

Biogenesis, assembly, and export of viral messenger ribonucleoproteins in the influenza A virus infected cell

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Abbreviations: vRNA, virion RNA; cRNA, complementary RNA; RNP, ribonucleoprotein; Pol II, RNA polymerase II; CTD, C-terminal domain; CBC, nuclear cap-binding complex; NXF1, nuclear export factor 1; REF/Aly, RNA and export factor-binding protein; TREX, transcription export complex; EJC, exon-junction complex; eIF4AIII, eukaryotic initiation factor 4AIII; UIF, UAP56-interacting factor; CRM1, chromosome region maintenance 1

The flow of genetic information from sites of transcription within the nucleus to the cytoplasmic translational machinery of eukaryotic cells is obstructed by a physical blockade, the nuclear double membrane, which must be overcome in order to adhere to the central dogma of molecular biology, DNA makes RNA makes protein. Advancement in the field of cellular and molecular biology has painted a detailed picture of the molecular mechanisms from transcription of genes to mRNAs and their processing that is closely coupled to export from the nucleus. The rules that govern delivering messenger transcripts from the nucleus must be obeyed by influenza A virus, a member of the *Orthomyxoviridae* that has adopted a nuclear replication cycle. The negative-sense genome of influenza A virus is segmented into eight individual viral ribonucleoprotein (vRNP) complexes containing the viral RNA-dependent RNA polymerase and single-stranded RNA encapsidated in viral nucleoprotein. Influenza A virus mRNAs fall into three major categories, intronless, intron-containing unspliced and spliced. During evolutionary history, influenza A virus has conceived a way of negotiating the passage of viral transcripts from the nucleus to cytoplasmic sites of protein synthesis. The major mRNA nuclear export NXF1 pathway is increasingly implicated in viral mRNA export and this review considers and discusses the current understanding of how influenza A virus exploits the host mRNA export pathway for replication.

Introduction

The assembly, nuclear export, and translation of messenger ribonucleoproteins (mRNPs) are indispensable for gene expression. The emergence of the nuclear envelope during evolutionary history separated the genetic material that is transcribed into messenger RNA (mRNA) from that of the cytoplasmic translational

machinery, coercing mRNP complexes to undergo a dynamically changing repertoire of proteins to define processing, localization, and turnover of mRNAs (reviewed in ref. 1). Recent advances in the field have led to an understanding of the complicated molecular details of metazoan mRNP formation and export (reviewed in refs. 2 and 3). Eukaryotic precursor mRNAs (pre-mRNAs) are synthesized by the DNA-dependent RNA polymerase II (Pol II) transcriptional machinery and then the recruitment of a plethora of RNA-binding and modifying proteins (capping, splicing, and polyadenylation factors) orchestrate the successive stages in mRNP assembly. Pre-mRNAs acquire a 7-methylguanosine cap at the 5' end and are subsequently spliced, cleaved, and 3' polyadenylated. Following maturation into an export-competent mRNP, the complex must traverse the nuclear pore complex (NPC) to the cytoplasm for translation, a progression that involves the recruitment of specific mediating nuclear export factors (reviewed in refs. 4 and 5). The NPC is a 125 MDa macromolecular complex that is 125 nm in diameter and is embedded within the nuclear envelope. The NPC is formed of over 30 nucleoporins, a sub-class of which contain multiple Phe-Gly (FG) repeats which interact with export factors for mediating nucleocytoplasmic transport (reviewed in refs. 6 and 7). In order for nuclear replicating viruses to arise and subsist, they have had to evolve strategies to exploit the host mRNP biogenesis and export pathways for ensuring that their transcripts are efficiently delivered to the cytoplasm for gene expression and replication of the virus (reviewed in refs. 8–10).

Unlike the majority of RNA viruses, the genome of influenza A virus, a member of the *Orthomyxoviridae*, is transcribed and replicated in the nucleus of the host cell (reviewed in refs. 11–13). Infection of a permissive cell begins with attachment by the viral haemagglutinin (HA) glycoprotein to α -2,3 or α -2,6-linked sialic acids on the surface of cells (Fig. 1). The virus particle is subsequently internalized into acidifying endosomes, which causes a conformational change in the haemagglutinin glycoprotein, exposing a fusion peptide that facilitates the fusion between viral and endocytic membranes, releasing the viral genome into the cytoplasm.¹³ The genome of influenza A virus consists of

