Biology of Influenza A Virus

TIMOTHY K. W. CHEUNG AND LEO L. M. POON

Department of Microbiology, Queen Mary Hospital, University of Hong Kong, Hong Kong, China

ABSTRACT: The outbreaks of avian influenza A virus in poultry and humans over the last decade posed a pandemic threat to human. Here, we discuss the basic classification and the structure of influenza A virus. The viral genome contains eight RNA viral segments and the functions of viral proteins encoded by this genome are described. In addition, the RNA transcription and replication of this virus are reviewed.

KEYWORDS: acidic polymerase protein (PA) avian influenza; basic polymerase protein 1 (PB1) basic polymerase protein 2 (PB2) genomic RNAs; vRNA; influenza A; Orthomyxoviridae; virology

INTRODUCTION

Influenza A viruses are medically important viral pathogens still causing significant mortality and morbidity throughout the world. By far the most catastrophic impact of influenza during the last century was the pandemic of Spanish flu in 1918, which cost more than 40 million lives. The outbreaks of avian influenza H5N1/1997 and H5N1/2004 in the geographical region are reminders of the unpredictability of this pathogen and the potential for emergence of new pandemic viruses. Thus, there remains a need for a better control of this infectious agent. A solid understanding of the underlying biology of influenza is therefore a key to better influenza pandemic preparedness. In this article, we will discuss some of the basic virology of the virus.

ORTHOMYXOVIRUSES

Influenza A virus is classified within the family of Orthomyxoviridae (from the Greek orthos, meaning “standard, correct,” and myxa, meaning “mucus”). Orthomyxoviruses are negative-sense, single-stranded, enveloped ribonucleic acid (RNA) viruses with a segmented genome. The genomic RNAs (vRNA)
TABLE 1. Comparison among different classes of influenza viruses (Influenza A, B, and C viruses)\(^5\)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Influenza A</th>
<th>Influenza B</th>
<th>Influenza C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of gene segment</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Surface glycoprotein(s)</td>
<td>HA and NA</td>
<td>HA and NA</td>
<td>HEF (Hemagglutinin-Esterase-Fusion)</td>
</tr>
<tr>
<td>Host range</td>
<td>Wide (humans, pigs, horses, whales, seals, and birds)</td>
<td>Humans and seals</td>
<td>Mainly humans, but also found in swine</td>
</tr>
</tbody>
</table>

function as templates for messenger RNAs (mRNA) and complementary RNAs (cRNA) syntheses. There are four genera in the family of *Orthomyxoviridae*: Influenza virus A, Influenza virus B, Influenza virus C, and Thogotovirus. However, recent sequence analysis of infectious salmon anemia virus has suggested a new genus within the family of *Orthomyxoviridae*.\(^4\)

**CLASSIFICATION OF INFLUENZA VIRUSES**

The influenza A, B, and C viruses can be distinguished on the basis of antigenic differences between their nucleoproteins (NP) and matrix proteins (M).\(^5\) Both influenza A and B viruses contain 8 RNA genomic segments, whereas influenza C virus contains only 7 RNA genomic segments\(^6\) (TABLE 1). All of these viruses can naturally infect humans. However, only influenza A virus has been responsible for all influenza pandemics.\(^7\)

Based on the antigenic variation of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins, influenza A viruses are further subdivided into different subtypes. Currently, there are 16 known subtypes of HA\(^8\) and 9 known subtypes of NA.\(^9\) Interestingly, each of these subtypes can be isolated from aquatic birds, suggesting avian species are the natural hosts of influenza viruses.\(^10\) Of these viruses, only H1N1, H2N2, H3N2, H5N1, H7N7, and H9N2 subtypes have been isolated from human,\(^5,11–16\) indicating that there is likely to be a host restriction for influenza viruses.

