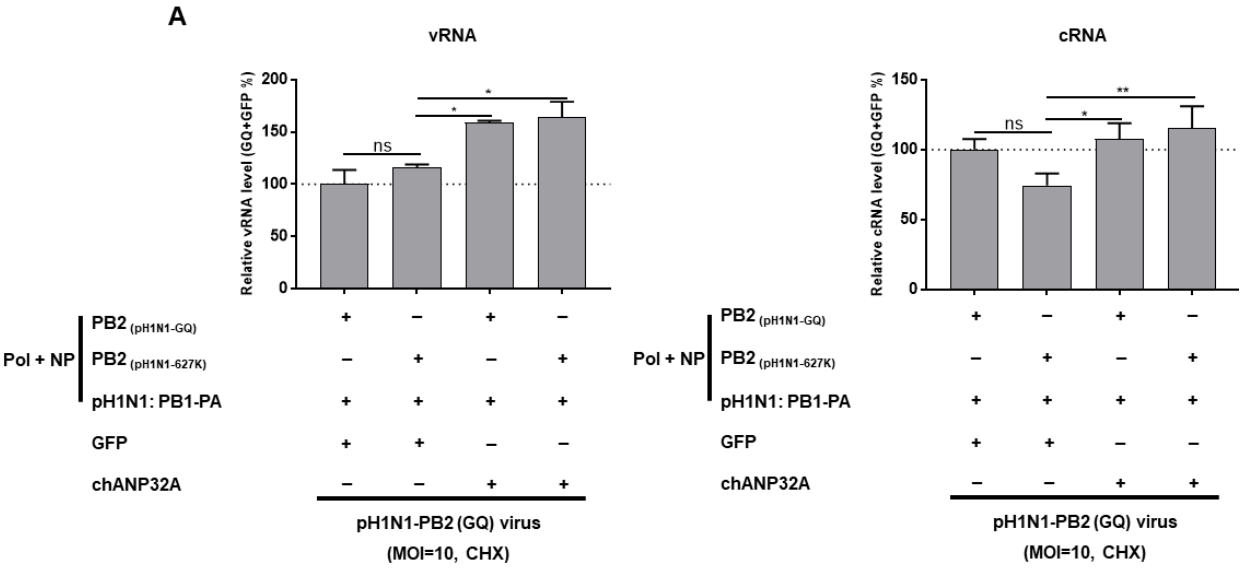
Figure 4.3 chANP32A specifically enhances the vRNA synthesis of avianized pH1N1 polymerase in humans.

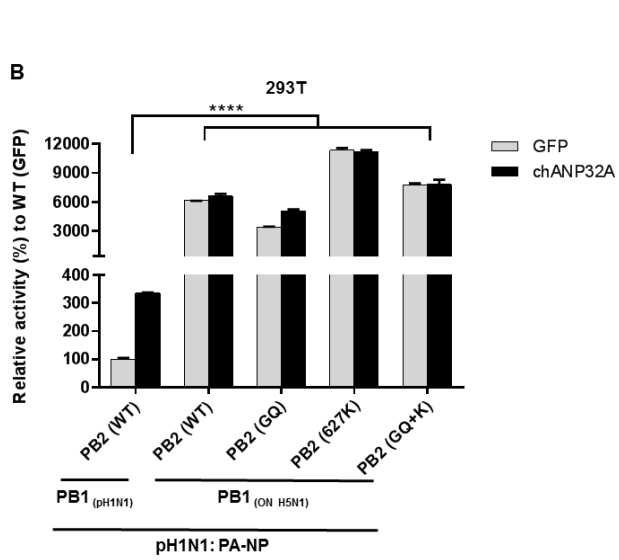
(A) chANP32A enhanced viral replication of avian-like polymerase in human cells. The avianized pH1N1 (GQ) polymerase was reconstituted in 293T cells with either GFP or chANP32A. At 48 h.p.t, the relative levels of vRNA and cRNA accumulations were measured by strand-specific real time PCR. The vRNA and cRNA levels produced by pH1N1 (GQ) polymerase with GFP was set as 100%. (B) chANP32A did not promote cRNA synthesis. 293T cells were co-transfected with plasmids expressing pH1N1-PB2, PB1a (mutant PB1 with D445A/D446A in catalytic domain [140]), PA, NP, along with chANP32A or GFP followed by infection of avianized pH1N1-PB2 (GQ) virus at an MOI of 10 in presences of cycloheximide. vRNA and cRNA levels at 6 h.p.i. were respectively measured by strand-specific real-time PCR. The ratio of cRNA and vRNA in cells pre-expressed with GFP was set as 100%. (C) chANP32A specifically facilitated the synthesis of nascent vRNA. 293T cells were pre-expressed with active pH1N1 polymerase, NP protein, together with chANP32A or GFP followed by infection of avianized pH1N1-PB2 (GQ) virus at an MOI of 10 in presences of cycloheximide. vRNA and cRNA levels at 6 h.p.i. were measured by strand-specific real-time PCR. Error bars represent the standard error of the mean of three biological replicates (±SD); the pattern of results are consistent in at least two independent experiments.

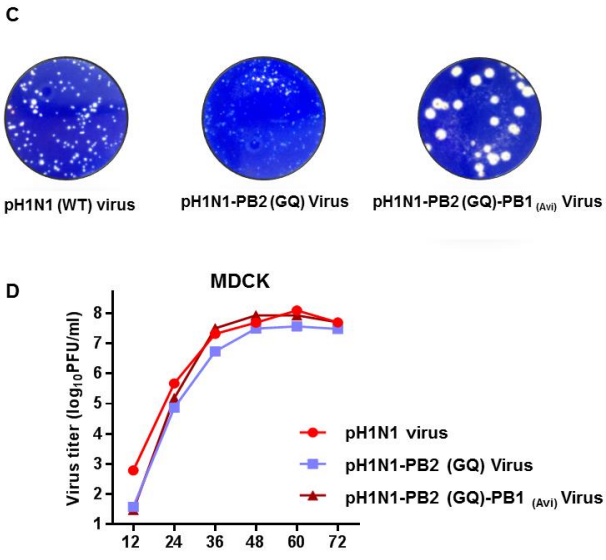
4.3.4. chANP32A enhances the activating effect of *trans*-polymerase

The synthesis of nascent vRNA is carried out by the cRNP-associated polymerase with the indispensable activation from the *trans*-polymerase (*trans*-activating effect) [66]. Since chANP32A could facilitate the vRNA synthesis, this enhancing effect could either be exerted on the cRNP-associated polymerase or the *trans*-activating effect. As shown in Figure. 4.4A (Bar 1 and 2), when the incoming vRNP carried the avian-like pH1N1 (GQ) polymerase, the amount of vRNA or cRNA produced by fully adapted cRNP-associated polymerases (627K) was at a comparable level as the avian-like cRNP-associated polymerase (GQ) did. Notably, by addition of chANP32A, the avian-like pH1N1 (GQ) polymerase even produced significantly higher level of vRNA and cRNA than the adapted pH1N1 (627K) polymerase did without chANP32A (Figure. 4.4A), implying that the enhancing effect of chANP32A is not on cRNA-associated polymerase

but on the *trans*-activating effect carried out by incoming vRNP-associated polymerase. To further corroborate this finding, we explored the effect of chANP32A on viral replication when a fully-adapted *trans*-activating polymerase carried by incoming vRNP is provided. To this end, we generated the reassortant pH1N1 virus, pH1N1-PB2 (GQ)-PB1 $_{(Avi)}$ Virus, the PB1 segment of which is from avian A/Turkey/Ontario/6213/1966 (H5N1) virus. As shown in Figure. 4.4B, the reassortant pH1N1 vRNP with avian-origin PB1 displayed significantly higher polymerase activity compared to pH1N1 (WT) polymerase. Addition of chANP32A cannot further enhance polymerase activities of these reassortant vRNPs (Figure. 4.4B), recapitulating the fully-adapted status mediated by avian-origin PB1. In MDCK cells, although acquisition of avian-origin PB1 did not markedly promote the viral growth kinetics (Figure. 4.4C), it apparently changed the plaque phenotype as observed by the increased radium of plaque size (Figure. 4.4D). To verify whether the enhancing effect of chANP32A on viral replication would be diminished when the defect in polymerase carried by incoming vRNP is restored, we set up the similar setting as mentioned above but with an adapted *trans*-activating polymerase containing avian-origin PB1. In this scenario, all these cRNP-associated polymerases with different signatures produced similar levels of vRNA and cRNA, despite the presence of chANP32A (Figure. 4.4E). Taken together, these results indicate that chANP32A can promote vRNA synthesis by facilitating the *trans*-activating effect exerted on cRNP-associated polymerase.