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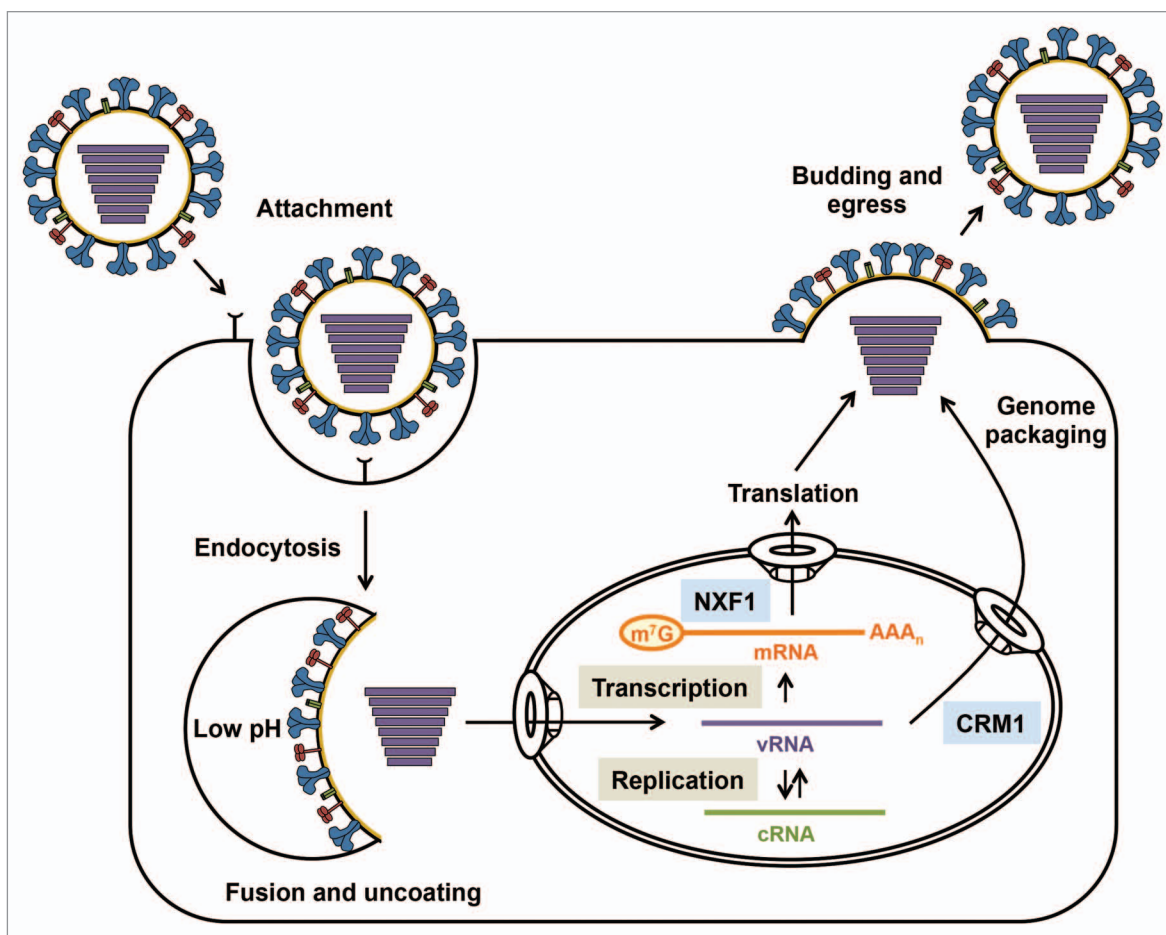


Figure 1. The replication cycle of influenza A virus. Influenza A virus infection commences with attachment via the viral HA glycoprotein to host cell receptors that contain terminal α -2,3-linked or α -2,6-linked sialic acids and viral entry by receptor-mediated endocytosis. Acidification of the endosome causes a conformational change in the viral HA protein, exposing a fusion peptide that facilitates viral-endosome membrane fusion, releasing viral RNP complexes into the host cell. Viral RNP complexes are subsequently imported into the host cell nucleus, where the RdRp transcribes and replicates the negative sense vRNA genome. Genomic vRNA is replicated via a complementary positive-sense cRNA that is encapsidated in nucleoprotein and the RdRp. Viral mRNAs are transported to the cytoplasm by the NXF1 pathway for translation of viral proteins. Progeny viral RNP complexes are exported to the cytoplasm via the CRM1 export pathway and are packaged into progeny virions at cell membrane. Progeny virions are released by budding.

eight individual negative-sense viral RNA (vRNA) segments and encodes 10 major proteins and several auxiliary polypeptides.^{14–19} Each RNA segment is bound by nucleoprotein (NP) and the 250 kDa viral heterotrimeric RNA-dependent RNA polymerase (RdRp) consisting of polymerase basic 2 (PB2), polymerase basic 1 (PB1), and polymerase acidic (PA) subunits to form a viral ribonucleoprotein (vRNP) complex (reviewed in refs. 13 and 20–22). Incoming vRNPs are imported into the host cell nucleus (reviewed in ref. 23) where they are transcribed into mRNA and replicated via a full-length complementary replicative intermediate (cRNA) by the viral RdRp (reviewed in refs. 20, 21, 24, and 25). Viral mRNAs are subsequently exported to the cytoplasm for translation to produce viral proteins. Progeny vRNPs assemble in the nucleus, followed by their nuclear export in a CRM1-dependent manner (reviewed in ref. 26) and transport across the cytoplasm to the cell membrane where virus assembly takes place. Progeny virions are released from the cell by budding, leading to the completion of the viral replication cycle (Fig. 1).