**STRUCTURE OF THE INFLUENZA A VIRUS**

Influenza virus is an enveloped virus and the virions are pleomorphic, with shapes ranging from small spherical to long filamentous. The viral morphology is a genetic trait and several viral proteins (HA, NA, M1, and M2) are known to have effects on the morphology of influenza virus particles.\(^17–22\) Roberts and Compans\(^23\) further demonstrated that the nature of the host cells also determines the morphology of influenza virus particles.
The influenza A viral particle contains a lipid envelope, which is derived from the host’s cell membrane during the viral budding process. Three viral proteins, HA, NA, and M2, are embedded in the lipid envelope. HA and NA are spike glycoproteins and they are anchored in the lipid bilayer by the short sequences of hydrophobic amino acids. Electron micrographs of influenza virus show that the HA spike and the NA spike are rod-shaped and mushroom-shaped, respectively. The HA is a homotrimer, which is responsible for the receptor binding and membrane fusion. The NA is a homotetramer whose function is to destroy receptors by hydrolyzing sialic acid groups from glycoproteins and to release the viral progeny. M2 protein is an integral membrane homotetramer, which functions as an ion channel for the acidification of the interior of the viral particle during viral infection. Under the viral lipid envelope there is a M1 protein layer.

Inside the virion, all eight vRNA segments are bound to the NP and to the influenza virus RNA polymerases to form ribonucleoprotein (RNP) complexes. Apart from M1, NP is the most abundant protein in the virion and it is thought to associate with the phosphate-sugar backbone of the vRNA in a sequence-independent manner. Each NP monomer interacts with approximately 20 nucleotides of the vRNA. The RNA polymerase complex is composed of three polymerase subunits (PB2, PB1, and PA). Electron micrographs of isolated RNPs indicated that both ends of the vRNA interact with each other to form a circular or supercoil structure and that the RNA polymerase interacts with one end of the RNP, suggesting that the RNA polymerase interacts with both ends of the vRNA within the viral particle. The NS2 protein is also present in virions in low amounts. It appears to function as a nuclear export protein for vRNA in infected cells.

GENOMIC ORGANIZATION OF THE INFLUENZA A VIRUS

The genome of the influenza A virus contains eight segments. Viral mRNAs from segments 1 and 3 to 6 are monocistronic. Viral mRNAs from segment 2 of some viral isolates contain an alternative open reading frame. In contrast, viral mRNAs derived from segments 7 or 8 can undergo alternative splicing for protein expressions. The sizes of the viral RNA segments and the proteins encoded are summarized in Table 2. Of these proteins, only the PB1-F2 protein from segment 2 (PB1 segment) and NS1 protein from segment 8 (NS segment) are nonstructural proteins.

Segment 1—Basic Polymerase Protein 2 (PB2)

Segment 1 of influenza A virus encodes one of the influenza viral polymerase subunits, PB2. It is widely accepted that PB2, PB1, PA, and NP form the
TABLE 2. The genome RNA and the corresponding encoded proteins of the influenza A/PR/8/34 virus

<table>
<thead>
<tr>
<th>vRNA segment</th>
<th>vRNA length (bps)</th>
<th>Encoded protein(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2341</td>
<td>PB2</td>
</tr>
<tr>
<td>2</td>
<td>2341</td>
<td>PB1 &amp; PB1-F2*</td>
</tr>
<tr>
<td>3</td>
<td>2233</td>
<td>PA</td>
</tr>
<tr>
<td>4</td>
<td>1778</td>
<td>HA</td>
</tr>
<tr>
<td>5</td>
<td>1565</td>
<td>NP</td>
</tr>
<tr>
<td>6</td>
<td>1413</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>1027</td>
<td>M1 and M2**</td>
</tr>
<tr>
<td>8</td>
<td>890</td>
<td>NS1 and NS2(NEP)**</td>
</tr>
</tbody>
</table>

*Generated by an alternative open reading frame of the mRNA of PB1.
** Generated by splice mRNA.

minimum set of proteins required for viral transcription and replication.\textsuperscript{37–39} PB2 contains a nuclear localization signal\textsuperscript{40,41} and it is transported into the nucleus of infected cells for viral transcription and replication.\textsuperscript{42} PB2 is an important protein for generating the cap structure for viral mRNAs. Studies of the influenza viral polymerase demonstrated that the PB2 subunit is a cap-binding protein.\textsuperscript{43–46} Further analyses of this protein indicated that the cap-binding site is probably located near the carboxyl terminus.\textsuperscript{47–49} Besides, PB2 has endonuclease activity\textsuperscript{50,51} and it uses host mRNAs to generate cap primers for viral mRNA synthesis.\textsuperscript{52–54} Consistent with this view, Nakagawa et al.\textsuperscript{55} demonstrated that a cell line that expresses PB1, PA, and NP, but not PB2, can synthesize transcription products lacking 5′ cap structures, showing that PB2 is an important polymerase subunit for cap-snatching. Although the cap-snatching mechanism is not entirely understood, the 5′ and 3′ end of the vRNA template are shown to stimulate cap-snatching\textsuperscript{56–60} and some of the aromatic residues in PB2 are shown to be critical for the process.\textsuperscript{61}

