Molecular mechanisms of transcription and replication of the influenza A virus genome

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Abstract Influenza A virus is one of the major pathogens that pose a large threat to human health worldwide and has caused pandemics. Influenza A virus is the *Orthomyxoviridae* prototype, and has 8 segmented negative-sense single-stranded RNA (vRNA) as its genome. Influenza virus RNA polymerase (RdRp) consists of three subunits PB2, PB1 and PA, and catalyzes both transcription and replication. Recently, intensive biochemical and structural analysis of its RdRp has been performed. In this paper, we review the details from the biochemical analysis of the purified influenza virus RdRp and the classical ribonucleoprotein complex, as well as piece together their structures to form an overall picture.

Keywords influenza virus, RNA polymerase, ribonucleoprotein complex, transcription, replication

Introduction

Influenza A virus is one of the major pathogens that pose a large threat to human health. Influenza A viruses cause disease in humans, pigs, and other mammals and birds (Webster et al., 1995). In the last century, influenza A virus caused at least three pandemics (Spanish influenza [H1N1] of 1918, Asian influenza [H2N2] of 1957, and Hong Kong influenza [H3N2] of 1968), costing hundreds of millions of human lives (Wright et al., 2007). The latest pandemic that broke out at the beginning of this century (2009), was caused by the novel swine-origin influenza virus A/H1N1 (S-OIV) and is still posing major economical and health threats to humans (<http://www.who.int/csr/disease/swineflu/en/index.html>). Besides the S-OIV pandemic, H5N1, a highly pathogenic avian influenza virus (HPAIV) that is causing a major epidemic among poultry (Gutiérrez et al., 2009), has also been causing severe and even fatal disease in humans in Asia, the Middle East, and Africa since its emergence in Hong Kong in 1997. Over the past decades, many studies have been performed to gain insight into the H5N1 virus and the virus-host interaction, which has resulted in many great

achievements. This article will review our current understanding of the molecular machinery involved in viral transcription and replication.

The influenza A virus is the *Orthomyxoviridae* prototype that has 8 segmented negative-sense single-stranded RNA (vRNA) as its genome (Palese and Shaw, 2007). This virus has a host cell-derived lipid membrane enveloping ribonucleoprotein (RNP) complexes. Each RNP is comprised of vRNA, RNA-dependent RNA polymerase (RdRp), and nucleoproteins (NP) (Fig. 1). The eight RNA genes encode 11 proteins: the RdRp subunits PB2, PB1 and PA; hemagglutinin (HA); NP; neuraminidase (NA); matrix protein 1 (M1); ion channel (M2); non-structural protein 1 (NS1), which is known as an interferon antagonist; nuclear export protein (NEP), which was previously named NS2; and PB1-F2, which is encoded in the alternate coding frame of PB1 in some strains.

In influenza virus-infected cells, viral RNPs are transported to the nuclei for viral genome transcription and replication. Influenza virus RdRp (EC 2.7.7.48) catalyzes both transcription (the synthesis of plus-strand mRNA containing the host mRNA-derived cap-1 structure at the 5'-terminus and a poly(A) tail at the 3'-terminus) and replication (the synthesis of full-length plus-strand complementary RNA [cRNA] from vRNA, and the cRNA-dependent synthesis of minus-strand vRNA) (Ishihama and Nagata, 1988). The viral RdRp also catalyzes polyadenylation at the 3'-termini of mRNA *in vitro*

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Figure 1 Schematic diagram of influenza virus RNP. Schematic diagram (A) and cryoelectron micrograph of influenza virus RNP (B). Influenza virus RdRp, consisting of PA, PB1, and PB2 subunits, binds to the 3'-promoter/origin and 5'-end of vRNA. vRNA surrounds the NP homotrimer (see Fig. 5). The cryoelectron micrograph was modified from Fig. 6B of Area et al. (2004).

(Pritlove et al., 1998). The activities identified for RNP were also detected for the purified influenza virus RdRp from insect cells (Honda et al., 2001, 2002; Jiang et al., 2010; Zhang et al., 2010a, 2010b).

The RdRp heterotrimer complex purified from influenza virus consists of three subunits, PB1, PB2, and PA (Honda et al., 1990). PB1 is the core of the RdRp assembly, and its N and C termini bind with the C and N termini of PA and PB2, respectively (Pérez and Donis, 1995; Toyoda et al., 1996a; Ohtsu et al., 2002). The structures of the subunit-binding sites were revealed recently (He et al., 2008; Obayashi et al., 2008; Sugiyama et al., 2009). No physical interaction between PA and PB2 was observed. PB1 is involved in RNA polymerization (Argos, 1988; Biswas and Nayak, 1994; Kobayashi et al., 1996; Toyoda et al., 1996a). PB2 is the cap-binding subunit (Li et al., 2001; Fechter et al., 2003; Guilligay et al., 2008). Recently, the cap-snatching endonuclease activity, which had been previously assigned to the PB1 subunit (Li et al., 2001), was identified in PA (Dias et al., 2009; Yuan et al., 2009). Although high-resolution structure data of RNP or RdRp have yet to be obtained, the functional domains of each RdRp subunit and the crystal structure of NP have been solved successfully.

