TRANSCRIPTION AND REPLICATION OF THE INFLUENZA A VIRUS GENOME

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Summary. – The genome of influenza A virus consists of eight segments of negative-strand viral RNA (vRNA). During the replication cycle of the virus, the genomic vRNA is transcribed into positive-strand mRNA and complementary RNA (cRNA) in the cell nucleus. The promoter for the synthesis of mRNA molecules is located in a partially double-stranded RNA structure formed by the 5′- and 3′-terminal sequences of genomic vRNA segments. The virus encoded RNA-dependent RNA polymerase complex has to interact with both ends of the vRNA in order to generate capped RNA primers by endonucleolytic cleavage of cellular pre-mRNAs for the initiation of viral mRNA synthesis. Conserved sequence elements in the 5′-end, e.g. a polymerase binding site and a U_{5-7} sequence are required for polyadenylation of virus-specific mRNAs. Polyadenylation occurs by reiterative copying of the U_{5-7} sequence by the viral RNA polymerase, which is bound to the 5′end of the vRNA template. The U_{5-7} sequence acts directly as a template for the poly(A)-tail. During the replication cycle of the virus, a "switch" from mRNA to cRNA synthesis occurs, but the mechanism by which this switch occurs remains unclear. The viral nucleoprotein and its interaction with the polymerase proteins and vRNA might play a role in this process. In contrast to transcription, the process of replication – the synthesis of cRNA and vRNA, which are known to occur in the absence of primers – is poorly understood.

Key words: influenza A virus; transcription; replication; polyadenylation; RNA polymerase

Introduction

Influenza viruses belong to the *Orthomyxoviridae* family. They are enveloped viruses with segmented, single-stranded RNA genome of negative polarity (Palese, 1977). Influenza A virus is the most extensively studied among influenza viruses and this review focuses on the transcription and replication strategies of influenza A viruses. It is likely that other members of the *Orthomyxoviridae* family, influenza B and C viruses, Thogoto and Dhori viruses employ similar replication strategies.

Replication cycle of the virus

Following binding of influenza virus particles to cells via interaction between the receptor-binding site of their haemagglutinin (HA) surface glycoprotein and the terminal sialic acid of the cell surface receptor, the attached virion is endocytosed by the cell. Inside the acidic endosomes, M2 protein in the viral envelope catalyses the entry of protons into virus particle. Exposure of the nucleocapsid inside the incoming virus to low pH may induce changes in the conformation of the nucleocapsid, thus priming dissociation

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Abbreviations: RNP = ribonucleoprotein; dsRNA = double-stranded RNA; vRNA = virion RNA; HA = haemagglutinin; mRNA = messenger RNA; M1 = matrix protein 1; M2 = matrix protein 2; NA = neuraminidase; NP = nucleoprotein; NS2 = non-structural protein 2; nt = nucleotide; PA = acidic polymerase protein; PB1 = basic polymerase protein 1; PB2 = basic polymerase protein 2; cRNA = complementary RNA