The nuclear replication cycle of influenza A virus has been a fundamental aspect in the evolution of the virus, permitting exploitation of a plethora of cellular functions that are offered by the nuclear compartment. In gaining admittance to the nucleus, influenza A virus has access to the host transcriptional machinery (reviewed in refs. 11 and 12), which may provide the viral polymerase access to a high concentration of pre-mRNAs and mRNA-processing enzymes for viral transcription at sites of host transcription. A nuclear replication cycle has also offered the virus the potential to exploit the host splicing apparatus for the expansion of its coding capacity through the generation of spliced viral gene transcripts.^{27–34} Importantly, nuclear replication might allow the virus to minimize detection of its RNAs by cytoplasmic RIG-I-like helicases and, thus, avoid the triggering of innate immune responses (reviewed in refs. 35 and 36). Clearly, adoption of a nuclear replication cycle has been an advantage to influenza A virus; however, in deploying this tactic, the virus faces the challenging task of delivering viral transcripts to the cellular

translational apparatus in the cytoplasm. In this review, we provide an overview of the current understanding of how influenza A virus has evolved to hijack the cellular machinery for this trade-off, through the integration of virally synthesized mRNA transcripts into the host mRNA export pathway.

Transcribing the Message: Host vs. Viral Synthesis of mRNA

Transcription initiation. Although host and viral mRNAs are structurally equivalent in that they have both a 5' cap and a 3' poly(A) tail, they are synthesized by dissimilar mechanisms and by different molecular machines. Host pre-mRNA synthesis is catalyzed by the Pol II holoenzyme resident in transcription factories (reviewed in ref. 37). Active RNA polymerases do not track as locomotives along their templates, but instead are fixed within transcription factories, discrete foci within the nucleus to which chromatin loops are tethered, containing a high concentration of Pol II and transcription factors (Fig. 2). Host pre-mRNAs are subsequently processed through the involvement of numerous RNA processing factors entering and exiting the factory. Reliant on the phosphorylation pattern of residues within the carboxyl terminal domain (CTD) of the RBP1 subunit of Pol II, pre-mRNAs acquire a 5' cap through the action of the capping enzyme complex containing a phosphatase, guanylyl transferase and a methyl transferase. Once a cap structure has been added to the pre-mRNA, the cap is bound by the cap binding complex (CBC), composed of CBC20 and CBC80 (reviewed in refs. 5 and 38). Influenza A virus exploits host-capped mRNA during viral transcription. The viral polymerase interacts with the CTD of Pol II that is phosphorylated at serine-5, early in the transcription cycle,^{12,39} and this may facilitate access to the 5' cap. The PB2 subunit of RdRp binds to the 5' cap of the nascent pre-mRNA and then the endonucleolytic activity residing in the PA subunit cleaves the cap 10–15 nucleotides downstream of the cap, thus generating a short capped RNA primer for extension by the PB1 subunit, using vRNA as a template.^{40–43} Like cellular mRNAs, viral mRNAs contain internal N6-methyladenosine residues, distinguishing influenza viral mRNAs from those produced by viruses that synthesize mRNAs in the cytoplasm and providing additional evidence that influenza virus mRNA synthesis occurs in the nucleus of infected cells.⁴⁴

Splicing. Following capping, splicing of metazoan pre-mRNAs in order to remove introns is important in the generation