It is generally accepted that this heterotrimer is required for the synthesis of vRNA, cRNA, and mRNA. However, some biochemical characters of the purified RdRp heterotrimer are controversial because of the differences in the way RdRp is purified as well as its purity. Kobayashi et al. (1996), Toyoda

et al. (1996b), and Nakagawa et al. (1996) demonstrated that PB1 alone could transcribe RNA using a dinucleotide AG primer, and Honda et al. (2002) showed that the PB2-PB1 complex was capable of transcription using AG and cap-1 RNA primers *in vitro*.

Most RNA viruses use their own RdRp for genome transcription and replication (Ball, 2007). The initiation mechanism is classified into two categories: primer-dependent and primer-independent (*de novo*) initiation (Kao et al., 2001; van Dijk et al., 2004). Influenza virus transcription is an example of primer-dependent initiation. However, how influenza virus replication is initiated has been a long-time dilemma. To date, no specific structure has been identified at the 5'-terminus of influenza virus genomes, and *de novo* initiation was proposed as the mechanism of replication until dinucleotide AG was synthesized *in vitro* by the purified RdRp (Deng et al., 2006c; Zhang et al., 2010a).

The 5'- and 3'-ends of influenza virus vRNA and cRNA are highly conserved and are essential for virus transcription and replication. Many models have been proposed to describe the secondary structure of the RNA ends, and among them, the special "cork screw" secondary structure of the 5'- and 3'-ends of influenza virus vRNA and cRNA has been accepted after a series of mutational studies regarding intra- and inter-strand complementarity and disparity (Baudin et al., 1994; Fodor et al., 1994; Neumann and Hobom, 1995; Poon et al., 1998; Flick and Hobom, 1999; Pritlove et al., 1999).

Although many host factors contribute to influenza virus genome transcription and replication (Hao et al., 2008; Nagata et al., 2008), the essential biochemical reaction of transcription and replication was reconstituted by the purified RdRp and short model RNA templates (Honda et al., 2002; Zhang et al., 2010a; Zhang et al., 2010b). In this review, we provide an overview of influenza genome transcription and replication.

Transcription initiation

Initiation of influenza virus transcription consists of three steps: 1) host cellular (pre-) mRNA cap-1 binding, 2) cleavage of capped RNA, and 3) addition of the first nucleotide G to the capped RNA primer. These are followed by RNA chain elongation (Fig. 2).

Once influenza virus RdRp binds to the host mRNA cap-1 structure ($m^7GpppNm$) and cleaves the host mRNA at approximately 10 to 13 nucleotides from the cap-1 structure, usually after purine residues (A or G), to generate the transcription primer (Plotch et al., 1981; Beaton and Krug, 1986; Fodor and Brownlee, 2002), transcription initiation begins from a G residue, which is complementary to the 2nd nucleotide of the 3'-end of vRNA (Kawakami et al., 1983; Fodor and Brownlee, 2002). Therefore, influenza virus mRNA contains host-derived heterogeneous sequences at

Figure 2 Schematic diagram of influenza virus transcription initiation and poly(A) tailing. A: Binding of PB1 subunit to the 5'-end of vRNA activates PB2 cap binding activity and PA endonuclease activity, which cleaves host cellular pre-mRNA to produce a 13–17 nucleotide long capped RNA primer. B: A guanosine residue is added to the capped RNA primer, initiating the viral transcription process. C: The vRNA template is read in the 3' to 5' direction. However, the 5'-end remains bound to the PB1 subunit. Therefore, RdRp begins to stutter-read upon encountering the U stretch, thus producing the poly(A) tail.

the 5'-ends (Beaton and Krug, 1981; Shaw and Lamb, 1984). Both purified viral RNP and RdRp use globin mRNA as primers for transcription (Honda et al., 2002; Zhang et al., 2010b). Experimentally, globin mRNA and alfalfa mosaic virus genome RNA, which have cap-1 structures at their 5'-ends, are used (Bouloy et al., 1978). The endonuclease activity of influenza virus RdRp strictly depends on the presence of the 7-methyl group on the guanyl cap (m^7G), but could be further stimulated by additional *O*-methylation of the ribosyl 2-hydroxyl group (Bouloy et al., 1980; Ulmanen et al., 1981; Kawakami et al., 1983). This cap-snatching initiation mechanism is shared by other segmented negative-sense RNA genome viruses, including bunyaviruses and arenaviruses (Kormelink et al., 1992; Huiet et al., 1993; Jin

and Elliott, 1993a, 1993b; Garcin et al., 1995; Duijsings et al., 2001).

