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Review

Introduction to molecular biology of influenza A viruses

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This minireview presents an overview of current knowledge on virion structure, genome organization and basic events in the development of influenza A virus. The processes of entry, transcription/replication and viral release are described. In this context, the roles of viral proteins (including recently discovered minor polypeptides) in the subsequent stages of viral development are also discussed.

Key words: *Orthomyxoviridae*, influenza A virus, virion structure, genome, entry, release, new polypeptides

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INTRODUCTION

A number of excellent reviews on influenza viruses has been published in recent years (Taubenberger & Morens, 2008; Krauss & Webster, 2010; Medina & Garcia-Sastre, 2011; Wahlgren J 2011; Iwasaki & Pillai, 2014; Spackman, 2014). This article summarizes basic facts on the molecular biology of influenza viruses and it should serve only as a reference article for the remaining articles in this special issue of Acta Biochimica Polonica. For more thorough review of influenza molecular biology we also suggest chapters on *Orthomyxoviridae* in the latest edition of Fields Virology (Shaw & Palese, 2013).

Influenza viruses are the enveloped RNA viruses belonging to the relatively small *Orthomyxoviridae* family. The single-stranded genomic RNA of this family has been termed negative in contrast to mRNA which, by convention, is called positive-strand RNA. At present, *Orthomyxoviridae* family consists of five genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogovirus* and *Isavirus*. The first four genera were established long time ago. The fifth genus is a relatively new addition to *Orthomyxoviridae*; it contains an infectious salmon anemia virus, which is a devastating pathogen in fish farms all over the world. It is likely that the sixth genus, *Influenzavirus D* will be soon created because the virus C/OK isolated from swine and cattle, provisionally included in the *Influenzavirus C* genus, exhibits only low sequence homology to the influenza C viruses (Hause *et al.*, 2014). From the epidemiological point of view it is *Influenzavirus A* genus that is the most important for humans. The only representative of this genus – influenza A virus is divided into many subtypes. According to the current nomenclature, description of the subtypes includes the host from which it was originally isolated, geographic location of the first isolation, strain number, year of isolation and, in parentheses, the serological characteristics of two viral proteins: hemagglutinin (HA) and neuraminidase (NA). For the strains isolated from humans, the name of the host is omitted. As an example, A/Sw/Taiwan/1/70 (H1N2) should be read: influenza A strain number 1, isolated from swine in Taiwan in 1970, which contains hemagglutinin HA1 and neuraminidase NA2. As for today, 18 serotypes of hemagglutinin and 11 serotypes of neuraminidase are described (Tong *et al.*, 2013).

INFLUENZA A VIRION STRUCTURE

Orthomyxovirus virions are usually close to being spherical, with diameter ranging from 100 – 200 nm (Fig. 1). However, some laboratory strains may form extended threadlike structures, which sometimes exceed 1000 nm. The virions are relatively unstable in the environment and influenza viruses are inactivated by heat, dryness, extremes of pH and detergents (Shaw & Palese, 2013). The outer lipid layer of influenza viruses originates from the plasma membranes of the host in which the virus was propagated (Nayak *et al.*, 2009). Outside of the lipid envelope, there are about 500 projections/virions in the form of spikes. About 80% of these projections resemble rods which are composed of hemagglutinin. The remaining projections are in the shape of mushrooms and they are built from molecules of neuraminidase. The viral outer membrane also contains some copies of the small M2 protein that form ion channels in the virion particles. The matrix protein M1, which is the most abundant protein in virions, underlies the lipid layer and plays an important role in the attachment of the ribonucleoprotein (RNP). The RNP core is a complex structure

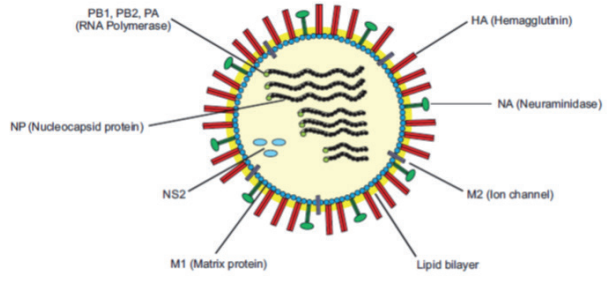

Figure 1. Schematic view of the influenza A virion structure.