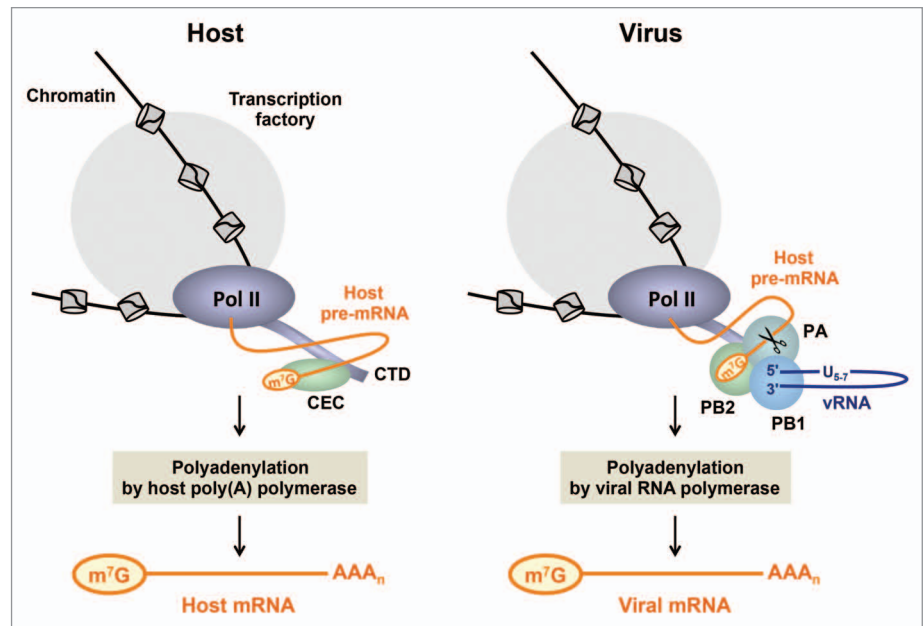


Figure 2. Host and viral transcription result in the synthesis of structurally indistinct mRNAs. In eukaryotic cells, DNA is packaged with histones to form nucleosomes in order to organize the genetic material into chromatin. Actively transcribed DNA by the DNA-dependent RNA polymerase II (Pol II) is tethered to transcription factories, sites containing a high concentration of Pol II and transcription factors, for the synthesis of mRNA. Dependent on the phosphorylation pattern of heptad repeats within the carboxyl terminal domain (CTD) of the largest subunit of Pol II, the mRNA transcript acquires a 7-methylguanosine cap by the action of the capping enzyme complex (CEC). Host mRNA is matured by splicing, endonucleolytic cleavage of the pre-mRNA, followed by polyadenylation of the 5' cleavage product by host poly(A) polymerase. Viral mRNA synthesis is proposed to occur in direct association with actively transcribing Pol II located in a transcription factory. The viral RdRp, consisting of the PB1, PB2, and PA subunits, associates with the CTD of Pol II and the PB2 subunit binds to the 7-methylguanosine cap of host pre-mRNA followed by endonucleolytic cleavage by the PA subunit, resulting in a capped RNA fragment that is used as a primer by the PB1 subunit to initiate transcription of the vRNA template. Viral mRNA is polyadenylated by the viral RdRp reiteratively copying the 5–7 uridine residues near the 5' end of the vRNA template. The nucleoprotein associated with vRNA has been omitted for simplicity in this figure.

of export-competent mRNPs and occurs co-transcriptionally (reviewed in refs. 45–49). Splicing is performed by the spliceosome, a complex of snRNA and proteins that coordinate the removal of introns between splice donor and acceptor sites. At the same time, association of pre-mRNA with an exon junction complex (EJC), composed of four key components, including eIF4AIII, Magoh, Y14, and MLN51, can regulate the fate of the transcript. The EJC can influence localization, stability, and translation of the spliced mRNA transcript (reviewed in ref. 50). Two of the smallest of the eight segments of influenza A virus produce both unspliced and spliced mRNA transcripts. The M segment gives rise to the M1 mRNA that encodes the M1 matrix protein. The M1 mRNA can be processed into various alternatively spliced mRNAs, the most important of which is the M2 mRNA encoding the M2 ion channel.^{30,31} Further spliced transcripts include the mRNA3 of unknown function³⁴ and, for some viral strains, the M4 mRNA encoding the M42 variant of the M2 ion channel with an antigenically distinct ectodomain and a putative 54 amino acid polypeptide that corresponds to an internally deleted version of M1.¹⁹ The NS segment gives rise to the unspliced NS1 mRNA that encodes the multifunctional

non-structural protein 1 (NS1), an interferon antagonist that inhibits cellular responses to infection (reviewed in ref. 51), and the spliced NS2/NEP transcript³³ encoding the non-structural protein 2/nuclear export protein, involved in vRNP nuclear export (reviewed in ref. 26). Recently, it was reported that another spliced transcript can be derived from the NS1 mRNA, resulting in the expression of NS3, a truncated version of NS1 with an internal deletion.¹⁷ Splicing of viral transcripts has been shown to be performed by the cellular splicing machinery (reviewed in ref. 32). The molecular details of this process are not fully understood, but it is believed to be regulated, involving viral and host proteins as well as *cis*-acting RNA signals. A study supporting a role for viral regulation revealed that the accumulation of spliced mRNA transcripts from the M segment was affected by the viral NS1 protein in an RNA binding-dependent manner.⁵² However, regulation of NS transcript splicing by the NS1 protein is more controversial with reports both in favor and against the involvement of the NS1 protein.^{53–55} Recently, it was proposed that influenza A virus utilizes suboptimal splicing to coordinate the timing of infection.⁵⁶ In particular, it was found that utilization of an erroneous splice site ensures the slow accumulation of the viral NS2/NEP while generating high levels of NS1. Modulation of this simple transcriptional event resulted in improperly timed export of vRNPs and lack of virus infection demonstrating that coordination of the influenza A virus lifecycle is set by a “molecular timer” that operates on the inefficient splicing of a virus transcript.⁵⁶