For a long time, the functional link between viral transcription and host cellular transcription by host DNA-dependent RNA polymerase II (RNA Pol II) was assumed (Bouloy et al., 1978; Mark et al., 1979; Fodor et al., 2000). In influenza virus-infected cells, influenza virus RdRp was found to associate with RNA Pol II, which transcribed host mRNA (Engelhardt et al., 2005). This association of viral transcription with RNA Pol II transcription seems logical since influenza virus transcription requires cap-1 mRNA as the primer and 2 viral mRNAs (M2 and NEP mRNAs) are spliced by the host's splicing mechanism.

Binding of the conserved sequence at the 5'-end of vRNA

activates PB2 cap-1 RNA binding activity and PA endonuclease activity; the 3'-end of vRNA only serves as a template for RNA chain elongation (Hagen et al., 1994; Rao et al., 2003). There was some controversy regarding endonuclease activation, where some studies claimed that endonuclease activity was activated by binding of the PB1 subunit to the 3'-end of vRNA, which required base-pairing between bases 11–13 in the 5'-terminus of vRNA and bases 10–12 in the 3'-terminus, and small hairpin loops in both the 5'- and 3'-termini (Hagen et al., 1994; Leahy et al., 2001a; Leahy et al., 2001b). Cap-1 RNA fragments that contained CA residues at the 3'-terminus were effectively used as primers both *in vivo* and *in vitro* (Beaton and Krug, 1981; Shaw and Lamb, 1984; Rao et al., 2003), and then a G residue was added to the capped primer, directed by the penultimate C at 3'-end of vRNA (Beaton and Krug, 1981; Kawakami et al., 1983; Fodor and Brownlee, 2002). The minimum cap-1 RNA chain length required for this priming activity was 9 nucleotides (Chung et al., 1994).

PA is the endonuclease subunit (Dias et al., 2009; Yuan et al., 2009; Zhao et al., 2009; Crépin et al., 2010), while PB1 polymerizes RNA chains. The 3'-end of the cap-1 RNA that is cleaved by PA must be transferred to the catalytic domain (SDD) of PB1 (Biswas and Nayak, 1994).

The vRNA promoter was reported to exclusively induce transcription initiation (Honda et al., 2001). However, influenza virus RdRp was also able to transcribe cRNA from the cap-1 primer (Jiang et al., 2010). The transcription activity of the cRNA template is much lower than that of the vRNA template, and the cRNA transcript did not contain a poly(A) tail due to the lack of a U stretch element (see below).

Poly(A) tailing

Both prokaryotic and eukaryotic cells harness special poly(A) polymerase to add the poly(A) tail to the 3'-end of mRNA. Typically, the poly(A) tail is added post-transcriptionally to the 3'-end of mRNA. In contrast, the influenza virus synthesizes the poly(A) tail by the viral RdRp itself.

In vitro, both RNP and the purified influenza virus RdRp can produce poly(A) tails with heterologous lengths (Plotch and Krug, 1977; Poon et al., 1998; Honda et al., 2002; Zhang et al., 2010b). The polyadenylation requires the U stretch RNA sequence signal, which is 5–7 U residues long and is 16 nucleotides from the 5'-terminus of vRNA (Robertson et al., 1981; Luo et al., 1991; Li and Palese, 1994; Poon et al., 2000), whereas cRNA without a U stretch cannot produce a poly(A) tail even though it is capable of utilizing globin mRNA for priming (Jiang et al., 2010). Moreover, the RNA duplex structure adjacent to the U stretch is essential for polyadenylation (Luo et al., 1991; Li and Palese, 1994).

The current model for polyadenylation suggests that during transcription, when reaching the U stretch, the poly(A) tail is

produced by influenza virus RdRp reiteratively reading the U stretch because of steric hindrance caused by the binding of RdRp to the 5'-terminus of vRNA (Pritlove et al., 1998; Poon et al., 1999; Zheng et al., 1999) (Fig. 2C). To support this model, mutations in the vRNA promoter regions reduce the binding affinity or change the binding pattern without obviously changing the binding ability (Fodor et al., 1994; Fodor et al., 1998; Poon et al., 1998; Pritlove et al., 1998, 1999). Like in other mRNAs, the poly(A) tail is indispensable for mRNA translation in cells, and mutation of the U stretch to the A stretch leads to a failure in mRNA export from the nucleus to cytoplasm (Poon et al., 2000). The mechanism of how RdRp chooses read-through or stuttering at the 5'-terminal U stretch remains to be studied because the biochemical reaction of poly(A) tailing is independent of cap-1-priming.

Replication initiation

The purified influenza virus RdRp catalyzed both dinucleotide AG-primed and *de novo* replication (Honda et al., 2002; Zhang et al., 2010b). However, whether virion RNP initiates vRNA-dependent replication with or without primers is still controversial (Seong et al., 1992; Vreede and Brownlee, 2007; Vreede et al., 2008). The switching from mRNA to cRNA synthesis may depend on NP (Shapiro and Krug, 1988; Newcomb et al., 2009) and newly synthesized RdRp.