A
vRNA
Relative vRNA level (GQ+GFP %)
200
150
100
50
0
ns
*
*
Pol + NP
PB2 (pH1N1-GQ)
PB2 (pH1N1-627K)
pH1N1: PB1-PA
GFP
chANP32A
+ – + –
– + – +
+ + + +
+ + – –
– – + +
pH1N1-PB2 (GQ) virus
(MOI=10, CHX)
cRNA
Relative cRNA level (GQ+GFP %)
150
100
50
0
ns
*
**
Pol + NP
PB2 (pH1N1-GQ)
PB2 (pH1N1-627K)
pH1N1: PB1-PA
GFP
chANP32A
+ – + –
– + – +
+ + + +
+ + – –
– – + +
pH1N1-PB2 (GQ) virus
(MOI=10, CHX)


B
293T
****
Relative activity (%) to WT (GFP)
12000
9000
6000
3000
400
300
200
100
0
GFP
chANP32A
PB2 (WT)
PB2 (WT)
PB2 (GQ)
PB2 (627K)
PB2 (GQ+K)
PB1 (pH1N1)
PB1 (ON H5N1)
pH1N1: PA-NP


C
pH1N1 (WT) virus
pH1N1-PB2 (GQ) Virus
pH1N1-PB2 (GQ)-PB1 (Avi) Virus
D
MDCK
Virus titer (log10PFU/ml)
8
7
6
5
4
3
2
1
12
24
36
48
60
72
pH1N1 virus
pH1N1-PB2 (GQ) Virus
pH1N1-PB2 (GQ)-PB1 (Avi) Virus

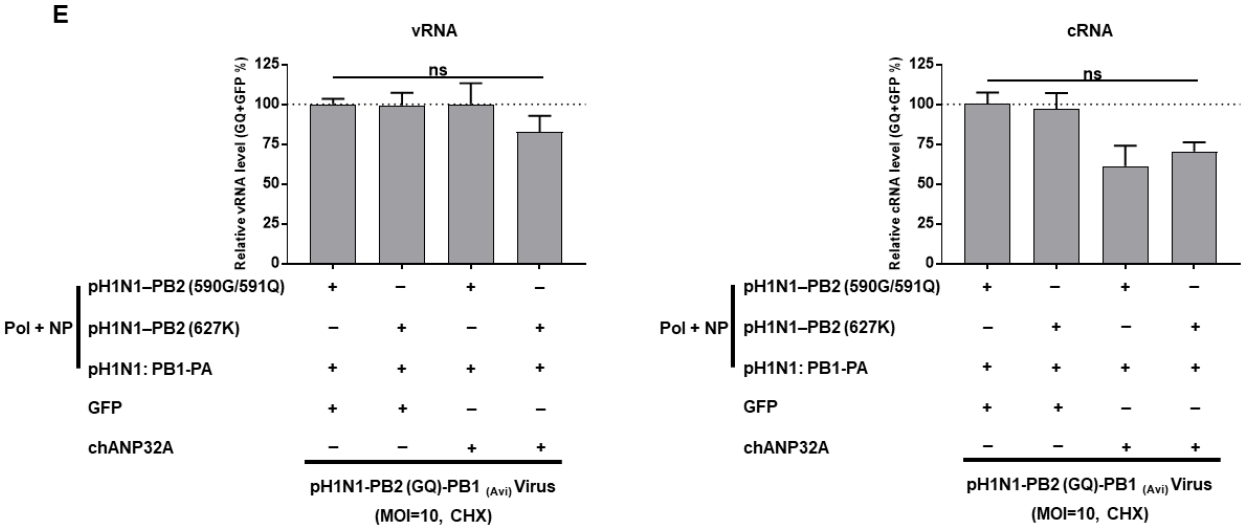


Figure 4.4 chANP32A enhances the activating effect of trans-polymerase

(A) The adaptation of cRNP-associated polymerase is not responsible for the enhanced vRNA synthesis by overexpression of chANP32A. 293T cells were pre-expressed with either pH1N1-PB2 (GQ) or pH1N1-PB2 627K along with pH1N1-PB2, PA, NP, chANP32A (GFP control) followed by infection of avianized pH1N1-PB2 (GQ) virus at an MOI of 10 in presences of cycloheximide. (B) Avian-origin PB1 renders fully adapted status to pH1N1 virus. Polymerase activities of reassortant pH1N1 vRNP with an avian-derived PB1 and different signatures in PB2 segment were respectively measured by luciferase assay. The activity of pH1N1 (WT) vRNP with GFP was set as 100%. (C) Plaque phenotypes of pH1N1 (WT) virus, pH1N1-PB2 (GQ) virus, and reassortant pH1N1-PB2 (GQ)-PB1 $_{(Avi)}$ Virus in MDCK cells. (D) Multicycle growth curve of pH1N1 (WT) virus, pH1N1-PB2 (GQ) virus, and reassortant pH1N1-PB2 (GQ)-PB1 $_{(Avi)}$ Virus in MDCK cells. (E) chANP32A promotes the *trans*-activating effect in vRNA synthesis. 293T cells were pre-expressed with same combination of viral polymerase and NP proteins with chANP32A or GFP as (A), while the subsequent infection was conducted using the reassortant pH1N1-PB2 (GQ)-PB1 $_{(Avi)}$ Virus carrying the fully-adapted polymerase. In above cRNA stabilization assays, vRNA and cRNA levels at 6 h.p.i. were respectively measured by strand-specific real-time PCR. In each setting, the vRNA and cRNA levels in cells pre-expressed with pH1N1 (GQ) polymerase and GFP were set as 100%. Error bars represent the standard error of the mean of three biological replicates (±SD); the pattern of results are consistent in at least two independent experiments.

4.4 DISCUSSION

The defect of avian-like polymerase is speculated in its incompatibility with mammalian host factors in cellular milieu [115, 122]. The species differences in ANP32A have been confirmed as one of the major components of host restrictions on viral polymerase complex. By differentiating the effect of chANP32A on PB2 627 residue, and 590, 591residues, we show that SR polymorphism, like PB2 E627K, is also to accommodate the species differences in ANP32A. Unlike previous study, we demonstrate that chANP32A can specifically enhance vRNA without altering the genuine vRNA assembly capacity. Instead, it is the stronger *trans*-activating effect mediated by chANP32A leads to the enhanced vRNA synthesis.

Using different combinations of adaptive mutations at PB2 590, 591, and 627 residues, we showed that ANP32A also underlies the selection of SR polymorphism in PB2 segment. In fact, it is reasonable to substantiate that SR polymorphism and E627K in PB2 segment share the same adaptive pathway as both of them have been demonstrated to restore the positively charged surface of PB2 627-domain [120]. Thus, it is attempting to investigate whether the polymerase supporting effect of chANP32A represents a global effect which could be applied to explain the recurrent selection of other adaptive mutations in viral polymerase genes.

Contrary to a recent finding that chANP32A could promote vRNP assembly, our results indicate that chANP32A actually cannot alter the genuine vRNP assembly capacity when the amount of vRNA template involved in vRNP assembly is constant. The application of split luciferase complementation assay allows us to dynamically evaluate the vRNP assembly capacity in different settings. Particularly, the tagged luciferase gene on PB1 and PA simultaneously disrupts the normal viral replication, which makes it possible to measure the authentic vRNP assembly capacity without interferes from replicating vRNA, a variable that could be easily changed by adaptive mutations or chANP32A. Therefore, our data together with a previous study suggests that the defective vRNP assembly actually is not responsible for the defects of avian influenza polymerase in human cells [116].

ANP32A is proved as an essential factor to the vRNA synthesis of influenza A virus even before its role in host restriction on viral polymerase complex has been recognized. Analogously, our results show that chANP32A can specifically enhance vRNA synthesis of avianized pH1N1

(GQ) polymerase, which agrees with the previous finding that the major defect of avian-like polymerase in human cells is vRNA synthesis [132]. The mechanism of vRNA synthesis is not fully clear yet, different models (*trans*-acting or *trans*-activating) have been proposed to explain the molecular details in this step [66, 91]. Based on other reports [66], the *trans*-activating model in which the vRNA synthesis is actually performed by cRNP-associated polymerase is more closely matched with the experimental data. Based on *trans*-activating model, we further pinpoint the advantage provided by chANP32A to the *trans*-activating process. It has been reported that ANP32A can only interact with the heterotrimeric polymerase complex instead of single polymerase subunit or and the binary sub-complex [129]. More importantly, the species specific interaction of ANP32A is independent of identifies of PB2 627 residue [131]. Possibly, ANP32A is involved in polymerase-polymerase interaction (such as recruitment of the *trans*-polymerase) which is critical to viral replication and transcription [173, 174]. If this is the case, the function of PB2 E627K is purely to compensate the impaired polymerase-polymerase interaction of avian influenza polymerase complex in human cells. In summary, we proposed a possible mechanism by which chANP32A to promote viral replication of avian-like polymerase by stimulating the *trans*-activating effect in the synthesis of nascent vRNA, which sheds light on the mechanism of influenza polymerase mammalian adaptation.