Polyadenylation. Polyadenylation of host and viral mRNA results in the addition of a poly(A) tail of 250–300 adenosines at the 3' terminus. However, the molecular mechanisms of polyadenylation between virus and host are remarkably distinct. During host transcription, two *cis* RNA sequence elements are required for polyadenylation, the poly(A) site 10–30 nucleotides upstream of a cleavage site,⁵⁷ which is bound by cleavage and polyadenylation specificity factor (CPSF), and the DSE element, which is bound by cleavage stimulation factor (CstF). Cleavage is mediated by these factors together with cleavage factors CFI and CFII. Polyadenylation is subsequently catalyzed by the poly(A) polymerase (PAP), initially at a slow rate, but later at a rapid rate in synergy with the poly(A) binding protein II (PABPII) (reviewed in ref. 58). In contrast to host polyadenylation, the viral RdRp is responsible for generating poly(A) tails on mRNA transcripts by stuttering on a stretch of 5–7 uridine residues, approximately 16 nucleotides from the 5' end of the vRNA template.⁵⁹ During transcription, it is believed that the RdRp remains bound to the 5' end of the vRNA template as the vRNA is threaded through in a 3'→5' direction.^{60,61} When the 5' end of the template approaches the catalytic center of the RdRp, steric hindrance results in reiterative stuttering on the stretch of uridines to produce a poly(A) tail. Firm evidence that the RdRp reiteratively copies the stretch of uridine residues came from experiments in which the uridine stretch was replaced with an adenine stretch, resulting in polyuridylated RNAs.^{62–64} Therefore, although catalyzed by different molecular machines, influenza A virus has evolved a strategy for synthesizing mRNA transcripts that are structurally

indistinguishable from host mRNA transcripts for deceiving the host cell into recognizing viral transcripts as native (Fig. 2).

Delivering the Message: Nuclear Export of Viral mRNA

The central dogma of molecular biology, DNA makes RNA makes protein, dictates that once mRNPs have been assembled in the nucleus of eukaryotic cells, they must be exported to the cytoplasm for translation. It was recently shown that the fate of Pol II transcripts is initially determined by the hnRNP C1/C2 complex that acts as a “molecular ruler” to classify Pol II transcripts as long (> 200 nt) mRNA transcripts that are destined to exit the nucleus via the mRNP export pathway, or short snRNA transcripts, which are exported by CRM1.⁶⁵ The export of mRNA is closely coupled to Pol II transcription and pre-mRNA maturation, during which mRNA acquires a myriad of RNA binding proteins, including the CBC, heterogeneous nuclear ribonucleoproteins (hnRNPs), poly(A) binding proteins, the EJC, serine/arginine-rich (SR) proteins, and the transcription export (TREX) complex. Nuclear mRNP export is facilitated by export receptors that bind to mRNP complexes and direct their export through the NPC. The chief export factor for host mRNA is the nuclear export factor 1 (NXF1), also known as TAP that forms a heterodimer with its cofactor p15 (also known as NXT1). NXF1-p15 can be recruited to cellular pre-mRNA through multiple mechanisms involving adaptor molecules.

Recruitment of NXF1 to cellular transcripts. NXF1 recruitment has been linked to splicing that occurs co-transcriptionally bridging nuclear export and transcription. Serine/arginine-rich (SR) proteins and the EJC that are deposited on cellular pre-mRNAs upstream of exon-exon junctions are believed to play a major role by providing a platform for the binding of NXF1-p15 (reviewed in refs. 3, 4, 46, 58, and 66–69). SR proteins have been shown to associate to specific sites within the nascent transcript and help the recruitment of spliceosomal components and the EJC for the removal of introns, and remaining associated with the transcript, subsequently aid the assembly of an mRNP molecule and act as a mediator in the recruitment of NXF1.^{45,47–49,70} NXF1 has also been shown to be recruited to intron-containing transcripts by the transcription export (TREX) complex made up of the THO complex (THOC1–7), UAP56, and Aly/REF, which is recruited in a splicing-dependent manner (reviewed in ref. 71). Unspliced transcripts and transcripts that do not contain introns must also be exported from the nucleus; for this, an alternative (ALREX) mechanism has been proposed whereby a TREX complex of Aly/REF, THO complex subunits, and UAP56 are recruited directly by the CBC, independent of splicing. The intronless or unspliced transcripts bound by the TREX complex are then exported via an interaction with NXF1 (reviewed in refs. 3 and 71). Export is also thought to be linked to polyadenylation (reviewed in ref. 58), with a newly identified TREX2 complex containing a germinal center-associated nuclear protein that was shown to interact with NXF1 potentially offering an alternative mechanism of export.⁷² In addition, the CPSF6 cleavage factor