The influenza virus was believed to replicate via *de novo* initiation because no specific structure had been identified at the 5'-termini of influenza viral genomes. However, like the Tacaribe virus, purified influenza virus RNP and RdRp can use dinucleotide AG as a primer to replicate the viral genome *in vitro* (Garcin and Kolakofsky, 1992). RdRp, but not RNP, is able to replicate vRNA without a primer (Plotch and Krug, 1978; Seong et al., 1992; Zhang et al., 2010b). In some viruses, *de novo* initiation is believed to start at the first residue of the 3'-terminus of the template and is immediately followed by elongation. While in other viruses, short RNA oligonucleotides are made by abortive RNA synthesis from either the 3'-terminal RNA sequence or the internal sequence, including *cis*-acting RNA replicating elements of the template (*cre*) (Paul et al., 2000; Yang et al., 2004). Subsequently, these oligonucleotides are used as primers for elongation in a process known as the prime-and-realign or jumping-back mechanism (Garcin and Kolakofsky, 1992; Paul et al., 2000).

Recently, internal initiation and the prime-and-realign model were also proposed for influenza viral cRNA replication (Deng et al., 2006c; Zhang et al., 2010a). A dinucleotide AG primer is synthesized from the internal UC sequence, which is located at the 4th and 5th positions of the 3'-cRNA end by influenza virus RdRp. The initiation complex, which consists of RdRp and the primer strand, pauses at the first U of "UUU" that is located at the 6th to 8th positions from the 3'-end of the cRNA UC sequence, and

leaves the cRNA and realigns to the 3'-cRNA end UC sequence. This initiation complex or AG is able to realign to the 3'-end of vRNA, which is the UC sequence. The heterotrimeric complex, but not the binary complex, was able to synthesize the pppApG dinucleotide (Deng et al., 2006b).

Using the purified RdRp, a similar internal initiation mechanism for vRNA replication was proposed (Zhang et al., 2010a). From the vRNA template, RdRp initiates at the 2nd position of the 3'-end (Fig. 3). According to this model, a nucleotide residue (A, G, or U are preferred) is added to the 3'-end of the vRNA by host terminal nucleotidyl transferase activity. The initiation complex is not stable at the "UUUU" of vRNA which is located at the 4th to 7th positions from its 3'-end. Influenza virus RdRp may not bind well to the U-stretch, or may not read-through it easily as observed for the poly(A) tailing mechanism (Li and Palese, 1994).

The low purity of the influenza virus RdRp purified by the tandem affinity purification (TAP) approach may account for the contrary model, which proposes a terminal initiation for this replication process (Deng et al., 2006c). Different from other viral RdRps for which only the initiating nucleotide is required at high concentration (Testa and Banerjee, 1979; Kao and Sun, 1996; Luo et al., 2000), influenza virus RdRp requires high concentrations of the first three NTPs (ATP,

GTP, and CTP) for efficient initiation (Vreede et al., 2008). The kinetics study using the purified RdRp showed that influenza virus RdRp has low binding affinity to nucleotide substrates than other viral RdRps (Zhang et al., 2010b). However, the purified RdRp showed that only a high concentration of UTP (>1 mmol/L) was required for cRNA template initiation (Zhang et al., 2010a).

The second step of influenza virus genome replication is the complete copying of cRNA into vRNA. Mutational analyses of vRNA and cRNA promoters demonstrated that viral RdRp uses an internal initiation on the cRNA promoter, generating a dinucleotide that can be realigned to the first two nucleotide residues at the 3'-end of cRNA (Deng et al., 2006c; Zhang et al., 2010a). Our study further indicated that a high UTP concentration, which base pairs with the 3rd A residue at the 3'-cRNA end, together with the "UUU" sequence immediately downstream of the 2nd U(4)C(5) sequence at the 3'-end, is essential for the transfer of nascent AG to the 1st UC sequence at the 3'-end (Zhang et al., 2010a).

Ternary structures of influenza virus RdRp and NP

An RNP complex consisting of influenza virus vRNA, RdRp, and NP is the template for transcription and replication. The

Figure 3 Schematic diagram of influenza virus replication initiation. Two steps of replication are carried out by different initiation mechanisms. A: vRNA → cRNA, host cellular terminal nucleotidyl transferases (TNT) add one nucleotide residue to the 3'-end of vRNA. Viral RdRp reads vRNA immediately after the addition of this extra nucleotide, thereby initiating cRNA replication. B: cRNA → vRNA, viral RdRp synthesizes pppApG directed by the 2nd UC sequence from the 3'-cRNA end. The pppApG motif realigns with the first UC from the 3'-cRNA end because of the instability of the pppApG-cRNA-protein complex. RdRp begins to elongate nascent RNA when the UTP concentration is high.

crystallography of NP (Ye et al., 2006) and ternary structures of RdRp subunit fragments were determined (Tarendeau et al., 2007; Guilligay et al., 2008; He et al., 2008; Obayashi et al., 2008; Dias et al., 2009; Kuzuhara et al., 2009b; Sugiyama et al., 2009; Yuan et al., 2009). RdRp and RNP structures were analyzed by cryoelectron microscopy (Area et al., 2004; Coloma et al., 2009).