Several studies indicated that PB2, PB1, and PA form a polymerase complex for viral transcription and replication. Immunoprecipitation assays on influenza viral polymerase demonstrated that PB2 is associated with the PB1 subunit.\textsuperscript{62} Analysis of deletion mutants of PB2 indicated that the amino terminus of this protein is a binding site for PB1.\textsuperscript{63} More recently, functional analysis of PB2 protein has shown that this polymerase subunit contains a novel binding site for PB1 subunit and two regions for binding nucleoprotein (NP) with regulatory interactions potential.\textsuperscript{64}

Apart from the above biological functions, PB2 is also suggested to be a major determinant in controlling the pathogenicity of influenza A virus.\textsuperscript{65} Using reverse genetics techniques, an introduction of a mutation at position 627 in the PB2 protein was shown to alter the virulence of H5N1/97 viruses in mice.\textsuperscript{65}
Segment 2—Basic Polymerase Protein 1 (PB1)

The PB1 RNA polymerase subunit is encoded by segment 2. Several lines of evidence indicated that PB1, itself, is an RNA polymerase. Firstly, photochemical cross-linking assays demonstrated that both the substrate,\textsuperscript{66} the elongated RNA product,\textsuperscript{44} and the vRNA template\textsuperscript{67–70} could be cross-linked to PB1, suggesting that PB1 carries the site for RNA polymerization. Secondly, amino acid sequence comparison with other RNA polymerases showed that the PB1 contains the four conserved motifs of RNA-dependent RNA polymerases\textsuperscript{71} and mutations in these motifs abolished the polymerase activity.\textsuperscript{72} Thirdly, nuclear extracts from cells expressing PB1 protein alone would transcribe model vRNA templates.\textsuperscript{73}

Several studies have described the functional domains of PB1 involved in interaction with the other polymerase subunits. Immunoprecipitation studies of the influenza virus RNA polymerase suggested that PB1 contains independent binding sites for PB2 and PA.\textsuperscript{62} Deletion mutant analyses of PB1 suggested that the amino- and carboxyl-termini of PB1 are binding sites for the PA and PB2 polymerase subunits, respectively.\textsuperscript{63,74} The nuclear localization signal of PB1 was mapped to a region near the amino terminus.\textsuperscript{75} The PB1 subunit plays a key role in both the assembly of three polymerase protein subunits and the catalytic function of RNA polymerization. Recently, Honda \textit{et al}.\textsuperscript{37} proposed that the catalytic specificity of PB1 subunit is modulated to the transcriptase by binding PB2 or the replicase by interaction with PA.

Segment 3—Acidic Polymerase Protein (PA)

The PA protein is encoded by segment 3 and is the smallest subunit of the influenza RNA polymerase complex. Like the other influenza viral polymerase subunits, it contains nuclear localization signals\textsuperscript{76} required for transport into the nucleus.\textsuperscript{42} PA is known to be essential for viral transcription and replication\textsuperscript{38,39} and mutations near the carboxyl terminus inhibit transcription.\textsuperscript{77} Fodor \textit{et al}.\textsuperscript{78,79} demonstrated that a single amino acid mutation in the PA subunit of the influenza virus RNA polymerase inhibits endonucleolytic cleavage of capped RNAs and promotes the generation of defective interfering RNAs. Furthermore, Fodor and Smith\textsuperscript{80} illustrated that the PA subunit is required for efficient nuclear accumulation of the PB1 subunit of the influenza A virus RNA polymerase complex. Besides, amino acid sequence comparison with other known proteins suggested that the PA has helicase and ATP-binding activities.\textsuperscript{47} However, the exact functions of PA are still poorly understood.