CHAPTER 5. GENERAL DISCUSSION

5.1 SUMMARY OF THE PROJECT

It has long been observed that avian-origin PB1 segments are recurrently selected in reassortment events of previous pandemic viruses [137]. However, the underlying mechanism behind this phenomenon is enigmatic. In this project, by using the most recent pH1N1 virus naturally lacking avian-origin PB1 segment, we demonstrate that acquiring an avian-origin PB1 indeed can promote transcription as well as replication of pH1N1 polymerase within mammalian cells. In the replication process, our results indicate that avian-origin PB1 can specifically enhance the synthesis of nascent vRNA in a *trans*-activating manner. The adaptive strategies deployed by the virus are thought to accommodate incompatibilities between avian-like polymerase and host factors within mammalian hosts. ANP32A has recently been identified as one of the major host factors underlying host restriction of the viral polymerase complex [130]. Our finding that

ANP32A from avian species can specifically facilitate the vRNA synthesis by promoting the *trans*-activation effect recapitulates that adaptation mediated by avian-origin PB1 in reassortment.

The replication mechanism (especially vRNA synthesis) of influenza A virus is not fully elucidated yet. Using polymerases bearing different adaptive signatures in cRNA stabilization assays, our data implies that the incoming vRNP-associated polymerase might serve as the *trans*-activating polymerase to cooperatively perform the vRNA synthesis with cRNP-associated polymerase. More importantly, the adaptive signatures carried by the incoming vRNP-associated polymerase, instead of cRNP-associated polymerase, play the dominant role in determining the consequences of vRNA synthesis. Although there is no direct *in vitro* biochemical studies in this project to further verify our proposed RNP-RNP interaction, it is still reasonable to argue that that the incoming vRNP-associated polymerases with different adaptive mutations determine the first round of replication, which in turn would be crucial to the subsequent ongoing replication.

Coherently, our results pertaining to ANP32A lead us to the same conclusion that the defect of avian-like polymerase is in vRNA synthesis, probably due to the weak *trans*-activating effect of the *trans*-polymerase. Up to date, the molecular mechanism of how ANP32A regulates the viral polymerase activities is unknown. Our study shed light on a possible direction which is that ANP32A mediated the interaction between the *trans*-activating polymerase and cRNP-associated polymerase, thereby enhancing the trans-activating effect and promoting the vRNA synthesis. Indeed, there is emerging data showing that the polymerase-polymerase interaction might be critical to the RNA synthesis of influenza A virus [173, 174]. If this is the case, it is tempting to speculate that the general function of canonical adaptive mutation in polymerase genes is to maintain the efficient interaction between polymerase complexes.

In summary, we demonstrate the adaptive role of avian-origin PB1 in pandemic formation, and reveal the molecular mechanism by which avian-origin PB1 enhances viral polymerase activity in reassortment with human strain. Moreover, our data also incorporates the function of ANP32A, the recent identified polymerase supporting host factor, into the adaptation model we proposed for avian-origin PB1. These knowledge would promote our understanding of basic influenza replication mechanism as well as its polymerase adaptation mechanism.

5.2 FUTURE DIRECTIONS

First, it is worthwhile to pinpoint the exact avian-signatures in PB1 segment. Sequence alignment results indicate that there are twenty conserved amino acid substitutions between avian-origin PB1 segments and the 2009 pH1N1 PB1 segment (human-derived). These mutations are scattered among different functional domains of the entire PB1 segments. We indeed constructed a serial of chimeric PB1 segment to screen the critical avian-signatures. The mutations in N-terminal domain (1-240) of avian-origin PB1 segment play the major role in the enhanced polymerase activity. However, due to the compounding effect of multiple different mutations, we did not pinpoint the avian-signatures to specific mutations.

Second, it would be interesting to test the actual effect of avian-origin PB1 on the virulence or transmissibility of 2009 pH1N1 virus. Although we did not observe the stark increase in viral replication in cell cultures, the actual infection in animal model could be distinct from what we observed in cell culture. Additionally, the growth kinetics in cell culture cannot reflect the changes in viral transmissibility which is a critical factor in pandemic formation.

Third, further studies on the polymerase-polymerase interaction could provide more insights into the fundamental mechanism of viral replication and mammalian adaptation of viral polymerase complex. In this project, our results suggest that acquisition of avian-origin PB1 or overexpression can both facilitate the *trans*-activating effect in vRNA synthesis, which might involve the interaction between two different polymerase complexes. Therefore, establishing a system to observe the interaction between two different trimeric polymerase complexes could provide more direct evidences about the role of adaptive mutations or host factors in mammalian adaptation of viral polymerase complex. Such a method could also be used to further corroborate our findings.

REFERENCES

1. Bernard N; Knipe, D.M.D.M.-H., Peter M, *Fields virology.* p. 1187.
2. Tong, S., et al., *New world bats harbor diverse influenza A viruses.* PLoS Pathog, 2013. **9**(10): p. e1003657.
3. Matsuzaki, Y., et al., *Clinical features of influenza C virus infection in children.* J Infect Dis, 2006. **193**(9): p. 1229-35.
4. Hause, B.M., et al., *Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses.* PLoS Pathog, 2013. **9**(2): p. e1003176.
5. Noda, T., *Native morphology of influenza virions.* Front Microbiol, 2011. **2**: p. 269.
6. Scheiffele, P., et al., *Influenza viruses select ordered lipid domains during budding from the plasma membrane.* J Biol Chem, 1999. **274**(4): p. 2038-44.
7. Lamb, R.A., S.L. Zebedee, and C.D. Richardson, *Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface.* Cell, 1985. **40**(3): p. 627-33.
8. Enami, M. and K. Enami, *Influenza virus hemagglutinin and neuraminidase glycoproteins stimulate the membrane association of the matrix protein.* J Virol, 1996. **70**(10): p. 6653-7.
9. Te Velthuis, A.J. and E. Fodor, *Influenza virus RNA polymerase: insights into the mechanisms of viral RNA synthesis.* Nat Rev Microbiol, 2016. **14**(8): p. 479-93.
10. Richardson, J.C. and R.K. Akkina, *NS2 protein of influenza virus is found in purified virus and phosphorylated in infected cells.* Arch Virol, 1991. **116**(1-4): p. 69-80.
11. Hutchinson, E.C., et al., *Conserved and host-specific features of influenza virion architecture.* Nat Commun, 2014. **5**: p. 4816.
12. Rogers, G.N. and J.C. Paulson, *Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin.* Virology, 1983. **127**(2): p. 361-73.
13. Connor, R.J., et al., *Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates.* Virology, 1994. **205**(1): p. 17-23.
14. Shinya, K., et al., *Avian flu: influenza virus receptors in the human airway.* Nature, 2006. **440**(7083): p. 435-6.
15. Suzuki, Y., et al., *Sialic acid species as a determinant of the host range of influenza A viruses.* J Virol, 2000. **74**(24): p. 11825-31.
16. Rogers, G.N., et al., *Differential sensitivity of human, avian, and equine influenza A viruses to a glycoprotein inhibitor of infection: selection of receptor specific variants.* Virology, 1983. **131**(2): p. 394-408.
17. Glaser, L., et al., *A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity.* J Virol, 2005. **79**(17): p. 11533-6.
18. Dunham, E.J., et al., *Different evolutionary trajectories of European avian-like and classical swine H1N1 influenza A viruses.* J Virol, 2009. **83**(11): p. 5485-94.

19. Ito, T., et al., *Molecular basis for the generation in pigs of influenza A viruses with pandemic potential.* J Virol, 1998. **72**(9): p. 7367-73.

20. Lakadamyali, M., M.J. Rust, and X. Zhuang, *Endocytosis of influenza viruses.* Microbes Infect, 2004. **6**(10): p. 929-36.

21. Skehel, J.J. and D.C. Wiley, *Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin.* Annu Rev Biochem, 2000. **69**: p. 531-69.

22. Huang, R.T., R. Rott, and H.D. Klenk, *Influenza viruses cause hemolysis and fusion of cells.* Virology, 1981. **110**(1): p. 243-7.

23. Pinto, L.H. and R.A. Lamb, *The M2 proton channels of influenza A and B viruses.* J Biol Chem, 2006. **281**(14): p. 8997-9000.