Influenza virus RdRp is a complex of three subunits, PA, PB1, and PB2, that have a combined molecular weight of approximately 250 kDa (Torreira et al., 2007). The ternary structures of influenza virus RdRp fragments have been revealed recently, although the crystal structure of the entire molecule has not been solved yet (Fig. 4).

PA directly interacts with PB1 (Pérez and Donis, 1995; Toyoda et al., 1996a). Crystal structures of the PA C-terminal fragment, PA_C (239–716), which interacted with the PB1 N-terminal peptide, were resolved (He et al., 2008; Obayashi et al., 2008). The crystal structure data show that PA_C (239–716) consists of 13 α -helices, 9 β -strands, and several loops/turns, and that PB1_N harbors a small 3_{10} helix by P5 to K11 with an LLFL motif critical for the interaction with PA. The 3 α -helices, $\alpha 10$, $\alpha 11$, and $\alpha 13$, form the jaws of a clamp, grasping the N terminus of PB1, which is wrapped by the β -hairpin loop of $\beta 8$ and $\beta 9$ (Fig. 4B). The interaction between PA and PB1 depends on a series of hydrogen bonds and hydrophobic contacts. Amino acid residues I621 to E623 of PA form anti-parallel β -sheet-like interactions with residues D2 to N4 of PB1. Residues E623, Q408, W706, Q670, and R673 in PA form hydrogen bonds with the carbonyl oxygen atoms of D2, V3, F9, L10, and V12 in PB1, and E623, N412, I621, P620, and N670 form hydrogen bonds to the backbone nitrogen atoms of D2, V3, N4, L8, and A14 in PB1, respectively. Hydrophobic interactions contribute greatly to PA and PB1 binding. PB1 P5 lies between F411 and W706, and PB1 L8 is packed with the M595, W619, V636, and L640 side chains.

The crystal structure of amino acid residues 197–209 in the PA N-terminal domain was solved (Dias et al., 2009; Yuan et al., 2009). This region possesses manganese ion-dependent endonuclease activity. PA residues 197–209 had an α/β architecture with five mixed β -strands forming a twisted plane surrounded by seven α -helices (Fig. 4C). This domain contains the structural motif (P)DXN(D/E)XK, which is characteristic of the catalytic core of a broad family of nucleases. The active site features a cluster of three acidic residues (E80, D108, and E119) and a catalytic lysine (K134). The acidic residues, together with H41, which are conserved in influenza A, B, and C viruses, coordinate two manganese ions in a configuration consistent with a two metal-dependent reaction mechanism that is observed in many other nucleases (Dias et al., 2009; Yuan et al., 2009).

The N-terminus of the PB2 subunit interacts with the C-terminus of the PB1 subunit (González et al., 1996; Toyoda et al., 1996a; Poole et al., 2007). This interaction was analyzed by crystallography (Fig. 4D) (Sugiyama et al., 2009). Both PB1-C and PB2-N consist of three α -helices, but neither

polypeptide adopts a stable tertiary structure alone. Helix 1 of PB2-N lies against helices 2 and 3 of PB1-C, and helix 1 of PB1-C is held among all three helices of PB2-N. Unlike the hydrophobic interaction featured in PA_C and PB1_N binding, the majority of the interaction energy appears to be contributed by the four salt bridges E2 and K698, R3 and D725, R3 and K698, and E6 and K698, as well as key apolar contacts, such as I4 and L7.

PB2 is the cap-1-binding subunit (Blaas et al., 1982b). The cap binding action of PB2 was verified by UV cross-linking experiments, mutational analysis, and crystal structural study (Ulmanen et al., 1981; Blaas et al., 1982a, 1982b; Ulmanen et al., 1983; Fechter et al., 2003; Guilligay et al., 2008). However, the crystallography and biochemical analysis data are inconsistent. Amino acids 242–252 and 533–577 were identified from cross-linking experiments (Honda et al., 1999; Li et al., 2001), and 363F and 404F were identified from mutational analysis (Fechter et al., 2003). Residues 323F, 325F, 361E, 363F, 404F, 357H, and 376K were identified around m⁷GTP from the co-crystallization of PB2 318–483 and m⁷GTP (Guilligay et al., 2008). The N- and C-terminal domains of PB2 might interact with the cap binding site to hold the cap-1.