Interestingly, PA is found to induce proteolysis in infected cells,\textsuperscript{81} but this property is not related to any known viral function and the significance of these findings is yet to be determined. Functional analysis of PA deletion mutants identified the amino-terminal one-third of this protein to be responsible for
the protease activity. When PA is expressed in cells in the absence of the other polymerase subunits, it induces a general proteolysis of both viral and cellular coexpressed proteins. This might explain the failure to establish a PA-expressing cell line. In addition, the proteolytic activity of PA seems to require the nuclear localization signals of the PA, suggesting that nuclear transport is required for proteolytic activity. This might also explain the observations that the localization of PA in the nucleus is closely correlated with chromatin condensation and aberrant nuclear morphology. Sanz-Ezquerro et al. demonstrated that PA is a phosphorylated protein. Thus, the biological functions of PA protein might be regulated by a phosphorylation process.

Segment 4—Hemagglutinin (HA)

Segment 4 of influenza A virus encodes the HA. In viral particles, HA proteins associate as homotrimers. It is responsible for the binding of viral particles to sialic acid-containing receptors on the cell surface. It also mediates the fusion of the viral and cellular membrane. In addition, it is also the major target for neutralizing antibodies. The HA is synthesized as a precursor polypeptide, HA0. This precursor polypeptide is post-translationally cleaved into two disulphide-linked subunits, HA1 and HA2. The cleavage of the HA0 is a prerequisite for viral infectivity. This process liberates the “fusion peptide” at the amino terminus of HA2 for membrane fusion. In addition, this cleavage also allows the native HA molecule to undergo a conformational change, a process that is triggered by an acidic environment and is essential for membrane fusion. In general, the HA0 is believed to be cleaved by trypsin-like proteases extracellularly. However, the presence of multiple basic amino acids within the cleavage site allows the protein to be cleaved by intracellular proteases, for example, furin, which are ubiquitously expressed in most tissues. Hence, influenza viruses containing HA with multiple basic amino acids near the cleavage site are regarded as highly pathogenic and deletion of the multiple basic amino acids in the connective peptide could reduce the virulence of the highly pathogenic viruses.

The HA receptor-binding site is a pocket that locates at the globular head domain of the HA1. The amino acid sequence in the pocket region controls receptor-binding specificity. For example, an HA molecule that contains a glutamine residue at position 226 prefers to bind to α2,3-linked sialic acid. Whereas, an HA molecule with a leucine residue at position 226 prefers to bind to α2,6-linked sialic acid.

Segment 5—Nucleoprotein (NP)

NP is encoded by segment 5. The protein is a phosphorylated basic protein and has a net positive charge at neutral pH.
components for transcription and replication.\textsuperscript{38,39} The amino terminus of NP protein contains an RNA-binding domain. As a result, it has been suggested that the NP encapsidates the viral RNA in a sequence nonspecific manner.\textsuperscript{95,96} Since the NP only binds to the backbone of the viral RNA, dissociation of the NP from the RNA template is not thought to be required for viral transcription and replication.\textsuperscript{28} A temperature-sensitive mutant of NP has been shown to fail to synthesize complementary RNA (cRNA), but not mRNA, at the nonpermissive temperature.\textsuperscript{97} Thus, this suggested that NP has a role in controlling the "switching" of RNA polymerase from transcription to replication. However, the mechanism by which NP might control such switching is not understood. Based on \textit{in vitro} antibody depletion experiments, Shapiro and Krug\textsuperscript{97} suggested that the NP binds to the RNA template and acts as an anti-termination factor for replication.\textsuperscript{97,98} In contrast, based on immunoprecipitation assays and mutational analyses, Biswas \textit{et al.}\textsuperscript{99} and Mena \textit{et al.}\textsuperscript{100} suggested that the NP somehow regulates viral RNA replication by interacting with the viral polymerase subunits. However, others suggested that the transcription and replication of influenza are controlled via other mechanisms.\textsuperscript{101} The NP has also been shown to be important for vRNA nuclear transport.\textsuperscript{102–104} It contains nuclear localization signal(s)\textsuperscript{105} and is a shuttle protein.\textsuperscript{104,105} During the early stage of viral infection, the transport of incoming vRNPs from the viral particle into the nucleus is believed to be mediated by NP.\textsuperscript{102–104} Whereas, in the late infection stage, progeny vRNAs associated with NP, M1, and NS2 are exported to the cytoplasm for viral packaging.\textsuperscript{102,103,106}