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Abbreviations: cRNA, complementary copy of the vRNA; HA, hemagglutinin; M1, matrix protein; NA, neuraminidase; NEP, nuclear export protein; RNP, ribonucleoprotein; ssRNA, single-stranded RNA; vRNA, viral RNA.

composed mostly of the NP protein, which wraps eight different RNA segments of the influenza A genome. Additionally, RNPs contain about 50 copies per virion of RNA-dependent RNA polymerase, which in case of influenza A virus is a complex of three proteins: PB1, PB2 and PA (Noda *et al*., 2006; Boivin *et al*., 2010).

INFLUENZA A GENOME ORGANIZATION

Influenza A's eight segments of single-stranded RNA (ssRNA) of negative polarity contain about 14 000 nucleotides. Historically, the gene assignment of gene products to particular segments was as follows:

Segment 1 – PB2
Segment 2 – PB1
Segment 3 – PA
Segment 4 – HA
Segment 5 – NP
Segment 6 – NA
Segment 7 – M1 and M2
Segment 8 – NS1 and NS2/NEP

Recently some new gene products were added to this list. They are described in the last subchapter of this article.

The first three largest segments encode three subunits of the RNA polymerase which, as mentioned above, are found in virions associated with viral RNA. Proteins encoded by segments 4–7 and one protein encoded by segment 8 — NS2/NEP are also found in the viral particle. Only segment 8 encodes a protein, NS1, which is a non-structural protein. The organization of the eight RNA segments inside the virion is not fully understood. Electron microscopy studies indicate that each RNA segment is individually wrapped by the NP protein forming helical structures. The mechanism of packaging of eight different segments into a single virion shell still remains to be elucidated.

PROPAGATION OF INFLUENZA A VIRUS

Historically, influenza viruses were first isolated from swine in 1930 and from humans three years later. For a few decades embryonated chicken eggs were the only reactors where influenza viruses could be grown. This is still a method of choice for the cultivation of the virus for vaccine production, where massive quantities of the virus are needed. Some subtypes of influenza A do not replicate well in chicken embryos and they require adaptation to growth in the allantoic cavity. The quality of the virus replication in embryonated eggs can be assessed by the ability of the infected allantoic fluid to agglutinate erythrocytes. At present, influenza viruses are often grown in tissue cultures originating from kidney cells (most often these are Madin-Darby canine kidney cells — MDCK) (Govorkova *et al*., 1999; Tree *et al*., 2001). However, for most HA subtypes preliminary treatment with trypsin is necessary to produce infectious viral particles. This protease cleaves hemagglutinin into active subunits, which is a prerequisite for the efficient propagation of the virus.

THE ROLE OF PROTEINS ENCODED BY INFLUENZA A GENOME IN THE SUBSEQUENT STAGES OF VIRAL MULTIPLICATION

Stages of influenza A replication are essentially the same as for the majority of other ssRNA viruses of negative polarity. One can distinguish the following major processes leading to the production of viral progeny:

- Virus adsorption, entry and uncoating
- Messenger RNA synthesis and replication of viral RNA
- Post-transcriptional processing of viral mRNA
- Translation and post-translational processing of viral proteins
- Virus assembly and release from the cells

Each of these processes has one or more viral proteins playing a key role in the accomplishment of the successful outcome of the subsequent steps of viral amplification. However, it has to be pointed out that some cellular factors play a crucial role in allowing the virus to achieve this final aim.

Virus adsorption, entry and uncoating

Influenza A virus utilizes sialic acids on the surface of cells as viral receptors (Skehel & Wiley, 2000). Hemagglutinins of influenza viruses replicating in different species show some preference for particular glycosidic linkages of sialic acids. Human viruses' hemagglutinins bind very well to sialic acids linked to galactose by alfa 2,6 glycosidic bond. On the other hand, avian viruses preferentially bind to sialic acids connected with galactose by alfa 2,3 glycosidic bond (Matrosovich *et al*., 1997; Wilks *et al*., 2012). Alfa 2,6 linkage of sialic acids to galactose is found mostly in human trachea, while sialic acid alfa 2,3 galactose sugar moieties are found mostly in gut epithelium of birds. This fact explains the preference of different viral subtypes for particular host species, but it should be pointed out that it is a preference for receptor and not absolute specificity. It may be bypassed by high viral inoculum (as was the case with most infections of humans with influenza H5N1) or by a few point mutations in the hemagglutinin gene.