24. Harrison, S.C., *Viral membrane fusion.* Nat Struct Mol Biol, 2008. **15**(7): p. 690-8.

25. O'Neill, R.E., et al., *Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import.* J Biol Chem, 1995. **270**(39): p. 22701-4.

26. Martin, K. and A. Helenius, *Transport of incoming influenza virus nucleocapsids into the nucleus.* J Virol, 1991. **65**(1): p. 232-44.

27. Chou, Y.Y., et al., *Colocalization of different influenza viral RNA segments in the cytoplasm before viral budding as shown by single-molecule sensitivity FISH analysis.* PLoS Pathog, 2013. **9**(5): p. e1003358.

28. Eisfeld, A.J., G. Neumann, and Y. Kawaoka, *At the centre: influenza A virus ribonucleoproteins.* Nat Rev Microbiol, 2015. **13**(1): p. 28-41.

29. Plotch, S.J., et al., *A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription.* Cell, 1981. **23**(3): p. 847-58.

30. Poon, L.L., et al., *Direct evidence that the poly(A) tail of influenza A virus mRNA is synthesized by reiterative copying of a U track in the virion RNA template.* J Virol, 1999. **73**(4): p. 3473-6.

31. Fodor, E., *The RNA polymerase of influenza a virus: mechanisms of viral transcription and replication.* Acta Virol, 2013. **57**(2): p. 113-22.

32. Hutchinson, E.C. and E. Fodor, *Transport of the influenza virus genome from nucleus to nucleus.* Viruses, 2013. **5**(10): p. 2424-46.

33. Akarsu, H., et al., *Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2).* EMBO J, 2003. **22**(18): p. 4646-55.

34. Neumann, G., M.T. Hughes, and Y. Kawaoka, *Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1.* EMBO J, 2000. **19**(24): p. 6751-8.

35. O'Neill, R.E., J. Talon, and P. Palese, *The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins.* EMBO J, 1998. **17**(1): p. 288-96.

36. Perez, D.R. and R.O. Donis, *The matrix 1 protein of influenza A virus inhibits the transcriptase activity of a model influenza reporter genome in vivo.* Virology, 1998. **249**(1): p. 52-61.

37. Chua, M.A., et al., *Influenza A virus utilizes suboptimal splicing to coordinate the timing of infection.* Cell Rep, 2013. **3**(1): p. 23-9.

38. Sakaguchi, A., et al., *Nuclear export of influenza viral ribonucleoprotein is temperature-dependently inhibited by dissociation of viral matrix protein.* Virology, 2003. **306**(2): p. 244-53.

39. Cao, S., et al., *A nuclear export signal in the matrix protein of Influenza A virus is required for efficient virus replication.* J Virol, 2012. **86**(9): p. 4883-91.

40. Shimizu, T., et al., *Crucial role of the influenza virus NS2 (NEP) C-terminal domain in M1 binding and nuclear export of vRNP.* FEBS Lett, 2011. **585**(1): p. 41-6.

41. Faleiro, L. and Y. Lazebnik, *Caspases disrupt the nuclear-cytoplasmic barrier.* J Cell Biol, 2000. **151**(5): p. 951-9.

42. Momose, F., et al., *Visualization of microtubule-mediated transport of influenza viral progeny ribonucleoprotein.* Microbes Infect, 2007. **9**(12-13): p. 1422-33.

43. Amorim, M.J., et al., *A Rab11- and microtubule-dependent mechanism for cytoplasmic transport of influenza A virus viral RNA.* J Virol, 2011. **85**(9): p. 4143-56.

44. Momose, F., et al., *Apical transport of influenza A virus ribonucleoprotein requires Rab11-positive recycling endosome.* PLoS One, 2011. **6**(6): p. e21123.

45. Avilov, S.V., et al., *Influenza A virus progeny vRNP trafficking in live infected cells studied with the virus-encoded fluorescently tagged PB2 protein.* Vaccine, 2012. **30**(51): p. 7411-7.

46. Hutchinson, E.C., et al., *Genome packaging in influenza A virus.* J Gen Virol, 2010. **91**(Pt 2): p. 313-28.

47. Harris, A., et al., *Influenza virus pleiomorphy characterized by cryoelectron tomography.* Proc Natl Acad Sci U S A, 2006. **103**(50): p. 19123-7.

48. Noda, T., et al., *Architecture of ribonucleoprotein complexes in influenza A virus particles.* Nature, 2006. **439**(7075): p. 490-2.

49. Fournier, E., et al., *A supramolecular assembly formed by influenza A virus genomic RNA segments.* Nucleic Acids Res, 2012. **40**(5): p. 2197-209.

50. Noda, T., et al., *Three-dimensional analysis of ribonucleoprotein complexes in influenza A virus.* Nat Commun, 2012. **3**: p. 639.

51. Gavazzi, C., et al., *An in vitro network of intermolecular interactions between viral RNA segments of an avian H5N2 influenza A virus: comparison with a human H3N2 virus.* Nucleic Acids Res, 2013. **41**(2): p. 1241-54.

52. Brown, D.A. and J.K. Rose, *Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface.* Cell, 1992. **68**(3): p. 533-44.

53. Chen, B.J., et al., *Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles.* J Virol, 2007. **81**(13): p. 7111-23.

54. Leser, G.P. and R.A. Lamb, *Influenza virus assembly and budding in raft-derived microdomains: a quantitative analysis of the surface distribution of HA, NA and M2 proteins.* Virology, 2005. **342**(2): p. 215-27.

55. Rossman, J.S. and R.A. Lamb, *Influenza virus assembly and budding.* Virology, 2011. **411**(2): p. 229-36.

56. Ali, A., et al., *Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein.* J Virol, 2000. **74**(18): p. 8709-19.

57. Chen, B.J., et al., *The influenza virus M2 protein cytoplasmic tail interacts with the M1 protein and influences virus assembly at the site of virus budding.* J Virol, 2008. **82**(20): p. 10059-70.

58. Rossman, J.S., et al., *Influenza virus M2 protein mediates ESCRT-independent membrane scission.* Cell, 2010. **142**(6): p. 902-13.

59. Iwatsuki-Horimoto, K., et al., *The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly.* J Virol, 2006. **80**(11): p. 5233-40.

60. Palese, P., et al., *Characterization of temperature sensitive influenza virus mutants defective in neuraminidase.* Virology, 1974. **61**(2): p. 397-410.

61. Palese, P. and R.W. Compans, *Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action.* J Gen Virol, 1976. **33**(1): p. 159-63.

62. Compans, R.W., J. Content, and P.H. Duesberg, *Structure of the ribonucleoprotein of influenza virus.* J Virol, 1972. **10**(4): p. 795-800.

63. Arranz, R., et al., *The structure of native influenza virion ribonucleoproteins.* Science, 2012. **338**(6114): p. 1634-7.

64. Moeller, A., et al., *Organization of the influenza virus replication machinery.* Science, 2012. **338**(6114): p. 1631-4.

65. Ortega, J., et al., *Ultrastructural and functional analyses of recombinant influenza virus ribonucleoproteins suggest dimerization of nucleoprotein during virus amplification.* J Virol, 2000. **74**(1): p. 156-63.

66. York, A., et al., *Isolation and characterization of the positive-sense replicative intermediate of a negative-strand RNA virus.* Proc Natl Acad Sci U S A, 2013. **110**(45): p. E4238-45.

67. Deng, T., et al., *In vitro assembly of PB2 with a PB1-PA dimer supports a new model of assembly of influenza A virus polymerase subunits into a functional trimeric complex.* J Virol, 2005. **79**(13): p. 8669-74.

68. Pflug, A., et al., *Structure of influenza A polymerase bound to the viral RNA promoter.* Nature, 2014. **516**(7531): p. 355-60.

69. Portela, A. and P. Digard, *The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication.* J Gen Virol, 2002. **83**(Pt 4): p. 723-34.

70. Beaton, A.R. and R.M. Krug, *Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5' capped end.* Proc Natl Acad Sci U S A, 1986. **83**(17): p. 6282-6.

71. Shapiro, G.I. and R.M. Krug, *Influenza virus RNA replication in vitro: synthesis of viral template RNAs and virion RNAs in the absence of an added primer.* J Virol, 1988. **62**(7): p. 2285-90.

72. Turrell, L., et al., *The role and assembly mechanism of nucleoprotein in influenza A virus ribonucleoprotein complexes.* Nat Commun, 2013. **4**: p. 1591.

73. Resa-Infante, P., et al., *Structural and functional characterization of an influenza virus RNA polymerase-genomic RNA complex.* J Virol, 2010. **84**(20): p. 10477-87.

74. Ng, A.K., et al., *Structure of the influenza virus A H5N1 nucleoprotein: implications for RNA binding, oligomerization, and vaccine design.* FASEB J, 2008. **22**(10): p. 3638-47.