\textbf{Segment 6—Neuraminidase (NA)}

Segment 6 of influenza A virus encodes the NA. The three-dimensional structure of the NA revealed that the NA monomer consists of four domains: a box-shaped globular head, a thin stalk, a transmembrane domain, and a cytoplasmic domain.\textsuperscript{107} The NA is a surface glycoprotein and the glycosylation of the NA might be an important determinant (but not the sole determinant) of the neurovirulence of influenza viruses.\textsuperscript{108} It exists as a homotetramer,\textsuperscript{109} which has receptor-destroying activity to cleave the α–ketosidic linkage between a terminal sialic acid and an adjacent D-galactose or D-galactosamine residue.\textsuperscript{110} The function of NA activity in the influenza virus life cycle is still not entirely clear. Liu \textit{et al.}\textsuperscript{111} demonstrated that a NA-deficient virus is infectious in cell culture and in mice, suggesting that the NA molecule is not required for viral entry, replication, and assembly. However, when the NA activity is inhibited, progeny viral particles attach to each other and/or to the cell surface to form large aggregates.\textsuperscript{112,113} Therefore, it is generally assumed that NA has an important role in releasing progeny viral particles from infected cells. Recent studies have indicated that the conserved cytoplasmic tail of NA might control virion morphology and virulence.\textsuperscript{20,21,114}
Segment 7—Matrix Proteins (M1 and M2)

Segment 7 of influenza A virus encodes two proteins, M1 and M2. The M1 protein is a colinear transcription product of segment 7. In contrast, the M2 protein is encoded by the spliced mRNA of segment 7.

M1 Protein

In the viral particle, the M1 protein forms a layer to separate the RNP from the viral membrane and it interacts with both the vRNA and protein components of RNP in assembly and disassembly of influenza A viruses. The M1 protein is reported to have several functions for the virus. First, it binds to RNA in a sequence nonspecific manner and inhibits viral transcription. It contains a nuclear localization signal and seems to regulate vRNP nuclear transport. When it binds to vRNP, it promotes vRNP nuclear export and inhibits vRNP nuclear import. Several studies demonstrated the nuclear export of viral ribonucleoproteins is associated with the matrix protein. It has been proposed that the vRNA and M1 protein together promote the self-assembly of influenza virus NP into the typical quaternary helical structure of the vRNP and the interaction of NP with vRNA and M1 in a system devoid of other viral proteins may lead to translocation of vRNP from the nucleus to the cytoplasm. It was previously reported that M1 protein synthesized at high temperature (41°C) is unable to interact with vRNP and the transfer of vRNP into the cytoplasm from the nucleus is blocked, suggesting that the association between M1 and vRNP is essential for the nuclear export of vRNP. Hirayama et al. found that the induction of heat shock protein 70 (HSP70) by prostaglandin A1 (PGA1) at 37°C caused the suppression of virus production by preventing M1 protein from associating with vRNP and thus inhibiting the nuclear export of viral proteins. In addition, the M1 protein binds to the cell membrane and seems to have an effect on viral assembly, budding, and viral morphology. Structural analysis of a knockout mutant of influenza virus M1 protein indicated that the amino-terminal domain of this protein carries the nuclear localization sequence (NLS) motif and is important for membrane binding, self-polymerization, and nuclear export of vRNPs. Recently, it has been suggested that the matrix protein has a potential to bind and inhibit the amidolytic activity of the viral RNA polymerase PA subunit.

M2 Protein

The M2 protein is an integral membrane protein and exists as a disulphide-bonded homotetramer. The M2 tetramer has ion channel activity.
CHEUNG & POON