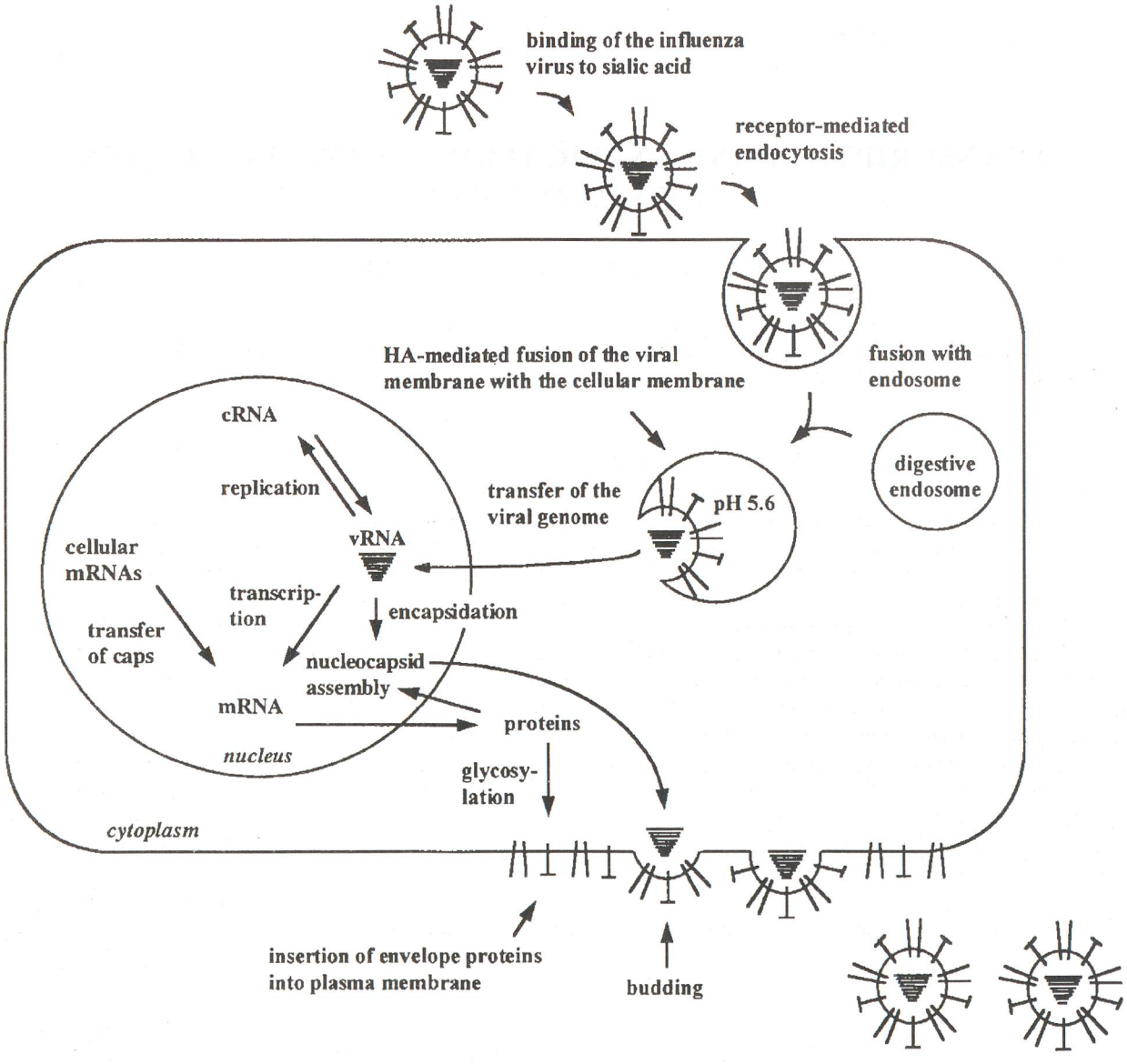

Fig. 1
Replication cycle of influenza A virus

of the viral ribonucleoproteins (vRNPs) from matrix protein 1 (M1) (Helenius, 1992). The low pH inside the endosomes induces a conformational change in HA, which mediates fusion of the viral envelope and the host membrane. Upon the fusion, vRNPs are released into the cell cytoplasm. Thereafter, the vRNPs enter the nucleus where they are transcribed and replicated by the viral RNA-dependent RNA polymerase complex (Ishihama, 1988; Krug, 1983; Krug *et al.*, 1989; Lamb and Choppin, 1983; McCauley and Mahy, 1983).