75. Ye, Q., R.M. Krug, and Y.J. Tao, *The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA.* Nature, 2006. **444**(7122): p. 1078-82.

76. Chan, W.H., et al., *Functional analysis of the influenza virus H5N1 nucleoprotein tail loop reveals amino acids that are crucial for oligomerization and ribonucleoprotein activities.* J Virol, 2010. **84**(14): p. 7337-45.

77. Baudin, F., et al., *Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent.* EMBO J, 1994. **13**(13): p. 3158-65.

78. Scholtissek, C. and H. Becht, *Binding of ribonucleic acids to the RNP-antigen protein of influenza viruses.* J Gen Virol, 1971. **10**(1): p. 11-6.

79. Neumann, G. and G. Hobom, *Mutational analysis of influenza virus promoter elements in vivo.* J Gen Virol, 1995. **76 (Pt 7)**: p. 1709-17.

80. Tiley, L.S., et al., *Sequence-specific binding of the influenza virus RNA polymerase to sequences located at the 5' ends of the viral RNAs.* J Virol, 1994. **68**(8): p. 5108-16.

81. Fodor, E., D.C. Pritlove, and G.G. Brownlee, *The influenza virus panhandle is involved in the initiation of transcription.* J Virol, 1994. **68**(6): p. 4092-6.

82. Flick, R., et al., *Promoter elements in the influenza vRNA terminal structure.* RNA, 1996. **2**(10): p. 1046-57.

83. Hsu, M.T., et al., *Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle.* Proc Natl Acad Sci U S A, 1987. **84**(22): p. 8140-4.

84. Bae, S.H., et al., *Structural features of an influenza virus promoter and their implications for viral RNA synthesis.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10602-7.

85. Fodor, E., D.C. Pritlove, and G.G. Brownlee, *Characterization of the RNA-fork model of virion RNA in the initiation of transcription in influenza A virus.* J Virol, 1995. **69**(7): p. 4012-9.

86. Flick, R. and G. Hobom, *Interaction of influenza virus polymerase with viral RNA in the 'corkscrew' conformation.* J Gen Virol, 1999. **80 (Pt 10)**: p. 2565-72.

87. Pflug, A., et al., *Structural insights into RNA synthesis by the influenza virus transcription-replication machine.* Virus Res, 2017. **234**: p. 103-117.

88. Crow, M., et al., *Mutational analysis of the influenza virus cRNA promoter and identification of nucleotides critical for replication.* J Virol, 2004. **78**(12): p. 6263-70.

89. Thierry, E., et al., *Influenza Polymerase Can Adopt an Alternative Configuration Involving a Radical Repacking of PB2 Domains.* Mol Cell, 2016. **61**(1): p. 125-37.

90. Tomescu, A.I., et al., *Single-molecule FRET reveals a corkscrew RNA structure for the polymerase-bound influenza virus promoter.* Proc Natl Acad Sci U S A, 2014. **111**(32): p. E3335-42.

91. Jorba, N., R. Coloma, and J. Ortin, *Genetic trans-complementation establishes a new model for influenza virus RNA transcription and replication.* PLoS Pathog, 2009. **5**(5): p. e1000462.

92. Koppstein, D., J. Ashour, and D.P. Bartel, *Sequencing the cap-snatching repertoire of H1N1 influenza provides insight into the mechanism of viral transcription initiation.* Nucleic Acids Res, 2015. **43**(10): p. 5052-64.

93. Gu, W., et al., *Influenza A virus preferentially snatches noncoding RNA caps.* RNA, 2015. **21**(12): p. 2067-75.

94. Li, M.L., P. Rao, and R.M. Krug, *The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits.* EMBO J, 2001. **20**(8): p. 2078-86.

95. Poon, L.L., E. Fodor, and G.G. Brownlee, *Polyuridylated mRNA synthesized by a recombinant influenza virus is defective in nuclear export.* J Virol, 2000. **74**(1): p. 418-27.

96. Bier, K., A. York, and E. Fodor, *Cellular cap-binding proteins associate with influenza virus mRNAs.* J Gen Virol, 2011. **92**(Pt 7): p. 1627-34.

97. Wahl, M.C., C.L. Will, and R. Luhrmann, *The spliceosome: design principles of a dynamic RNP machine.* Cell, 2009. **136**(4): p. 701-18.

98. Dubois, J., O. Terrier, and M. Rosa-Calatrava, *Influenza viruses and mRNA splicing: doing more with less.* MBio, 2014. **5**(3): p. e00070-14.

99. Yamayoshi, S., et al., *Identification of a Novel Viral Protein Expressed from the PB2 Segment of Influenza A Virus.* J Virol, 2016. **90**(1): p. 444-56.

100. Lamb, R.A., C.J. Lai, and P.W. Choppin, *Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins.* Proc Natl Acad Sci U S A, 1981. **78**(7): p. 4170-4.

101. Inglis, S.C. and C.M. Brown, *Spliced and unspliced RNAs encoded by virion RNA segment 7 of influenza virus.* Nucleic Acids Res, 1981. **9**(12): p. 2727-40.

102. Lamb, R.A., P.R. Etkind, and P.W. Choppin, *Evidence for a ninth influenza viral polypeptide.* Virology, 1978. **91**(1): p. 60-78.

103. Robb, N.C., et al., *Splicing of influenza A virus NS1 mRNA is independent of the viral NS1 protein.* J Gen Virol, 2010. **91**(Pt 9): p. 2331-40.

104. Vreede, F.T., T.E. Jung, and G.G. Brownlee, *Model suggesting that replication of influenza virus is regulated by stabilization of replicative intermediates.* J Virol, 2004. **78**(17): p. 9568-72.

105. Barrett, T., A.J. Wolstenholme, and B.W.J. Mahy, *Transcription and Replication of Influenza-Virus Rna.* Virology, 1979. **98**(1): p. 211-225.

106. Vreede, F.T. and G.G. Brownlee, *Influenza virion-derived viral ribonucleoproteins synthesize both mRNA and cRNA in vitro.* J Virol, 2007. **81**(5): p. 2196-204.

107. Deng, T., F.T. Vreede, and G.G. Brownlee, *Different de novo initiation strategies are used by influenza virus RNA polymerase on its cRNA and viral RNA promoters during viral RNA replication.* J Virol, 2006. **80**(5): p. 2337-48.

108. Subbarao, K., et al., *Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness.* Science, 1998. **279**(5349): p. 393-6.

109. Tumpey, T.M., et al., *Characterization of the reconstructed 1918 Spanish influenza pandemic virus.* Science, 2005. **310**(5745): p. 77-80.

110. Subbarao, E.K., W. London, and B.R. Murphy, *A single amino acid in the PB2 gene of influenza A virus is a determinant of host range.* J Virol, 1993. **67**(4): p. 1761-4.

111. Fernandez-Sesma, A., et al., *Influenza virus evades innate and adaptive immunity via the NS1 protein.* J Virol, 2006. **80**(13): p. 6295-304.

112. Steel, J., et al., *Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N.* PLoS Pathog, 2009. **5**(1): p. e1000252.

113. Massin, P., S. van der Werf, and N. Naffakh, *Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses.* J Virol, 2001. **75**(11): p. 5398-404.

114. Hatta, M., et al., *Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice.* PLoS Pathog, 2007. **3**(10): p. 1374-9.

115. Mehle, A. and J.A. Doudna, *An inhibitory activity in human cells restricts the function of an avian-like influenza virus polymerase.* Cell Host Microbe, 2008. **4**(2): p. 111-22.

116. Cauldwell, A.V., O. Moncorge, and W.S. Barclay, *Unstable polymerase-nucleoprotein interaction is not responsible for avian influenza virus polymerase restriction in human cells.* J Virol, 2013. **87**(2): p. 1278-84.

117. Paterson, D., et al., *Host restriction of influenza virus polymerase activity by PB2 627E is diminished on short viral templates in a nucleoprotein-independent manner*. J Virol, 2014. **88**(1): p. 339-44.

118. Smith, G.J., et al., *Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic.* Nature, 2009. **459**(7250): p. 1122-5.

119. Maines, T.R., et al., *Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals.* J Virol, 2005. **79**(18): p. 11788-800.

120. Mehle, A. and J.A. Doudna, *Adaptive strategies of the influenza virus polymerase for replication in humans.* Proc Natl Acad Sci U S A, 2009. **106**(50): p. 21312-6.

121. Yamada, S., et al., *Biological and structural characterization of a host-adapting amino acid in influenza virus.* PLoS Pathog, 2010. **6**(8): p. e1001034.

122. Moncorge, O., M. Mura, and W.S. Barclay, *Evidence for avian and human host cell factors that affect the activity of influenza virus polymerase.* J Virol, 2010. **84**(19): p. 9978-86.