The crystal structure of the PB2 cap-binding domain in complex with m⁷GTP shows that this domain has a compact, well-ordered α - β fold (Fig. 4E). It is characterized by an anti-parallel β -sheet of five strands ($\beta 1$, $\beta 2$, $\beta 5$, $\beta 6$, and $\beta 7$) packed against a bundle of four helices ($\alpha 1$ – $\alpha 4$) and flanked by a C-terminal moiety of short β -strands ($\beta 8$ – $\beta 12$) (Guilligay et al., 2008). Residue F404 in the C-terminus of the $\alpha 1$ helix stacks against the methylated base, and F323 from the $\beta 1$ strand stacks on the ribose. These two residues are part of a remarkable hydrophobic cluster of five phenylalanine residues, which also includes F325, F330, and F363. On the solvent side of the ligand, the sandwich is completed by H357 (from the C-terminal end of the $\beta 4$ strand), which stacks parallel to the base at a distance of approximately 3.5 Å. The key acidic residue is E361, which forms hydrogen bonds with the N1 and N2 positions of the guanine. K376 is also involved in base recognition by interacting with O6. There are two well ordered water molecules obscured in the ligand pocket that interact with E361, K376, and Q406, but do not directly interact with the ligand. Within hydrogen-bonding distance of the N2 of the base is either another water molecule or S320, depending on the particular molecule in the asymmetric unit. The N7 methyl group contacts with the side chains of Q406 and the carbonyl oxygen of F404 with the side chain of M431. The triphosphate is bent around toward the base with the α -phosphate interacting with H432 and N429 and the γ -phosphate interacting with residues H357, K339, and R355.

Influenza virus PB2 determines the host range and pathogenicity of influenza viruses, with amino acids 627 and 701 being most important (Hatta et al., 2001; Gabriel et al., 2005; Li et al., 2005; Gabriel et al., 2008; Resa-Infante et al., 2008). Amino acid 627 in PB2 is almost exclusively a K

Figure 4 The structure of influenza virus RNA polymerase. A: Schematic diagram of influenza virus RdRp. Functional domains, including nuclear localization signal (NLS), polymerase motif (Pol Motif), and nucleotide binding domain of PB1, are indicated. Ternary structures of RdRp solved are indicated in red. B: The PA (C)-PB1 (N) complex. PA (C) is indicated in cyan and PB1 (N) is indicated in green. The red part represents the PB1 LLFL motif. Alpha helices $\alpha 10$, $\alpha 11$, and $\alpha 13$ grasp PB1 (N), and beta sheets $\beta 8$ and $\beta 9$ wraps PB1 (N) PDB accession number: 2ZNL. C: The nuclease domain of PA. PDB accession number: 3EBJ. D: The PB2 (N)-PB1 (C) complex. The resolved complex structure was rotated to show the electrostatic interactions. Unlike the PA-PB1 complex, the interaction of which mainly depends on non-polar interactions, PB2 (N)-PB1 (C) predominantly interacts on the basis of electrostatic interactions. PDB accession number: 2ZTT. E: The cap binding domain of PB2. The sandwich structure is formed by the 5 phenylalanine residues and 1 histidine residue on either side with an m⁷GTP in between. PDB accession number: 2VQZ. F: Enhanced view of PB2 residue 627. (a) The electrostatic potential of PB2 3/3 was calculated using the GRASP program. Blue and red indicate basic and acidic surfaces, respectively. Basic regions (I) and (III) are separated by the ϕ loop (II). (b) The electrostatic potential of PB2 3/3 E627 was calculated using the GRASP program. Modified from Fig. 2 of Kuzuhara et al. (2009b). G: PB2 C-terminal NLS. PDB accession number: 3L56. Figures were rendered in PyMOL (<http://www.pymol.org/>).

in human influenza isolates, and an E in avian influenza, with the exception of S-OIV (Herfst et al., 2010). PB2 K627 causes high replication activity and high pathogenesis in mammalian systems (Hatta et al., 2001; Gabriel et al., 2005; Li et al., 2005; Gabriel et al., 2008; Mehle and Doudna, 2008; Kashiwagi et al., 2009; Kuzuhara et al., 2009a). Avian influenza PB2, which has E627, showed “cold-sensitivity” in mammalian cells (Massin et al., 2001). The C-terminal domain around PB2 residue 627 showed RNA binding

activity (Fig. 4F) (Kuzuhara et al., 2009b). A K627E mutation leads to charge conversion and dramatically reduces the RNA binding activity.

The C terminus of PB2 contains the classical nuclear localization signal (NLS) motif, KR $\times$ 12KRIR (Fig. 4G). The co-crystal structure of the PB2 NLS-containing domain (DPDE) with importin $\alpha 5$ (66–512) has been solved (Tarendeau et al., 2007). DPDE interacts with its C-terminal residues beyond K736 in an extended conformation, permit-

ting binding of the bipartite NLS to 2 distinct regions within the superhelical groove of importin $\alpha 5$. The strong side chain interactions of three basic residues in both the minor (737-RKR-739) and major (752-KRIR-755) sites that are within discrete pockets of importin $\alpha 5$ are supplemented by additional hydrogen bonds, notably between the NE1 atoms of importin $\alpha 5$ tryptophans W149, W191, W234, and W360 and the DPDE NLS peptide main chain carbonyl oxygens. The folded region of the DPDE domain packs against importin $\alpha 5$; in particular, K718 forms 3 hydrogen bonds with G284, N286, and T325 in importin $\alpha 5$.