The assembly of nucleocapsids is initiated by binding of newly synthesized vRNAs to nucleoprotein (NP) in the cell nucleus. However, how the individual vRNA segments bound to NP are assembled together to form the nucleocapsid is not understood. M1 protein appears to play a role, possibly by binding to vRNA (Wakefield and Brownlee, 1989). M1 protein accumulates in the nucleus and interacts with nucleocapsids, promoting their migration out of the nucleus (Martin and Helenius, 1991). In addition, NS2 protein (renamed nuclear export protein (NEP)) was shown to mediate the nuclear export of vRNAs by acting as an adaptor between viral RNP complexes and the nuclear export machinery of the cell (O'Neill *et al.*, 1998). The nucleocapsids covered by M1 protein move towards the cell

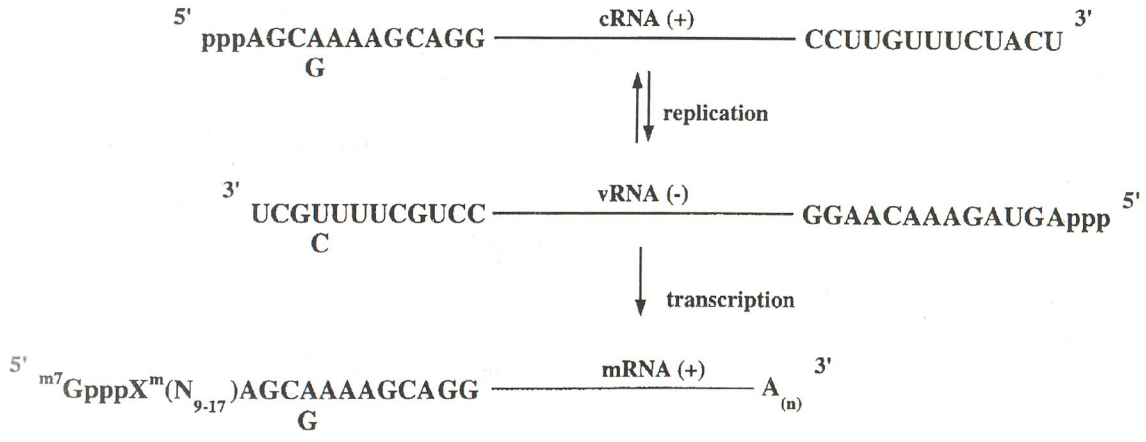

Fig. 2

Relationships between the three types of RNA molecules synthesized by the influenza A virus RNA polymerase in infected cells

The conserved 13 nucleotides at the 5′-end and the 12 nucleotides at the 3′-end of each of the vRNA segment are shown. The cap structure and the 9–17 heterologous nucleotides (nt 9–17) at the 5′-end of mRNA are derived from host cell pre-mRNAs.

membrane, where they are enclosed by an envelope, containing the viral surface glycoproteins (Martin and Helenius, 1991). Interactions between M1 protein and the cytoplasmic domains of HA, neuraminidase (NA) or M2 protein may play a role in promoting budding. NA plays a role in preventing virus aggregation after budding from the infected cell. This function is mediated by the enzymatic activity of NA, which is responsible for the removal of receptors, i.e. sialic acids from the virus surface (Liu *et al.*, 1995). A schematic diagram of the replication cycle of influenza A virus is given in Fig. 1.