123. Gabriel, G., A. Herwig, and H.D. Klenk, *Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus.* PLoS Pathog, 2008. **4**(2): p. e11.

124. Hudjetz, B. and G. Gabriel, *Human-like PB2 627K influenza virus polymerase activity is regulated by importin-alpha1 and -alpha7.* PLoS Pathog, 2012. **8**(1): p. e1002488.

125. Gabriel, G., et al., *Differential use of importin-alpha isoforms governs cell tropism and host adaptation of influenza virus.* Nat Commun, 2011. **2**: p. 156.

126. Reilly, P.T., et al., *Cracking the ANP32 whips: important functions, unequal requirement, and hints at disease implications.* Bioessays, 2014. **36**(11): p. 1062-71.

127. York, A., E.C. Hutchinson, and E. Fodor, *Interactome analysis of the influenza A virus transcription/replication machinery identifies protein phosphatase 6 as a cellular factor required for efficient virus replication.* J Virol, 2014. **88**(22): p. 13284-99.

128. Bradel-Tretheway, B.G., et al., *Comprehensive proteomic analysis of influenza virus polymerase complex reveals a novel association with mitochondrial proteins and RNA polymerase accessory factors.* J Virol, 2011. **85**(17): p. 8569-81.

129. Sugiyama, K., et al., *pp32 and APRIL are host cell-derived regulators of influenza virus RNA synthesis from cRNA.* Elife, 2015. **4**.

130. Long, J.S., et al., *Species difference in ANP32A underlies influenza A virus polymerase host restriction.* Nature, 2016. **529**(7584): p. 101-4.

131. Domingues, P. and B.G. Hale, *Functional Insights into ANP32A-Dependent Influenza A Virus Polymerase Host Restriction.* Cell Rep, 2017. **20**(11): p. 2538-2546.

132. Manz, B., et al., *Adaptive mutations in NEP compensate for defective H5N1 RNA replication in cultured human cells.* Nat Commun, 2012. **3**: p. 802.

133. de Jong, M.D., et al., *Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia.* Nat Med, 2006. **12**(10): p. 1203-7.

134. Tarendeau, F., et al., *Structure and nuclear import function of the C-terminal domain of influenza virus polymerase PB2 subunit.* Nat Struct Mol Biol, 2007. **14**(3): p. 229-33.

135. Liu, Q., et al., *Combination of PB2 271A and SR polymorphism at positions 590/591 is critical for viral replication and virulence of swine influenza virus in cultured cells and in vivo.* J Virol, 2012. **86**(2): p. 1233-7.

136. Taubenberger, J.K., et al., *Characterization of the 1918 influenza virus polymerase genes.* Nature, 2005. **437**(7060): p. 889-93.

137. Kawaoka, Y., S. Krauss, and R.G. Webster, *Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics.* J Virol, 1989. **63**(11): p. 4603-8.

138. Wendel, I., et al., *The avian-origin PB1 gene segment facilitated replication and transmissibility of the H3N2/1968 pandemic influenza virus.* J Virol, 2015. **89**(8): p. 4170-9.

139. Xu, C., et al., *Amino acids 473V and 598P of PB1 from an avian-origin influenza A virus contribute to polymerase activity, especially in mammalian cells.* J Gen Virol, 2012. **93**(Pt 3): p. 531-40.

140. Biswas, S.K. and D.P. Nayak, *Mutational analysis of the conserved motifs of influenza A virus polymerase basic protein 1.* J Virol, 1994. **68**(3): p. 1819-26.

141. Da Costa, B., et al., *Temperature-Sensitive Mutants in the Influenza A Virus RNA Polymerase: Alterations in the PA Linker Reduce Nuclear Targeting of the PB1-PA Dimer and Result in Viral Attenuation.* J Virol, 2015. **89**(12): p. 6376-90.

142. Mehle, A., et al., *Reassortment and mutation of the avian influenza virus polymerase PA subunit overcome species barriers.* J Virol, 2012. **86**(3): p. 1750-7.

143. Xu, G., et al., *Prevailing PA Mutation K356R in Avian Influenza H9N2 Virus Increases Mammalian Replication and Pathogenicity.* J Virol, 2016. **90**(18): p. 8105-14.

144. Bussey, K.A., et al., *PA residues in the 2009 H1N1 pandemic influenza virus enhance avian influenza virus polymerase activity in mammalian cells.* J Virol, 2011. **85**(14): p. 7020-8.

145. Watanabe, Y., et al., *The changing nature of avian influenza A virus (H5N1).* Trends Microbiol, 2012. **20**(1): p. 11-20.

146. Naffakh, N., et al., *Genetic analysis of the compatibility between polymerase proteins from human and avian strains of influenza A viruses.* J Gen Virol, 2000. **81**(Pt 5): p. 1283-91.

147. Taubenberger, J.K. and D.M. Morens, *1918 Influenza: the mother of all pandemics.* Emerg Infect Dis, 2006. **12**(1): p. 15-22.

148. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiol Rev, 1992. **56**(1): p. 152-79.

149. Smith, G.J., et al., *Dating the emergence of pandemic influenza viruses.* Proc Natl Acad Sci U S A, 2009. **106**(28): p. 11709-12.

150. Taubenberger, J.K., *The origin and virulence of the 1918 "Spanish" influenza virus.* Proc Am Philos Soc, 2006. **150**(1): p. 86-112.

151. Worobey, M., G.Z. Han, and A. Rambaut, *Genesis and pathogenesis of the 1918 pandemic H1N1 influenza A virus.* Proc Natl Acad Sci U S A, 2014. **111**(22): p. 8107-12.

152. Garten, R.J., et al., *Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans.* Science, 2009. **325**(5937): p. 197-201.

153. Lowen, A.C., *Constraints, Drivers, and Implications of Influenza A Virus Reassortment.* Annu Rev Virol, 2017. **4**(1): p. 105-121.

154. te Velthuis, A.J.W. and E. Fodor, *Influenza virus RNA polymerase: insights into the mechanisms of viral RNA synthesis.* Nature Reviews Microbiology, 2016. **14**(8): p. 479-493.

155. Naffakh, N., et al., *Host restriction of avian influenza viruses at the level of the ribonucleoproteins.* Annu Rev Microbiol, 2008. **62**: p. 403-24.

156. Manz, B., M. Schwemmle, and L. Brunotte, *Adaptation of avian influenza A virus polymerase in mammals to overcome the host species barrier.* J Virol, 2013. **87**(13): p. 7200-9.

157. Taubenberger, J.K. and J.C. Kash, *Influenza virus evolution, host adaptation, and pandemic formation.* Cell Host Microbe, 2010. **7**(6): p. 440-51.

158. Chen, L.M., et al., *Genetic compatibility and virulence of reassortants derived from contemporary avian H5N1 and human H3N2 influenza A viruses.* PLoS Pathog, 2008. **4**(5): p. e1000072.

159. Munster, V.J., et al., *Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets.* Science, 2009. **325**(5939): p. 481-3.

160. Sun, Y., et al., *High genetic compatibility and increased pathogenicity of reassortants derived from avian H9N2 and pandemic H1N1/2009 influenza viruses.* Proc Natl Acad Sci U S A, 2011. **108**(10): p. 4164-9.

161. Ping, J., et al., *Low-pathogenic avian influenza virus A/turkey/Ontario/6213/1966 (H5N1) is the progenitor of highly pathogenic A/turkey/Ontario/7732/1966 (H5N9).* J Gen Virol, 2012. **93**(Pt 8): p. 1649-57.

162. Skowronski, D.M., et al., *Avian Influenza A(H7N9) Virus Infection in 2 Travelers Returning from China to Canada, January 2015.* Emerg Infect Dis, 2016. **22**(1): p. 71-4.

163. Liu, G., et al., *Influenza A Virus Panhandle Structure Is Directly Involved in RIG-I Activation and Interferon Induction.* J Virol, 2015. **89**(11): p. 6067-79.

164. Hoffmann, E., et al., *A DNA transfection system for generation of influenza A virus from eight plasmids.* Proc Natl Acad Sci U S A, 2000. **97**(11): p. 6108-13.

165. Deng, Q., et al., *Application of a split luciferase complementation assay for the detection of viral protein-protein interactions.* J Virol Methods, 2011. **176**(1-2): p. 108-11.

166. Ying, B.W., D. Fourmy, and S. Yoshizawa, *Substitution of the use of radioactivity by fluorescence for biochemical studies of RNA.* RNA, 2007. **13**(11): p. 2042-50.

167. Kawakami, E., et al., *Strand-specific real-time RT-PCR for distinguishing influenza vRNA, cRNA, and mRNA.* J Virol Methods, 2011. **173**(1): p. 1-6.