NP is the predominant protein in RNP. The stoichiometry of NP and RNA was calculated as one nucleoprotein per 24 nucleotides of RNA (Ortega et al., 2000). NP is arginine-rich with a net positive charge reflecting its RNA binding activity and accounting for its ability to coat viral RNA. The NP NLS, responsible for nuclear RNP import (O'Neill et al., 1995; Cros et al., 2005), is comprised of a strong unconventional NLS at the extreme N-terminus (Neumann et al., 1997; Wang et al., 1997), a weaker bipartite NLS located between residues 198 and 216 (Weber et al., 1998), and a third NLS between residues 320 and 400 (Wang et al., 1997; Bullido et al., 2000). NP is essential for RNA chain elongation or antitermination (Beaton and Krug, 1986; Honda et al., 1988) by stabilizing the template RNA-nascent RNA-RdRp complex (Vreede et al., 2004). Recently, *in vitro* results demonstrated that NP can strengthen the *de novo* replication process independent of its RNA-binding activity (Newcomb et al., 2009).

The crystal structure of the NP trimer was solved (Ye et al., 2006). The overall shape of an NP unit structure resembles a crescent with a head and body domain (Fig. 5). Oligomerization of the influenza virus NP is mediated by a flexible tail loop that is inserted inside a neighboring molecule. An RNA binding groove is formed between the body and head, whose

surface is rich in a large number of basic residues, including R65, R150, R152, R156, R174, R175, R195, R199, R213, R214, R221, R236, R355, K357, R361, and R391, interacting with the phosphodiester backbone. An aromatic residue, Y148, resides in one end of the groove and may stack with a nucleotide base. vRNA, binding to this groove, would be greatly exposed on the external surface of NP molecules, consistent with the fact that influenza virus RNA coated with NPs is RNase-sensitive (Baudin et al., 1994). The crystal structure showed only the unconventional NLS is located to the outer periphery highly accessible to solvent. The bipartite NLS may not function as an NLS, because of the extremely short distance between the two motifs.

Host and viral factors

A large number of host factors have been identified to be involved in influenza viral replication and transcription processes by a variety of methodologies such as yeast two-hybrid screening (Huarte et al., 2001), cell-free reconstitution system (Momose et al., 2002), yeast-based influenza virus replicon system (Naito et al., 2007a), proteomics-based approaches (Mayer et al., 2007), and large-scale RNAi screening (Hao et al., 2008; Karlas et al., 2010; König et al., 2010). Moreover, the interactions between host factors and the viral RdRp subunits or NP were demonstrated to play critical roles in the host range of virus infection (Gabriel et al., 2008; Mehle and Doudna, 2008).

PA physically interacts with hCLE, which is homologous to a family of transactivators (Huarte et al., 2001). hCLE interacts with and positively modulates RNA Pol II (Pérez-Gonzalez et al., 2006). Influenza virus RdRp interacts with the C-terminal domain of the large subunit of RNA Pol II

Figure 5 Charge distribution on the NP surface. Figures were rendered in PyMOL (PDB accession number 2IQH).

(Engelhardt et al., 2005). However, no evidence has been collected to demonstrate the logical relationship among the interactions of PA and hCLE. Analysis using inhibitors of transcription initiation and elongation by Pol II indicates that only the initiation reaction is required for viral mRNA synthesis (Chan et al., 2006). Taken together, influenza virus RdRp utilizes Pol II to make nascent capped RNA and then snatches the pre-mRNA to produce a primer.

RanBP5 interacts with PB1 and the PB1-PA dimer, and transports them into the nucleus. RNAi-knockdown of RanBP5 inhibited PB1-PA dimer accumulation in the nucleus (Deng et al., 2006a). Ebp1 specifically interacts with the PB1 catalytic domain to inhibit RNA synthesis (Honda et al., 2007).

NPI-1/importin α 1/karyopherin α 1, NPI-3/importin α 2/karyopherin α 2, and NPI-5/RAF-2p48 are NP-interacting proteins (O'Neill et al., 1995; O'Neill and Palese, 1995; Momose et al., 2001). The former two proteins are able to transport RNP into the nucleus by binding to NP (O'Neill et al., 1995). Recently, the interaction between NPI-1 and NP was reported to determine the host range of virus infection (Gabriel et al., 2008). NPI-5 was proposed to facilitate NP RNA assembly, thereby enhancing viral RNA synthesis (Momose et al., 2001).