for pH regulation. Takeuchi et al.\textsuperscript{137} suggested that the amino acid residues in the transmembrane domain of the M2 protein, Histidine (His37) and Tryptophan (Trp41), are essential for the pH-regulated proton conductance. In the endosome of infected cells, the ion channel activity of M2 allows acidification of the interior of the incoming viral particle. The acidification of the viral particle is believed to be essential for viral replication, because it allows incoming vRNPs to dissociate from M1 proteins for nuclear import.\textsuperscript{102,123} On the other hand, the ion channel activity of M2 is also reported to maintain a high pH in the Golgi vesicles so as to stabilize the native conformation of newly synthesized HA during the intracellular transport for viral assembly.\textsuperscript{138} pH perturbations of acidic compartments via the influenza M2 proton channel affected the protein traffic along the secretory pathway.\textsuperscript{139,140} It has been shown that M2 proteins, together with the M1 matrix proteins, are important determinants in filamentous viral particle formation.\textsuperscript{22} However, recent study indicated that the M1 matrix protein would be the sole determinant to control the filamentous phenotype of influenza A virus.\textsuperscript{18} Analyses of deletion mutants of M2 protein indicated that different domains of the ion channel take part in various viral processes. For example, the amino terminus of M2 protein is important for its incorporation into the virions,\textsuperscript{141} whereas the carboxyl terminus is responsible for viral replication.\textsuperscript{142} Moreover, several reports have shown that vaccine candidates for influenza viruses can be developed by targeting the extracellular domain,\textsuperscript{143,144} as well as by deleting the transmembrane domain of M2 proteins.\textsuperscript{145} The amino terminus of M2 protein has been shown to induce antibodies with inhibitory activity and protective immunity against the influenza virus replication.\textsuperscript{146,147} A synthetic multiple antigenic vaccine that contains extracellular domain of M2 protein may induce influenza type A virus-specific resistance in mice.\textsuperscript{148} Besides, Thomas et al.\textsuperscript{149} reported that the phosphorylation status of a highly conserved phosphorylation site of the M2 protein (Serine 64) does not affect the intracellular transport of the protein in the infected cells. In addition, this study has also shown that the loss of phosphorylation on M2 proteins has not compromised the replication of influenza virus \textit{in vivo}. Mutation analysis on M2 protein reported by Watanabe et al.\textsuperscript{150} finds that influenza A virus without the M2 transmembrane domain, which is responsible for ion channel activity, can undergo multiple cycles of replication. In contrast, study of M2 mutant generated by Takeda et al.\textsuperscript{151} suggests that M2 ion channel is essential for efficient viral replication in tissue culture.

\textit{Segment 8—Nonstructural Proteins (NS1 and NS2)}

Segment 8 of influenza A virus encodes two proteins, NS1 and NS2.\textsuperscript{152,153} The NS1 protein is a colinear transcription product of segment 8. In contrast, the NS2 is encoded by the spliced mRNA of segment 8.
NS1 Protein

The NS1 protein is the only nonstructural protein of influenza virus. It exists as an oligomer\textsuperscript{154} and accumulates mainly in the nucleus.\textsuperscript{155} The NS1 protein seems to regulate cellular and viral protein expression by binding to different RNA molecules. In many \textit{in vitro} studies, NS1 has been shown to bind to a wide range of RNA molecules, such as poly(A)-containing cellular RNA,\textsuperscript{156} vRNA,\textsuperscript{157} vRNP,\textsuperscript{158} double-stranded RNA (dsRNA),\textsuperscript{159} and small nuclear RNA (snRNA).\textsuperscript{160} It is also known to have inhibitory effects on splicing,\textsuperscript{161,162} cellular mRNAs nuclear export,\textsuperscript{156,161,163–165} cellular mRNA polyadenylation by interacting with the cellular 3′ end processing machinery\textsuperscript{166,167} and dsRNA protein kinase (PKR) activation.\textsuperscript{168} In addition, NS1 protein also appears to enhance viral protein expression by stimulating the translation of viral mRNA.\textsuperscript{164,169,170} However, an influenza virus lacking the NS1 gene was generated in interferon-deficient cells,\textsuperscript{171} suggesting that the NS1 protein is not absolutely essential for the viral life cycle in cell culture. Recently, NS1 protein of H5N1/97 was found to make the virus less susceptible to the antiviral effects of interferons and tumor necrosis factor alpha. In addition, the NS gene of H5N1/97 was shown to be a potent inducer of proinflammatory cytokines in human macrophages,\textsuperscript{172} suggesting the unusual severity of human H5N1/97 disease might be due to the “cytokine storm” induced by the virus.