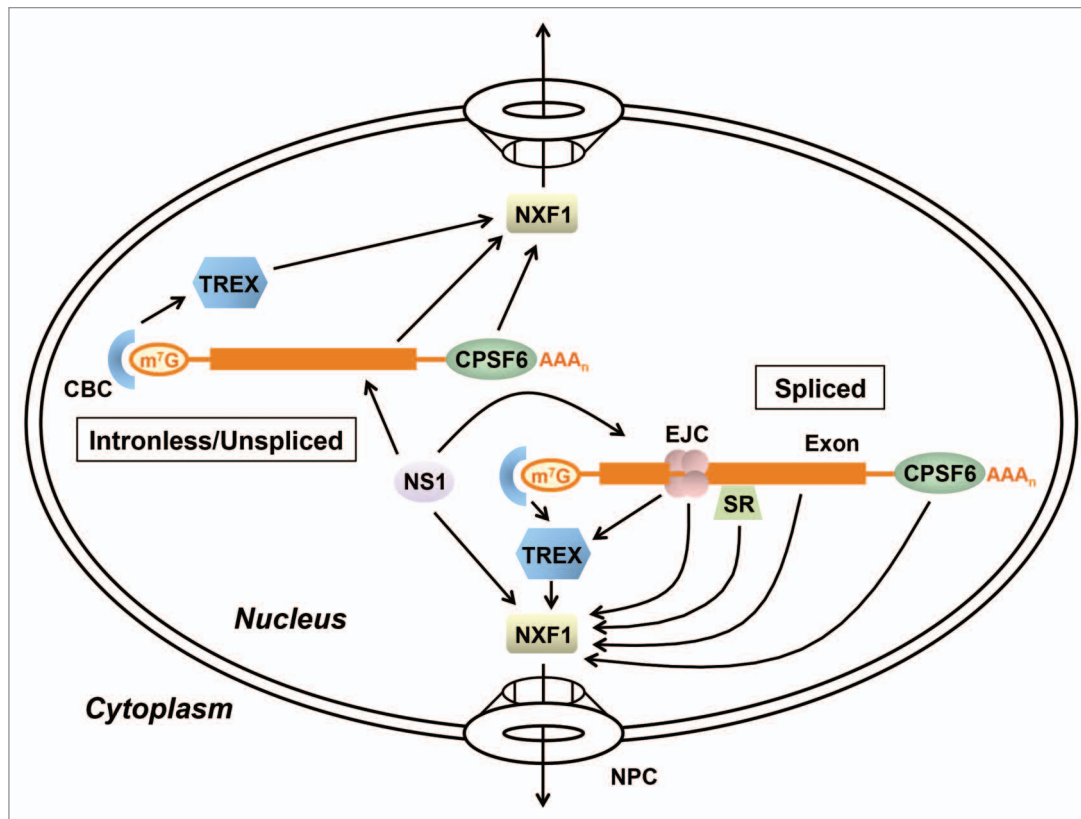


Figure 3. Assembly and export of influenza A virus mRNPs. Intronless and unspliced intron-containing viral mRNA transcripts can recruit the cellular nuclear export factor NXF1 through factors binding to the 5' cap structure or the 3' poly(A) tail. The nuclear cap binding complex (CBC) associated with the 5' cap recruits the transcription export (TREX) complex that subsequently recruits NXF1. NXF1 can also be recruited through poly(A) tail binding factors (CPSF6). Spliced viral mRNA transcripts can also recruit NXF1 through these factors. In addition, these spliced transcripts can recruit NXF1 through the exon junction complex (EJC) and serine/arginine-rich (SR) proteins, which are deposited on spliced mRNAs co-transcriptionally in a splicing-dependent manner. Alternatively, the viral NS1 protein could direct the export of both intronless/unspliced and spliced mRNA transcripts via NXF1. NXF1 was also shown to bind directly to viral mRNA.

involved in polyadenylation has been shown to interact with NXF1.⁷³

NXF1 is involved in the nuclear export of viral transcripts. There is a significant body of evidence in support of the view that host and viral transcription are not separate processes within the cell and that there is a functional and physical link between Pol II and the viral RdRp.³⁹ The interaction between host and viral transcription machineries may not only provide access to a supply of 5' capped RNA primers for the initiation of transcription, but it could potentially provide access of the nascent viral mRNA to all of the cellular proteins and enzymes that mediate pre-mRNA maturation and export of cellular pre-mRNAs. The interaction between the two transcription machineries would allow access to the NXF1-p15 export pathway (Fig. 3). Insight into the use of the NXF1-p15 export pathway by influenza A virus came from studies that showed that treatment of infected cells with 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB), a drug that inhibits Pol II elongation, leads to reversible nuclear retention of HA, M1, and NS1 mRNA, suggesting that mRNA export is dependent on host transcription. Therefore, a model has been proposed that cellular factors are recruited to viral mRNAs during Pol II transcription, but this does not occur if

Pol II transcription is blocked.⁷⁴ Evidence has been published that shows NXF1 interacting with viral mRNAs during infection.⁷⁵ Influenza A virus mRNA and NXF1 protein were found to co-localize in infected MDCK cells. The authors were also able to co-immunoprecipitate influenza A virus mRNAs with the NXF1 protein. NXF1 has also been identified in a number of RNAi screens⁷⁶⁻⁸⁰ as a candidate host protein necessary for influenza A virus replication. A study to directly test the involvement of the NXF1 pathway in viral mRNA export has been published⁸¹ in which prior to infection with influenza A virus, cells were treated with siRNAs directed against NXF1, Aly/REF, or UAP56. A differential dependence on NXF1 between viral genes was concluded from this study. It was observed using fluorescence in situ hybridization (FISH) that transcripts encoding late gene products, HA, M1 and M2 mRNAs showed a greater dependence on NXF1 than those viral transcripts encoding gene products that are expressed early in infection (PB1, PB2, PA, and NP). Knockdown of NXF1 also led to a reduction in virus growth. A 60% reduction in Aly/REF had very little effect on viral mRNA export; however, an 80% reduction in UAP56 severely impaired export of M1, M2, and NS mRNAs. Aly/REF is a major adaptor protein for cellular mRNA export, and therefore, one would