After binding to the receptor, influenza viruses are internalized in endocytic compartments. The main mechanism is clathrin-mediated endocytosis, although non-clathrin, non-caveolae pathway has been also attributed to influenza entry (Lakadamyali *et al*., 2004; Grove & Marsh, 2011). Low pH treatment in endosomes dramatically changes hemagglutinin structure, which becomes susceptible to protease digestion. As a consequence, hemagglutinin is cleaved into HA1 and HA2 subunits, which are connected together by disulfide bonds. The cleaved hemagglutinin undergoes further structural changes, which are evidenced by exposure of the fusion peptide at the N-terminus of the HA2. This in turn leads to the interaction of hemagglutinin with membranes of endosomes. During entry, virions must induce signalling to facilitate successful trafficking via the endocytic pathway (Elbahesh *et al*., 2014).

As a consequence of these subsequent processes, contents of the virion, that is its genetic material in the form of a ribonucleoprotein complex, are released into cytoplasm of the cell (Stegmann, 2000; Sieczkarski & Whittaker, 2005). Apart from hemagglutinin, the M2 protein which forms ion channels, also plays an important role in the release of the genetic material from the virion. It mediates the influx of H^+ ions from the endosome into viral particle. As a result, the interaction between ribonucleoprotein and matrix M1 protein is greatly weakened (Martin & Helenius, 1991; Pinto & Lamb, 2006; Wang *et al*., 2011). Recent findings suggest that all eight segments are transported as one moiety to the nucleus (Chou *et al*., 2013).

mRNA synthesis and replication of virion RNA

RNA synthesis of influenza virus occurs in the nucleus. The genomic segments of the virus never exist as naked RNA and it is the NP protein which wraps viral RNA. Ribonucleoprotein complexes are not able to diffuse passively through nuclear pores, so they must rely on cellular mechanisms of nuclear transport. Proteins belonging to the family of alfa importins recognize nuclear localization signals (NLS) on the NP protein and play an important role in the transport of RNA complexes (Cros & Palese, 2003).

Once in the nucleus, genomic ssRNA of negative polarity (vRNA) is used as a template for the syntheses of two classes of ssRNAs of positive polarity: messenger RNAs (mRNAs) and full-length complementary copies (cRNAs). Messenger RNAs are the incomplete copies of the template and they are capped and polyadenylated. During transcription, multimeric viral polymerase interacts with the host polymerase II (Engelhardt *et al.*, 2005). This interaction leads to the phenomenon called "cap-snatching", that is the removal of a cap from newly synthetized cellular mRNAs (Plotch *et al.*, 1979; Bouloy *et al.*, 1979; Bouloy *et al.*, 1980; Krug, 1981). The PB2 subunit of viral polymerase is responsible for cap binding. The PB1 subunit for a long time was regarded as an endonuclease cleaving the 5' capped oligonucleotide (10–13 nucleotides long), which serves as a primer for the initiation of transcription. However, recent crystallographic data suggest that endonuclease activity should be rather attributed to the PA subunit (Boivin *et al.*, 2010). This is one of the facts that contradicts the theory that particular activities of the polymerase can be dissected and attributed to a single subunit. It is rather more likely that three subunits of the polymerase act cooperatively and the synthesis of RNAs is a concerted action of all subunits. Transcription continues until approximately 15 nucleotides from the 5' end of the vRNA (Li *et al.*, 2001). Here, a stretch of uridine residues is localized which serves as a signal for polyadenylation (also catalyzed by viral polymerase). Some mRNA transcripts undergo splicing; this phenomenon leads to the increase of the coding capacity of the segments. Splicing was most extensively studied for transcripts originating from segments 7 and 8. Splicing can be observed in the absence of viral proteins, which suggests that this process is executed by cellular machinery (Lamb & Lai, 1984).