NS2 Protein

In early studies, it was believed that the NS2 protein was a nonstructural protein. However, further studies have indicated that NS2 is incorporated into viral particles in low amounts.\textsuperscript{33,34} Based on studies of NS2 mutants, Odagiri \textit{et al.}\textsuperscript{173} suggested that NS2 plays a role in promoting normal replication of the genomic RNAs by an unknown mechanism. In addition, the carboxyl-terminal region of NS2 contains a M1 protein-binding site\textsuperscript{34,174} suggesting that NS2 might regulate and cooperate with the function of M1. Based on the evidence that the NS2 protein contains a nuclear export signal and facilitates the vRNP export, O’Neill \textit{et al.}\textsuperscript{35} have proposed to rename this protein as NEP (viral nuclear export protein). Subsequence studies also confirmed that this protein is essential for vRNP nuclear export.\textsuperscript{175}

**TRANSCRIPTION AND REPLICATION OF INFLUENZA VIRUS RNA**

The genome of influenza virus is negative-stranded RNA.\textsuperscript{36} vRNAs are templates for both cRNA and mRNA syntheses. Unlike other negative-sense, single-stranded RNA viruses, the transcription and replication site for influenza
virus genome is in the nucleus of infected cells. All eight vRNA segments contain 12 and 13 conserved nucleotides at their 3’ and 5’ ends, respectively. In addition, each RNA segment also contains 2 to 3 segment-specific nucleotides near each end. These RNA sequences are partially complementary and can form a panhandle structure. Viral mRNA is a capped and polyadenylated transcript. It is an incomplete copy of the vRNA, lacking approximately 17 bases of the complementary viral 3’ sequence. In contrast, cRNA is a faithful copy of vRNA and acts as a template for further synthesis of vRNA. Unlike vRNA and mRNAs, cRNAs are not transported to the cytoplasm during viral infection. In general, it is believed that all the polymerase subunits (PB2, PB1, and PA) and NP are required for both viral transcription and replication.

**VIRAL TRANSCRIPTION OF INFLUENZA VIRUS**

Transcription of mRNA is initiated by a capped RNA fragment, which is cleaved from host mRNA by a cap-snatching mechanism. The PB2 polymerase subunit binds to the 5’ end of host cell mRNA and cleaves it at about 10–15 nucleotides downstream from the cap structure after predominantly an A or a G residue. This specific cleavage requires the presence of a methylated cap structure in the RNA substrate and the endonuclease activity of PB2 is stimulated by the vRNA. Several capped RNA or dinucleotides (e.g., ApG) are known to be used as primers for transcription in vitro. However, of these primers, the cap 1 structure (m7GpppXm) from mammalian cellular mRNA has been shown to be the preferred primer for viral transcription. Interestingly, although the viral mRNAs also contain the cap 1 structure, they seem to be protected from endonucleolytic cleavage. These observations suggested that the viral polymerase complex selectively uses host mRNA for viral mRNA synthesis.

After endonuclease cleavage, the short-capped oligonucleotide is used by the viral polymerase as a primer for transcription. Initiation of viral transcription requires the interaction between the 5’ and 3’ conserved sequences of vRNA and several different secondary structure models of these promoter sequences have been proposed. Early studies suggested that base pairing of the 3’ terminal nucleotide of the cleaved primer with the 3’ terminal U residue (i.e., position 1) of the vRNA template was not required for transcription initiation. However, subsequently, it became clear that this interaction has an effect on the position of transcription initiation. Transcription is terminated at a track of 5–7 U residues approximately 17 nucleotides from the 5’ end of vRNA and a poly(A) tail is then added to the mRNA transcript. RNPs isolated from viral particles can synthesize polyadenylated transcripts in vitro, indicating that the polyadenylation of influenza virus mRNA is a host-independent process. Currently, it is generally
believed that the viral RNA polymerase reiteratively copies the U-track at the 5′ end of vRNA to polyadenylate mRNAs\textsuperscript{196} and several polyadenylation models have been proposed.\textsuperscript{29,68,181,190,194,197} In vivo studies have indicated that the U-track is required for polyadenylation of viral mRNA.\textsuperscript{190,198} Mutational analysis showed that polyadenylated mRNA synthesized by a recombinant influenza virus with mutated U-track to A-track at the 5′ end of vRNA was defective in nuclear export.\textsuperscript{199} This result confirms that the poly(A) tail generated by U-track, together with the cap structure, are normally essential features for mRNA nuclear export\textsuperscript{200,201} and mRNA stabilization.\textsuperscript{202,203}