expect a greater dependence of viral mRNA export on Aly/REF than observed in this study. However, a more efficient knock-down could reveal a greater dependence of mRNA export on this host factor. Alternatively, influenza A virus may have evolved to adopt redundant pathways as studies have revealed Aly/REF to be dispensable for mRNA export.^{82,83} Despite the evidence that the NXF1 export pathway is involved in the export of some viral mRNA transcripts, further dissection is required to fully understand the molecular mechanisms of how viral mRNA transcripts are integrated into the cellular export pathway.

Recruitment of NXF1 to viral transcripts. NXF1 can be recruited to cellular transcripts by multiple mechanisms depending on whether the transcript is spliced or not. Six out of the eight of the influenza A virus genome segments (PB2, PB1, PA, HA, NP, and NA) encode transcripts that do not undergo splicing, while the M and NS segments encode transcripts that can be spliced. Thus, influenza A virus transcripts can be classified into three categories, intronless, intron-containing unspliced and spliced. Therefore, the question arises how are the three different categories of viral transcripts assembled into mRNPs and are exported?

Nuclear export of spliced viral mRNAs could be analogous to that of cellular mRNA transcripts involving the binding of EJC and subsequent recruitment of NXF1-p15, while intronless and unspliced viral transcripts might use the alternative RNA export pathway (ALREX) (Fig. 3). In this pathway, NXF1 is recruited, independently of splicing, through the TREX complex that binds to the 5' end of the transcript through interaction with the CBC. More specifically, the 20 kDa subunit of the CBC (CBC20) binds to the 5' cap, while the 80 kDa subunit of CBC recruits Aly/REF, a component of the TREX complex. In support of this pathway being used by influenza virus transcripts it was found that viral mRNAs associate with CBC.⁸⁴ Alternatively, two members of the evolutionarily conserved SR (serine/arginine-rich) family of splicing factors, 9G8 and SRp20, have been shown to promote the export of intronless cellular mRNAs⁸⁵ by directly interacting with NXF1. Thus, it is possible that intronless influenza virus mRNAs could recruit NXF1 through binding SF proteins. The splicing factor ASF/SF2, a member of the SF family of proteins, previously implicated in the regulation of splicing of the viral M1 mRNA,⁸⁶ was also found to directly interact with NXF1, potentially mediating the recruitment of cellular export receptors to viral mRNA.

Very recent evidence suggests that NXF1 is directly recruited to influenza virus mRNAs, raising the possibility that influenza virus RNAs contain *cis*-acting signals recognized by NXF1.⁸⁷ It was shown that influenza virus RNAs directly bind to NXF1 and that NXF1, in cooperation with the nuclear pore component Nup62, facilitates influenza virus RNA export from the nucleus to the cytoplasm. It was also found that the omega-3 polyunsaturated fatty acid (PUFA)-derived lipid mediator protectin D1 (PD1) inhibits the recruitment of virus RNAs to NXF1 resulting in markedly attenuated influenza virus replication. Type D retroviruses such as Mason-Pfizer monkey virus (MPMV) have evolved a similar strategy for ensuring that viral mRNA is exported from the nucleus; the viral RNA contains a constitutive transport element (CTE) that can be recognized by the NXF1 protein for nuclear export.^{88,89} Although the precise mechanisms

involved in this process during influenza infection remain to be clarified, this study raises the possibility that targeting the interactions of influenza virus with the mRNA export pathways might lead to the development of antiviral therapies.

NXF1-independent export pathways. During evolution, alternative, NXF1-independent nuclear export pathways were available to influenza A virus for nuclear export of viral mRNA. The best characterized alternative export pathway is the CRM1 export pathway used by small nuclear RNAs (snRNA) and rRNAs (reviewed in refs. 4 and 90). Human Immunodeficiency Virus (HIV), a member of the *Retroviridae*, similar to influenza A virus in that it has a nuclear replication cycle, exploits the CRM1 export pathway in order to express viral proteins. Rev, a virally encoded accessory protein, contains a nuclear export signal (NES) that is recognized by the CRM1 export receptor and acts in *cis* to direct the export of viral mRNA. Rev Response Elements (RREs) present within the viral mRNA are bound directly by Rev, and consequently, the transcript is exported into the cytoplasm (reviewed in refs. 9 and 10). A number of studies have investigated the potential involvement of the CRM1 pathway in viral mRNA export during influenza A virus infection using a known inhibitor of CRM1, leptomycin B. However, none of these studies have demonstrated that viral mRNA export is sensitive to leptomycin B.^{74,75,81,91} In contrast, influenza virus genomic vRNA nuclear export is sensitive to leptomycin B,⁹¹⁻⁹³ in agreement with CRM1 being involved in their nuclear export through an interaction with the viral NS2/NEP (reviewed in ref. 26). In addition to NXF1 and CRM1-mediated nuclear export of RNAs, a number of alternative RNA export pathways are known to function in the host cell for the export of tRNAs (Exportin-t) and miRNAs (Exportin-5).⁹⁴⁻⁹⁹ However, there is no evidence to suggest that these export pathways play a direct role in the nuclear export of influenza virus RNAs.