The switch from mRNA synthesis to the synthesis of full-length complementary copy (cRNA) of the vRNA has been intensively studied by many laboratories. For the initiation of the full-length cRNA, no capped primer is necessary and the cRNA chain is not prematurely terminated and polyadenylated, as is the case of viral mRNA synthesis. The precise mechanism of the switch from one to another mode of RNA synthesis is not fully understood; some theories, which were proposed to explain the switch have been described (Gonzalez & Ortin, 1999). Obviously, the polymerase subunits may have different contribution to these two processes, but it has been suggested that the soluble NP protein and some host proteins play an important role in this process (Momose *et al.*, 2002).

The next step of replication of influenza genomic segments is the copying of vRNA on the template of positive strand cRNAs. This process also generates full-length products which assemble with NP and polymerase subunits and form RNP complexes. Such complexes are finally exported into the cytoplasm; the M1 protein and NS2 protein (also called nuclear export protein – NEP) play a vital role in the translocation of these macromolecular structures (O'Neill *et al.*, 1998; Huang *et al.*, 2001).

Virus assembly and release

It has been postulated that assembly of viral components occurs preferentially in lipid rafts (Zhang *et al.*, 2000; Takeda *et al.*, 2003; Leser & Lamb, 2005). Lipid rafts are non-ionic lipid structures, rich in sphingolipids, which reside inside plasma membranes. This site of assembly of influenza virus is supported by the results of examination of the lipid content of purified viral particles. Additionally, hemagglutinin and neuraminidase glycoproteins are incorporated into the lipid rafts (Kundu *et al.*, 1996; Scheiffele *et al.*, 1997; Rossman & Lamb, 2011). Before being directed into lipid rafts, these two glycoproteins are post-translationally modified. The modifications take place in the endoplasmic reticulum and in the Golgi apparatus. In the endoplasmic reticulum these proteins become correctly folded and glycosylated. They are also assembled into oligomers: hemagglutinin into trimers and neuraminidase into tetramers. Subsequent modifications of the glycan chains of these proteins and their esterification with fatty acids occurs in the Golgi apparatus (Veit *et al.*, 1991). Signals for the association of these glycoproteins with lipid rafts are located in their transmembrane domains (Barman *et al.*, 2001). RNPs are probably transported to the region of assembly at the apical plasma membrane independently of other structural proteins. Chou and colleagues concluded on the basis of FISH analysis that eight viral segments gather together in the cytoplasm, which is facilitated by endosomal protein Rab 11. All eight segments probably form a supercomplex before arrival at the plasma membrane binding sites (Chou *et al.*, 2013).

Packaging of eight different RNA segments in the form of RNPs into virion shells is a phenomenon poorly understood, though many models have been proposed (Smith & Hay, 1982; Enami *et al.*, 1991; Bancroft & Parslow, 2002). Some facts suggest the presence of packaging signals at both ends of genomic segments, however, the precise sequences or structures responsible for specific packaging are still not well defined.

Both glycoproteins, neuraminidase and hemagglutinin, have affinity for sialic acids but they have opposite effect on the release of the virus from cells. Hemagglutinin anchors the virus to the cell membrane due to its interaction with sialic acid-containing receptors. Neuraminidase is required to remove sialic acids in order to allow virus to leave its host cells. Therefore, there must be a rigid balance between the activities of these two glycoproteins, so that maximum progeny of the virus can be produced.

Evolution of influenza A virus

The genetic changes of influenza A virus may be introduced by at least three evolutionary mechanisms:

- Changes due to the RNA polymerase errors
- Reassortment
- Recombination

The RNA polymerase of influenza viruses, similarly to other viral RNA polymerases, has no proofreading activity, so the mutation rate for this and other RNA viruses, due to the error-prone reading by RNA polymerases, is much higher than for DNA viruses where polymerases have proofreading properties. Hence, the mutation rate for RNA genomes can be up to 10^5 higher than for a viral DNA genome. Each round of replication may introduce more than one point mutation per replicated genome; these mutations may have neutral, negative or positive effect on virus vi-

ability. The most apparent effect of these gradual changes resulting from the positive selection is so-called antigenic drift in hemagglutinin and neuraminidase glycoproteins, which are localized on the outside of the virion. The antigenic drift, that is the gradual changes in the antigenicity of these two proteins, may finally lead to the establishment of viral variants which are no longer recognized and neutralized by antibodies produced against the parental strain. Therefore, such new antigenic variants may cause epidemics. These variants are usually dominant strains in human or animal populations for a few years and then they are replaced by new antigenic variants. Though this phenomenon is most evident for hemagglutinin and neuraminidase, obviously other genes undergo the same gradual changes as well, caused by polymerase error-prone copying.