Transcription and replication of vRNA

The genome of influenza A viruses consists of eight RNA molecules packaged into RNP complexes that contain, in addition to the RNA, NP and a polymerase complex (PB1, PB2, and PA) (Palese, 1977). NP constitutes the major protein component of the vRNP forming a core, around which the RNA is wrapped in a helical fashion (Baudin *et al.*, 1994; Ruigrok and Baudin, 1995). During the replication cycle of the virus, the viral genome (vRNA) serves as a template for the synthesis of two types of RNA molecules (Fig. 2). Firstly, it is used as a template for the synthesis of mRNA molecules (transcription). Secondly, it is replicated to cRNA molecules, which are then copied to new vRNA molecules (replication) (Krug *et al.*, 1989). Synthesis of the viral mRNAs requires a capped RNA primer generated from host mRNA by an endonuclease activity of the polymerase complex (Plotch *et al.*, 1981). The 9–17 nucleotides (nt) long capped RNA primer is incorporated at the 5′-end of the newly synthesized mRNA molecules (Bouloy *et al.*, 1979; Caton and Robertson, 1980; Dhar *et al.*, 1980; Krug *et al.*, 1979; Plotch *et al.*, 1979). The synthesis of mRNA is prematurely terminated at a sequence of 5–7 uridines 16–17 nt from the 5′-end of vRNA. At this site the viral RNA polymerase pauses and initiates the synthesis of a poly(A)-tail (Li and Palese, 1994; Luo *et al.*, 1991). Consequently, the mRNA molecules lack sequences complementary to the last 16–17 nt at the 5′-end of vRNA molecules (Hay *et al.*, 1977a). In contrast to mRNA, cRNA is a full-length copy of vRNA. It is neither capped nor polyadenylated (Skehel and Hay, 1978). Both cRNA and vRNA have a pppA at their 5′-terminus, which implies that the initiation of their synthesis occurs without a primer (Hay *et al.*, 1977b, 1982).

The viral RNA-dependent RNA polymerase complex, which is composed of three subunits, PB1, PB2, and PA proteins, is involved in both transcription and replication (Ishihama, 1988). PB1 functions as a polymerase and catalyses the sequential addition of nucleotide triphosphates to elongating RNA chains (Braam *et al.*, 1983; Ulmanen *et al.*, 1981). It contains the conserved motifs characteristic of RNA-dependent RNA polymerases (Argos, 1988; Biswas and Nayak, 1994; Poch *et al.*, 1989). PB2 protein binds to cap1 structures of host mRNA molecules (Blaas *et al.*, 1982a, 1982b; Ulmanen *et al.*, 1981) and it might be responsible for their endonucleolytic cleavage (Blok *et al.*, 1996; Shi *et al.*, 1995). PA has not been assigned a specific function in the replication cycle of the virus, although genetic evidence suggests that it is required for vRNA replication (Mahy *et al.*, 1981; Krug *et al.*, 1975). The PA subunit induces a generalized proteolytic process when expressed individually from cloned cDNA, but it is unclear whether

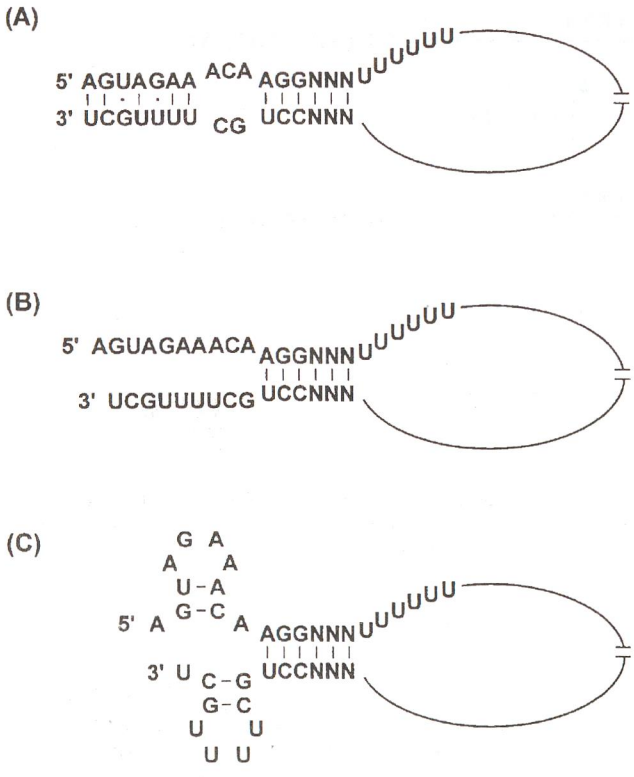

Fig. 3
Proposed secondary structures for the conserved 5′- and 3′-terminal sequences of vRNA formed upon binding of the RNA polymerase