168. McAuley, J.L., K. Zhang, and J.A. McCullers, *The effects of influenza A virus PB1-F2 protein on polymerase activity are strain specific and do not impact pathogenesis.* J Virol, 2010. **84**(1): p. 558-64.

169. Naffakh, N., et al., *Genetic analysis of the compatibility between polymerase proteins from human and avian strains of influenza A viruses.* Journal of General Virology, 2000. **81**: p. 1283-1291.

170. Belser, J.A., et al., *Pathogenesis of pandemic influenza A (H1N1) and triple-reassortant swine influenza A (H1) viruses in mice.* J Virol, 2010. **84**(9): p. 4194-203.

171. Herfst, S., et al., *Introduction of virulence markers in PB2 of pandemic swine-origin influenza virus does not result in enhanced virulence or transmission.* J Virol, 2010. **84**(8): p. 3752-8.

172. Nilsson, B.E., A.J.W. te Velthuis, and E. Fodor, *Role of the PB2 627 Domain in Influenza A Virus Polymerase Function.* Journal of Virology, 2017. **91**(7).

173. Jorba, N., E. Area, and J. Ortin, *Oligomerization of the influenza virus polymerase complex in vivo.* J Gen Virol, 2008. **89**(Pt 2): p. 520-4.

174. Chang, S., et al., *Cryo-EM structure of influenza virus RNA polymerase complex at 4.3 A resolution.* Mol Cell, 2015. **57**(5): p. 925-935.

175. Cauldwell, A.V., et al., *Viral determinants of influenza A virus host range.* J Gen Virol, 2014. **95**(Pt 6): p. 1193-210.

176. Hatta, M., et al., *Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses.* Science, 2001. **293**(5536): p. 1840-2.

177. Weber, M., et al., *Influenza virus adaptation PB2-627K modulates nucleocapsid inhibition by the pathogen sensor RIG-I.* Cell Host Microbe, 2015. **17**(3): p. 309-319.

178. Baker, S.F., M.P. Ledwith, and A. Mehle, *Differential Splicing of ANP32A in Birds Alters Its Ability to Stimulate RNA Synthesis by Restricted Influenza Polymerase.* Cell Rep, 2018. **24**(10): p. 2581-2588 e4.

APPENDIX A SUPPLEMENTARY INFORMATION

Table A1 List of primers for constructing plasmids pertaining to ON H5N1 virus.

Plasmids	Primer name	Sequences (5'-3')
pcDNA-ON H5N1-PB2	XhoI-ON6213 PB2 Fw	TAGACTCGAGACCATGGAGAGAATAAAAG AACTAAGAGACC
	KpnI-ON6213-PB2 Bw	GCTTGGTACCCTAATTGATGGCCATCCGAA TTCTTTTG
pcDNA-ON H5N1-PB1	XhoI-ON6213 PB1 Fw	TAGACTCGAGACCATGGATGTCAATCCGAC TTTACTC
	KpnI-ON6213-PB1 Bw	GCTTGGTACCCTATTTCTGCCGTCTGAGCTC TTC
pcDNA-ON H5N1-PA	XhoI-ON6213 PA Fw	TAGACTCGAGACCATGGAAGACTTTGTGCG ACAATG
	EcoRI-ON6213-PA Bw	TGGTGGAATTCCTATTTCAGTGCATGTGTAA GGAAGG
pcDNA-ON H5N1-NP	XhoI-ON6213 NP Fw	TAGACTCGAGACCATGGCGTCTCAAGGCAC C
	KpnI-ON6213-NP Bw	GCTTGGTACCTTAATTGTCATATTCCTCTGC ATTGTC
pcDNA-ON H5N1-PB1a (D445A, D446A)	ON-PB1 D445A, D446A Fw	GATGGCCTTCAATCCTCGGCGGCTTTTGCTC TAATTGTGAATGCACC
	ON-PB1 D445A, D446A Bw	GGTGCATTCACAATTAGAGCAAAAGCCGCC GAGGATTGAAGGCCATC
pcDNA-ON H5N1-PB1 (PB1-F2 KO)	ON H5N1 PB1-F2 S12 Stop codon Fw	CAGGATACACCATGGATACAGTAAACAGGA CACATCAATATTCAGAA
	ON H5N1 PB1-F2 S12 Stop codon Bw	TTCTGAATATTGATGTGTCCTGTTTACTGTA TCCATGGTGTATCCTG
	ON H5N1 PB1-F2 W58 Stop codon Fw	GTTATGCTCAAACAGACTGTGTTCTAGAGG CAATGGCTTTCCTTGAG
	ON H5N1 PB1-F2 W58 Stop codon Bw	CTCAAGGAAAGCCATTGCCTCTAGAACACA GTCTGTTTGAGCATAAC

Table A2 List of primers for constructing plasmids pertaining to pH1N1 virus.

Plasmids	Primer name	Sequences (5'-3')
pcDNA-pH1N1-PB2	XhoI-Halifax210-PB2 Fw	TAGACTCGAGACCATGGAGAGAATAAAAGAACTG
	BamHI-Halifax210-PB2 Bw	GCTCGGATCCCTAATTGATGGCCATCCGAATT
pcDNA-pH1N1-PB1	XhoI-Halifax210-PB1 Fw	TAGACTCGAGACCATGGATGTCAATCCGACTCTAC
	KpnI-Halifax210-PB1 Bw	GCTTGGTACCTTATTTTTGCCGTCTGAGTTCT
pcDNA-pH1N1-PA	XhoI-ON6213 PA Fw	TAGACTCGAGACCATGGAAGACTTTGTGCGACAATG
	KpnI-Halifax210-PA Bw	GCTTGGTACCCTACTTCAGTGCATGTGTGAGGA
pcDNA-pH1N1-NP	XhoI-Halifax210-NP Fw	TAGACTCGAGACCATGAGTGACATCGAAGCCATGG
	KpnI-Halifax210-NP Bw	GCTTGGTACCTCAACTGTCATACTCCTCTGC
pcDNA-pH1N1-PB2 (S590G, R591Q)	Hfx H1N1 PB2 S590G, R591Q Fw	CTTGTCCCTAAGGCAATCAGAGGCCAGTACAGTGGA TTCGTAAGGACA
	Hfx H1N1 PB2 S590G, R591Q Bw	GTCCTTACGAATCCACTGTACTGGCCTCTGATTGCCT TAGGGACAAG
pcDNA-pH1N1-PB2 (E627S)	Hfx H1N1 PB2 E627S Fw	CTTCTCCCCTTTGCTGCAGCTCCACCAAGCCAGAGTA GGATGCAATTTTCCTC
	Hfx H1N1 PB2 E627S Bw	GAGGAAAATTGCATCCTACTCTGGCTTGGTGGAGCT GCAGCAAAGGGGAGAAG
pcDNA-pH1N1-PB2 (E627L)	Hfx H1N1 PB2 E627L Fw	CTTCTCCCCTTTGCTGCAGCTCCACCACTGCAGAGTA GGATGCAATTTTCCTC
	Hfx H1N1 PB2 E627L Bw	GAGGAAAATTGCATCCTACTCTGCAGTGGTGGAGCT GCAGCAAAGGGGAGAAG
pcDNA-pH1N1-PB2 (E627I)	Hfx H1N1 PB2 E627I Fw	CTTCTCCCCTTTGCTGCAGCTCCACCAATTCAGAGTA GGATGCAATTTTCCTC
	Hfx H1N1 PB2 E627I Bw	GAGGAAAATTGCATCCTACTCTGAATTGGTGGAGCT GCAGCAAAGGGGAGAAG
pcDNA-Hfx-PB2 (E627K)	Hfx H1N1 PB2 E627K Fw	TTGCTGCTGCTCCACCAAAGCAGTCTAGAATGCAATT TTCCTCATTGACTGTG
	Hfx H1N1 PB2 E627K Bw	CACAGTCAATGAGGAAAATTGCATTCTAGACTGCTTT GGTGGAGCAGCAGCAA
pcDNA-pH1N1-PB1a (D445A, D446A)	HFX-PB1 D445A, D446A Fw	GTGGGATGGGCTCCAATCATCCGCCGCTTTTGCTCTC ATAGTGAATGCACC
	HFX-PB1 D445A, D446A Bw	GGTGCATTCACTATGAGAGCAAAAGCGGCGGATGAT TGGAGCCCATCCCAC

Table A2 List of primers for constructing plasmids pertaining to pH1N1 virus.