**VIRAL REPLICATION OF INFLUENZA VIRUS**

cRNA is a full-length copy of the vRNA and can be used as a template for vRNA synthesis. Unlike the products of transcription, which are capped at their 5′ end, the 5′ terminus of cRNA is pppA, suggesting that the synthesis of cRNA is a primer-independent process.\textsuperscript{183} In addition, cRNA is not polyadenylated. Although both cellular and viral factors have been suggested to have crucial roles in the transcription–replication regulation,\textsuperscript{99,184,204,205} the mechanism for the switching from transcription to replication is still poorly understood. Recently, Vreede \textit{et al.}\textsuperscript{101} demonstrated that there may be no switch regulating the initiation of RNA synthesis and proposed a model suggesting that nascent cRNA is degraded by host cell nucleases unless it is stabilized by newly synthesized viral RNA polymerase and NP proteins.

**EXPRESSION OF INFLUENZA VIRAL PROTEINS**

It is widely accepted that the replication and transcription of the influenza viral genome is a selective process.\textsuperscript{182,206} In infected cells, the synthesis of each mRNA molecule is known to vary over the time course of infection. However, the factors that control viral gene expression are not entirely understood. In general, the amount of viral proteins synthesized in infected cells is largely dependent on the amount of the corresponding mRNA in infected cells.\textsuperscript{182,184} Immediately after infection, primary transcription occurs.\textsuperscript{182} In this stage, all eight mRNAs are synthesized in equimolar amounts. This is followed by the second transcription stage. The second transcription stage can be further subdivided into early and late phases. In the early phase of the secondary transcription, NS1 and NP vRNA are preferentially synthesized. As a consequence, NS1 and NP are the predominant viral proteins in infected cells at this stage.\textsuperscript{182,184,206} The reason, however, for the preferential early expression of the NS1 and NP proteins is not known. It is possible that NP is required for the replication and transcription of viral RNA. NS1 might be required for the
regulation of cellular gene expression. During the late phase, vRNAs are synthesized in equivalent amounts, as required for progeny virus genome. At this stage, the NS1 protein is synthesized in a reduced level.\textsuperscript{182,184,206} In contrast, HA, NA, and M1 mRNAs are preferentially expressed.\textsuperscript{182,184,206} In general, most of the capped and polyadenylated viral mRNAs would be transported from the nucleus to the cytoplasm for protein synthesis. On the other hand, the membrane-bounded proteins, such as HA, NA, and M2, would pass the secretory pathway at the \textit{trans}-Golgi network for protein maturation. HA and NA proteins are post-translationally modified and transported to the cell surface for integration into the cell membrane.

**NUCLEAR EXPORT OF VIRAL RIBONUCLEOPROTEINS (VRNPS)**

Recent evidence suggests that the M1 protein is associated with the migration of ribonucleoproteins out of the nucleus for assembly into progeny viral particles in the cytoplasm.\textsuperscript{102,123–125} However, it was found that NS2 protein might also be involved in the translocation of vRNP for viral packaging in the cytoplasm.\textsuperscript{102,103} It is because M1 protein contains a nuclear localization domain\textsuperscript{122} that allows it to enter to and exit from the nucleus to regulate vRNP nuclear transport.\textsuperscript{102} When it binds to vRNP, it promotes vRNP nuclear export. Since the carboxyl-terminal region of NS2 contains an M1 protein-binding site,\textsuperscript{34,174} it is suggested that NS2 might regulate and cooperate with the function of M1 by interacting with the M1 protein for assisting the nuclear export of vRNPs.

**ACKNOWLEDGMENTS**

We acknowledge funding from the National Institute of Allergy and Infectious Disease, USA (AI95357), the Research Grant Council of Hong Kong (HKU 7343/04M), and the University of Hong Kong (seed funding for new staff, 2004–2005).

**REFERENCES**


into virions, virion morphology, and virulence in mice but is not essential for virus replication. J. Virol. 70: 873–879.


70. Li, M.L., B.C. Ramirez & R.M. Krug. 1998. RNA-dependent activation of primer RNA production by influenza virus polymerase: different regions of the same protein subunit constitute the two required RNA-binding sites. EMBO J. 17: 5844–5852.


Students make avian and human influenza A virus models and then infect a model lung cell to make a hybrid virus that has some avian and some human RNA segments and surface proteins. Activity Duration: 30-60 min. Cost: cost of supplies. Students will learn well not only about how the flu virus is different from other viruses, but also about the extensive biology of the flu virus. Rating: Recommended. show more.