RAF-1 and RAF-2 activate influenza virus RdRp activity, and RIF-1 inhibits viral RNA synthesis (Momose et al., 1996). Later, RAF-1 was identified as HSP90, which interacts with the PB2 subunit (Momose et al., 2002). Further, HSP90 was demonstrated to play roles in nuclear transport and assembly of influenza virus RdRp subunits by a series of co-immunoprecipitation experiments (Naito et al., 2007b). Together with previous advances in influenza virus RdRp assembly (Fodor and Smith, 2004; Deng et al., 2005), a new model has been proposed describing the transport and assembly of newly synthesized RdRp (Fig. 6). Intriguingly, PB2 mediates adaptation of avian influenza virus to mammalian hosts by a D701N mutation (Gabriel et al., 2005), which enhances the interaction with mammalian importin α 1 (Gabriel et al., 2008). Another position in PB2 that is important for host dependency and pathogenesis is residue 627 (Almond, 1977; Subbarao et al., 1993; Hatta et al., 2001; Chen et al., 2006). A host factor is speculated to inhibit the assembly of avian PB2, which has E627, into RNP (Mehle and Doudna, 2008).

MCM, a DNA replicative helicase, was found to stimulate *de novo*-initiated replication by stabilizing a replication complex during its transition from initiation to elongation using a biochemical compensation assay (Kawaguchi and

Figure 6 RNA polymerase assembly in infected cells. A: PB1 and PB2 enter the nucleus with the help of PA and HSP90, respectively. The RdRp heterotrimeric complex assembles in the nucleus, releasing HSP90. B: HSP90 escorts the nuclear import of the PB1 and PB2 binary complex by facilitating their association and interaction with the binary complex. Additionally, PA singly enters the nucleus, where the RdRp trimer forms and HSP90 is subsequently released. Modified from Naito et al. (2007b).

Figure 7 Network of host factors and viral factors interacting with RNP. Details are described in the text. The host factors in gray perform functions in viral replication or transcription by unknown mechanisms.

Nagata, 2007). NP is essential for RNA elongation (Honda et al., 1988; Shapiro and Krug, 1988). However, before viral NP synthesis after virus infection, some amount cRNA was detected (Vreede et al., 2004). Taken together, the MCM complex seems to ensure viral replication at an early stage of viral infection when the newly synthesized NP is absent.

More recently, genome-wide gene screening using siRNA libraries was performed intensively to get an overall picture of the interaction between virus and host cells. Many host factors affecting virus infection were identified, including the functions exerted during the viral replication process. *CSE1L* was required for vRNP nuclear import (König et al., 2010). Knockdown of *ATP6V0D1*, *NXF1*, and *COX6A1* abrogated influenza viral replication (Hao et al., 2008). Among them, *NXF1*, as well as *COPG*, *SON*, and *ATP6V0C*, localized with NP (Karlas et al., 2010).

In addition to host factors, viral factors are also involved in viral replication and transcription processes. M1 is a viral structural protein that covers vRNP in the virion. Newly synthesized M1 enters the nucleus and complexes with new RNP, which is essential for RNP export (Martin and Heleniust, 1991). In the context of viral RNA synthesis, M1 is shown to inhibit viral RNA synthesis (Zvonarjev and Ghendon, 1980; Ye et al., 1987; Hankins et al., 1989; Watanabe et al., 1996).

A full map of known host or viral factors interacting with RNP or its component is depicted in Fig. 7.

Conclusion

Influenza viruses are continually evolving, mutating randomly to generate diverse antigenic determinants. This challenges the control of influenza infection (Plotkin et al., 2002). Both vaccines and anti-influenza drugs play important roles in the control of influenza infection. The role of anti-influenza drugs have become more important than before in preparing for an influenza pandemic (Moss et al., 2010). Inappropriate immunization of influenza viruses may cause severe diseases (Monsalvo et al., 2011). Because of the high *K_m* value for influenza virus RdRp, nucleotide analogs will not effectively inhibit influenza virus replication (Zhang et al., 2010b). Recently, new small chemicals targeting NP (Kao et al., 2010) and RdRp (Su et al., 2010) that inhibit influenza virus replication. A pyrazinecarboxamide derivative, T-705 (Favipiravir), which targets influenza RdRp, has been developed (Furuta et al., 2002; Furuta et al., 2005). However, anti-influenza drugs are at risk of emergent mutants resistant to these drugs (Hurt et al., 2006; Beigel and Bray, 2008; Lackenby et al., 2008; Hayden, 2009). All of the H3N2 isolates were resistant to amantadine (Deyde et al., 2007), and H1N1 isolates were reported to be resistant to oseltamivir (Hauge et al., 2009; Moscona, 2009). S-OIV became resistant to oseltamivir quickly (Duan et al., 2010; Kiso et al., 2010). Viral polymerases are excellent targets for controlling viral infections (Oberge, 2006; Tsai et al., 2006). Crystallography of

influenza virus RdRp, together with intensive studies of the biochemical activity during replication and transcription and the interaction with host factors, will direct the development of effective drugs against influenza virus RdRp.

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