Role of viral proteins in nuclear export of viral transcripts. It is also necessary to consider the role of viral proteins in the recruitment of the host export machinery. There is evidence that the multi-functional NS1 protein of influenza A virus, an interferon antagonist, interacts with cellular proteins in the nuclear export pathway and with viral mRNAs (reviewed in refs. 51 and 100). NS1 has been shown to play a role in the splicing, transport, and enhancement of translation of viral mRNAs. The NS1 protein has also been shown to co-immunoprecipitate viral mRNA and to interact with NXF1,^{75,84,101} therefore it is conceivable that NS1 could act as an adaptor protein between viral transcripts and the cellular export proteins. However, as NS1 is a non-structural viral protein, viral mRNA export has to occur, at least initially, in the absence of NS1. It should also be noted that viral mRNA translation proceeds almost normally in IFN-deficient cells infected with an influenza virus lacking the NS1 gene, suggesting that although NS1 might have a regulatory function, it is clearly not essential.¹⁰² The viral RdRp has also been implicated as a viral factor involved in mRNP assembly and translation by associating with viral mRNA through an interaction with the 5' cap structure and the conserved 5' terminal sequence present in all viral mRNAs downstream of the capped RNA primer sequence.¹⁰³ However, more recently it was shown, using RNA co-immunoprecipitation assays, that the CBC but not viral RdRp associates with viral mRNA, and therefore, this

interaction may serve to act as a mechanism of recruitment for the TREX complex and NXF1.⁸⁴ Furthermore, this study concluded that viral mRNA is also bound by the eukaryotic translation initiation factor 4E (eIF4E). These data are contrary to the proposal that all 5' termini of viral mRNAs are occupied by the viral RdRp in order to prevent the use of viral mRNAs as cap donors during viral transcription.¹⁰⁴ These data also argue against the viral RdRp being involved in the regulation of splicing of M1 mRNA¹⁰⁵ and translation as a functional replacement of eIF4E.¹⁰³ Viral RdRp and vRNPs have also been shown to interact with various host splicing factors, including SFPQ/PSF and UAP56, which may mediate recruitment of export factors for viral mRNA nucleocytoplasmic export.^{79,106-109} The use of a virally encoded adaptor protein that can co-opt the host export machinery for the export of viral mRNA has been recognized in other nuclear replicating viruses, for example, two members of the *Herpesviridae*, Herpes Simplex Virus 1 (HSV-1) and Human Cytomegalovirus (HCMV). These herpes viruses encode ICP27 and a UL69 proteins, respectively, which are able to bind directly to viral mRNA and either Aly/REF or UAP56 and consequently direct export.^{10,110-112} Therefore, it is conceivable that influenza A virus proteins may act in an analogous fashion for the recruitment of nuclear export factors (Fig. 3).

Perspectives

Nuclear replicating viruses have had to evolve sophisticated mechanisms for ensuring that their viral genes are expressed efficiently to ensure the assembly of progeny virions and propagation of infection. Here we have reviewed the current understanding of how influenza A virus mRNPs are integrated into host nucleocytoplasmic export pathways for delivering viral mRNAs to the cellular translational machinery in the cytoplasm. Influenza A

viruses critically depend on host transcription and cellular export pathways; a deeper knowledge and understanding of the protein occupancy of viral mRNA would provide valuable insight into the biogenesis, nuclear export and translation of viral mRNP complexes. Much effort has been devoted to the investigation of protein-RNA interactions and the mRNA interactome by proteomic techniques involving mass spectrometry and future analyses employing these innovative techniques would help to expose the occupancy profile on viral mRNA transcripts.^{113,114} Furthermore, these studies would be complimented by exploration of localization and dynamics of exporting viral mRNP complexes through utilizing recent advances in mRNA labeling and detection by super resolution fluorescent microscopy and single molecule techniques.¹¹⁵ Recent advances in the field have made it now possible to visualize and analyze at the single mRNP level the pathway from chromatin association to interactions with the NPC and translocation.⁶⁸ Employing such techniques in the context of the influenza A lifecycle would help to identify and understand the principles and dynamics of the relationship between the key players in mRNP export and the virus. The implications of understanding the fundamental differences between host and viral mRNP assembly and export pathways could potentially offer the valuable opportunity of designing novel antiviral strategies against influenza A virus.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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