Much more abrupt changes in the genome content are caused by gene reassortment. This phenomenon can be defined as the rearrangement of viral gene segments occurring when a cell is infected with two different genomic variants of influenza A viruses. Reassortment usually takes place in animal species, which can be infected with strains of different origin, e.g. with avian and human strains. Very often it is swine that is such a reactor; after exchange of genomic segments, new strains with drastically changed properties may be established in the population. When hemagglutinin or neuraminidase gene segments undergo reassortment, the phenomenon is termed antigenic shift. It implies that major antigenic changes of these two most antigenic proteins may result in abrupt appearance of new strains, which are not recognized by human or animal immunological systems and they may give rise to pandemics. More detailed description of this unique phenomenon will be given in this issue of *Acta Biochimica Polonica* in an article describing swine influenza.

RNA recombination (both homologous and non-homologous) is not a common event in the evolution of influenza A viruses, though it probably takes place more often than it was believed in the past. Non-homologous recombination, which is more common, may occur between genes on different segments (Orlich *et al*., 1994; (Pasick *et al*., 2005). As the result of recombination, lower pathogenic strain is sometimes converted to a highly pathogenic strain (Hirst *et al*., 2004; Suarez *et al*., 2004).

NEW ADDITIONS TO THE LIST OF GENE PRODUCTS CODED FOR BY INFLUENZA A GENOME

Since the beginning of this century, several new influenza A proteins have been identified. The PB1-F2 protein discovered in 2001 is encoded by an open reading frame near the 5' end of the PB1 gene (Chen *et al*., 2001). This is a small, about 90 aa long, polypeptide which is found only in some strains of influenza A. PB1-F2 is translated from the fourth AUG codon in the second open reading frame (+1) of the PB1 gene. Apparently, this protein is an inducer of proapoptotic activity and it contributes to viral pathogenesis in mice (Zamarin *et al*., 2006). PB1-F2 has no polymerase activity, similarly to the third polypeptide encoded by PB1 segment, named PB1-N40. PB1-N40 is an N-terminal 39 aa polypeptide which is translated from the fifth AUG codon in frame with the PB1 start codon (Wise *et al*., 2009). The virus that lacks PB1-N40 has a slower replication rate, however, the more detailed role of this protein remains to be elucidated.

Novel polypeptides encoded by genomic segment 3 also have been recently identified. The first of them, PA-X, is encoded by an alternative reading frame of PA (Jagger *et al*., 2012). PA-X modulates host response and viral virulence (Schrauwen *et al*., 2014). It is expressed in majority of viral strains, though at a low level. It has been found that loss of PA-X changes the pathogenicity of the 1918 pandemic strain in experimental animals. Recently, two novel polypeptides which are truncated forms of the PA polymerase subunit, have been described (Muramoto *et al*., 2013). They are translated from the 11th and 13th in frame AUG codons in the PA mRNA. These two polypeptides, PA-N155 and PA-N182, do not exhibit polymerase activity, however mutants lacking these polypeptides replicate less efficiently in cell culture, and they are less pathogenic in mice than wild type viruses.

Still another protein recently discovered is M42, which is an M2-like protein, but with a different extra-cellular domain than M2 (Wise *et al*., 2012). The list of novel polypeptides encoded by influenza A is not closed. eNP and NS3 are other polypeptides postulated to be coded for by this virus. It remains to be elucidated whether these new additions to the list of influenza proteins play a significant role in viral pathogenicity and fitness.

CONCLUSIONS

Influenza remains one of the main public health threats of this century. Quickly increasing knowledge on molecular biology of influenza is a great help in search for means of prevention of future epidemics caused by this elusive pathogen. New antiviral drugs and novel approaches to construction of vaccines would not be possible without extensive molecular and bioinformatic data collected during recent years in many laboratories all over the world. We anticipate in the near future further breakthroughs in the prevention and therapy, like universal vaccines and inhibitors of viral transcription/replication, which may reduce the health burden caused by influenza.

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