Plasmids	Primer name	Sequences (5'-3')
pcDNA-pH1N1-PA (E410A)	HFX-PA E410A Fw	CTAGCAAGCTGGGTCCAAAATGCATTCAATAAG GCATGTGAATTGACTG
	HFX-PA E410A Bw	CAGTCAATTCACATGCCTTATTGAATGCATTTTG GACCCAGCTTGCTAG
pcDNA-pH1N1-PB2 (E627K, S590G, R591Q)	Hfx H1N1 PB2 S590G, R591Q Fw	CTTGTCCCTAAGGCAATCAGAGGCCAGTACAGT GGATTCGTAAGGACA
	Hfx H1N1 PB2 S590G, R591Q Bw	GTCCTTACGAATCCACTGTACTGGCCTCTGATTG CCTTAGGGACAAG
	Hfx H1N1 PB2 E627K Fw	TTGCTGCTGCTCCACCAAAGCAGTCTAGAATGC AATTTTCCTCATTGACTGTG
	Hfx H1N1 PB2 E627K Bw	CACAGTCAATGAGGAAAATTGCATTCTAGACTG CTTTGGTGGAGCAGCAGCAA
pcDNA-pH1N1-PB2 (E627S, S590G, R591Q)	Hfx H1N1 PB2 S590G, R591Q Fw	CTTGTCCCTAAGGCAATCAGAGGCCAGTACAGT GGATTCGTAAGGACA
	Hfx H1N1 PB2 S590G, R591Q Bw	GTCCTTACGAATCCACTGTACTGGCCTCTGATTG CCTTAGGGACAAG
	Hfx H1N1 PB2 E627S Fw	CTTCTCCCCTTTGCTGCAGCTCCACCAAGCCAGA GTAGGATGCAATTTTCCTC
	Hfx H1N1 PB2 E627S Bw	GAGGAAAATTGCATCCTACTCTGGCTTGGTGGA GCTGCAGCAAAGGGGAGAAG
pcDNA-pH1N1-PB2 (E627L, S590G,R591Q)	Hfx H1N1 PB2 S590G, R591Q Fw	CTTGTCCCTAAGGCAATCAGAGGCCAGTACAGT GGATTCGTAAGGACA
	Hfx H1N1 PB2 S590G, R591Q Bw	GTCCTTACGAATCCACTGTACTGGCCTCTGATTG CCTTAGGGACAAG
	Hfx H1N1 PB2 E627L Fw	CTTCTCCCCTTTGCTGCAGCTCCACCACTGCAGA GTAGGATGCAATTTTCCTC
	Hfx H1N1 PB2 E627L Bw	GAGGAAAATTGCATCCTACTCTGCAGTGGTGGA GCTGCAGCAAAGGGGAGAAG
pcDNA-pH1N1-PB2 (E627I, S590G,R591Q)	Hfx H1N1 PB2 S590G, R591Q Fw	CTTGTCCCTAAGGCAATCAGAGGCCAGTACAGT GGATTCGTAAGGACA
	Hfx H1N1 PB2 S590G, R591Q Bw	GTCCTTACGAATCCACTGTACTGGCCTCTGATTG CCTTAGGGACAAG
	Hfx H1N1 PB2 E627I Fw	CTTCTCCCCTTTGCTGCAGCTCCACCAATTCAGA GTAGGATGCAATTTTCCTC
	Hfx H1N1 PB2 E627I Bw	GAGGAAAATTGCATCCTACTCTGAATTGGTGGA GCTGCAGCAAAGGGGAGAAG

Table A2 List of primers for constructing plasmids pertaining to pH1N1 virus.

Plasmids	Primer name	Sequences (5'-3')
pHW-pH1N1-PB2 (590G/591Q)	Hfx H1N1 PB2 S590G, R591Q Fw	CTTGTCCCTAAGGCAATCAGAGGCCAGTAC AGTGGATTCGTAAGGACA
	Hfx H1N1 PB2 S590G, R591Q Bw	GTCCTTACGAATCCACTGTACTGGCCTCTGA TTGCCTTAGGGACAAG
	Ba-PB2-2341R	ATATGGTCTCGTATTAGTAGAAACAAGGTC GTTT
	Ba-PB2-1G	TATTGGTCTCAGGGAGCGAAAGCAGGTC
pHH21-pH1N1-vNA	Ba-NA-1413R	ATATGGTCTCGTATTAGTAGAAACAAGGAG TTTTTT
	BsaI-NA-4G Fw	TATTGGTCTCAGGGAGCGAAAGCAGGAGT

Table A3 List of primers for constructing plasmids used in split luciferase complementation assay (SLCA).

Plasmids	Primer name	Sequences (5'-3')
LN-PB1 $_{(ON\ H5N1)}$	XhoI-ON6213 PB1 Fw	TAGACTCGAGACCATGGATGTCAATCCGACTTTACTC
	KpnI-ON6213-PB1 Bw	GCTTGGTACCCTATTTCTGCCGTCTGAGCTCTTC
PB1 (pH1N1)-LC	XhoI-Halifax210-PB1 Fw	TAGACTCGAGACCATGGATGTCAATCCGACTCTAC
	NotI-HFX-PB1 Bw	CGCGGCGGCCGCTTTTTGCCGTCTGAGTTCTTC
PB1 (pH1N1)-LN	XhoI-Halifax210-PB1 Fw	TAGACTCGAGACCATGGATGTCAATCCGACTCTAC
	NotI-HFX-PB1 Bw	CGCGGCGGCCGCTTTTTGCCGTCTGAGTTCTTC
LN-PB1 (pH1N1)	XhoI-Halifax210-PB1 Fw	TAGACTCGAGACCATGGATGTCAATCCGACTCTAC
	KpnI-Halifax210-PB1 Bw	GCTTGGTACCTTATTTTTGCCGTCTGAGTTCT
PA (pH1N1)-LC	XhoI-ON6213 PA Fw	TAGACTCGAGACCATGGAAGACTTTGTGCGACAATG
	NotI-HFX-PA Bw	CGCGGCGGCCGCCTTCAGTGCATGTGTGAGGAA
LN-PA (pH1N1)	XhoI-ON6213 PA Fw	TAGACTCGAGACCATGGAAGACTTTGTGCGACAATG
	KpnI-Halifax210-PA Bw	GCTTGGTACCCTACTTCAGTGCATGTGTGAGGA
LC-PA (pH1N1)	XhoI-ON6213 PA Fw	TAGACTCGAGACCATGGAAGACTTTGTGCGACAATG
	KpnI-Halifax210-PA Bw	GCTTGGTACCCTACTTCAGTGCATGTGTGAGGA

Table A4 List of primers used in strand-specific real-time PCR.

Target	Primer name	sequences	Purpose
vRNA	vRNAtag-HFX-NA-Fw	GGCCGTCATGGTGGCGAATGAACACAAGA GTCTGAATGTGC	Reverse transcription
	vRNAtag-Fw	GGCCGTCATGGTGGCGAAT	Real-time PCR
	HFX-vNA-Bw	ACTAGAATCAGGATAACAGGAGC	Real-time PCR
cRNA	cRNAtag-PR8-NA-Bw	GCTAGCTTCAGCTAGGCATCAGTAGAAAC AAGGAGTTTTTTGAAC	Reverse transcription
	HFX-NA-cRNA-Fw	TGTATAAGACCTTGCTTCTGGG	Real-time PCR
	cRNAtag-Bw	GCTAGCTTCAGCTAGGCATC	Real-time PCR

Table A5 List of primers used in primer extension assay.

Target	Primer name	sequences
vRNA	5S rRNA Bw	TCCCAGGCGGTCTCCCATCC
c/mRNA	Hf09-c/mNA Bw	TTCCAATTGTCATACAGACCG
5S rRNA	Hf09-vNA Fw	GGTGCTGAGTTGCCATTTAC

Appendix B ACHIEVEMENTS DURING THE STUDY

B1. Presentations

Wang F, Liu G, Hlasny M, and Zhou Y. 2018. Avian-origin PB1 enhances RNA synthesis of 2009 pandemic H1N1 polymerase. Oral presentation. The Fifth International One Health Congress. Jun 22-25, 2018. Saskatoon, Canada.

Wang F, Liu G, Hlasny M, and Zhou Y. 2017. Avian-origin PB1 segment of influenza A virus confers a parallel selective advantage towards Virus adaptation and pandemic formation. Oral presentation. The Sixth ESWI Influenza Conference. Sept 10-13, 2017. Rega, Latvia.

B2. Scholarships

Western College of Veterinary Medicine Graduate Student Scholarship/Fellowship Award ($ 8000), 2017-2018
Saskatchewan Innovation & Opportunity Scholarship ($ 16000), 2016-2017
Western College of Veterinary Medicine Graduate Education Enhancement fund ($ 8000), 2015-2016

B3. Awards

Graduate Student Travel Award for the Fifth International One Health Congress from University of Saskatchewan ($ 750), 2018