(A) Panhandle structure (Hsu *et al.*, 1987). (B) RNA-fork structure (Fodor *et al.*, 1994). (C) Corkscrew structure (Flick *et al.*, 1996). The Watson-Crick base pairs (indicated by a line) and wobble base pairs (indicated by a dot) in the RNA structures are shown. "N" indicates segment-specific nucleotides. The U_6 poly(A)-site (Robertson *et al.*, 1981) at the 5′-end is also shown.

this activity is relevant for the replication process of influenza vRNA (Sanz-Esquerro *et al.*, 1995; Perales *et al.*, 2000).

The control of transcription and replication of viral genomic vRNA is not well understood. It is not known exactly which viral and host proteins are involved in transcription and replication of the viral genome, though considerable progress has been made in characterizing the viral RNA signals required for these processes (see below).

RNA signals important for transcription and replication

All vRNA segments of influenza A virus contain 12 and 13 non-coding conserved nucleotides at their 3′- and 5′-ends, respectively. These conserved sequences, along with an extra two or three segment-specific nucleotides at the 3′- and 5′-ends of vRNA, display inverted partial complementarity and they were proposed to form a partially double-stranded panhandle structure (Fig. 3A) (Desselberger *et al.*, 1980; Robertson, 1979; Skehel and Hay, 1978; Stoeckle *et al.*, 1987). The existence of panhandle structures in purified virus as well as in infected cells has been confirmed by psoralen cross-linking experiments (Hsu *et al.*, 1987).

Various *in vitro* and *in vivo* systems have been used to define the vRNA promoter, i.e. the RNA sequence required for the initiation of transcription and replication. These systems have allowed *in vitro* and *in vivo* reconstitution of RNP complexes, which where then used to transcribe model RNA templates (Li and Palese, 1992; Luytjes *et al.*, 1989; Neumann and Hobom, 1995; Parvin *et al.*, 1989; Piccone *et al.*, 1993; Seong and Brownlee, 1992a, 1992b; Yamanaka *et al.*, 1991). Luytjes *et al.* (1989) have demonstrated that the twenty-two 5′-terminal and the twenty-six 3′-terminal nucleotides of the influenza A virus vRNA are sufficient to provide the signals for RNA transcription, RNA replication, and packaging of vRNA into influenza virus particles. Based on further *in vitro* deletion analysis, the conserved 3′-end of vRNA was thought to suffice as the promoter for mRNA and cRNA synthesis (Parvin *et al.*, 1989; Seong and Brownlee, 1992a). Further studies, however, have shown that the conserved 5′-end of vRNA forms an integral part of vRNA promoter. The 5′-end contains a major polymerase binding site (Fodor *et al.*, 1994; Tiley *et al.*, 1994) and the polymerase requires both the 3′- and the 5′-end for its endonuclease activity (Hagen *et al.*, 1994).

Initiation of transcription

According to the current models for the initiation of transcription, the vRNA molecule adopts a circular panhandle conformation, which is formed as a consequence of base-pairing between the conserved 3′- and 5′-ends (Fig. 3A). The RNA fork model (Fig. 3B, Fodor *et al.*, 1994) proposed that a dsRNA structure is formed by conserved nt 10–12 at the 3′- end and nt 11′–13′ at the 5′-end (prime notation is used to distinguish 5′-residues from 3′-residues). This duplex region is extended by two or more often three additional base pairs specific for each segment, resulting in a total of 5–6 base pairs. Since such a short dsRNA structure is unlikely to be stable, it is proposed that the duplex region is stabilized by protein-RNA or protein-protein interactions dependent on the RNA polymerase subunits. On the basis of the results with single, double, and triple mutations in the proposed duplex region of the vRNA promoter it was concluded that, for the initiation of transcription, the secondary structure in this region is more important than the actual nucleotide sequence (Fodor *et al.*, 1995, 1998;

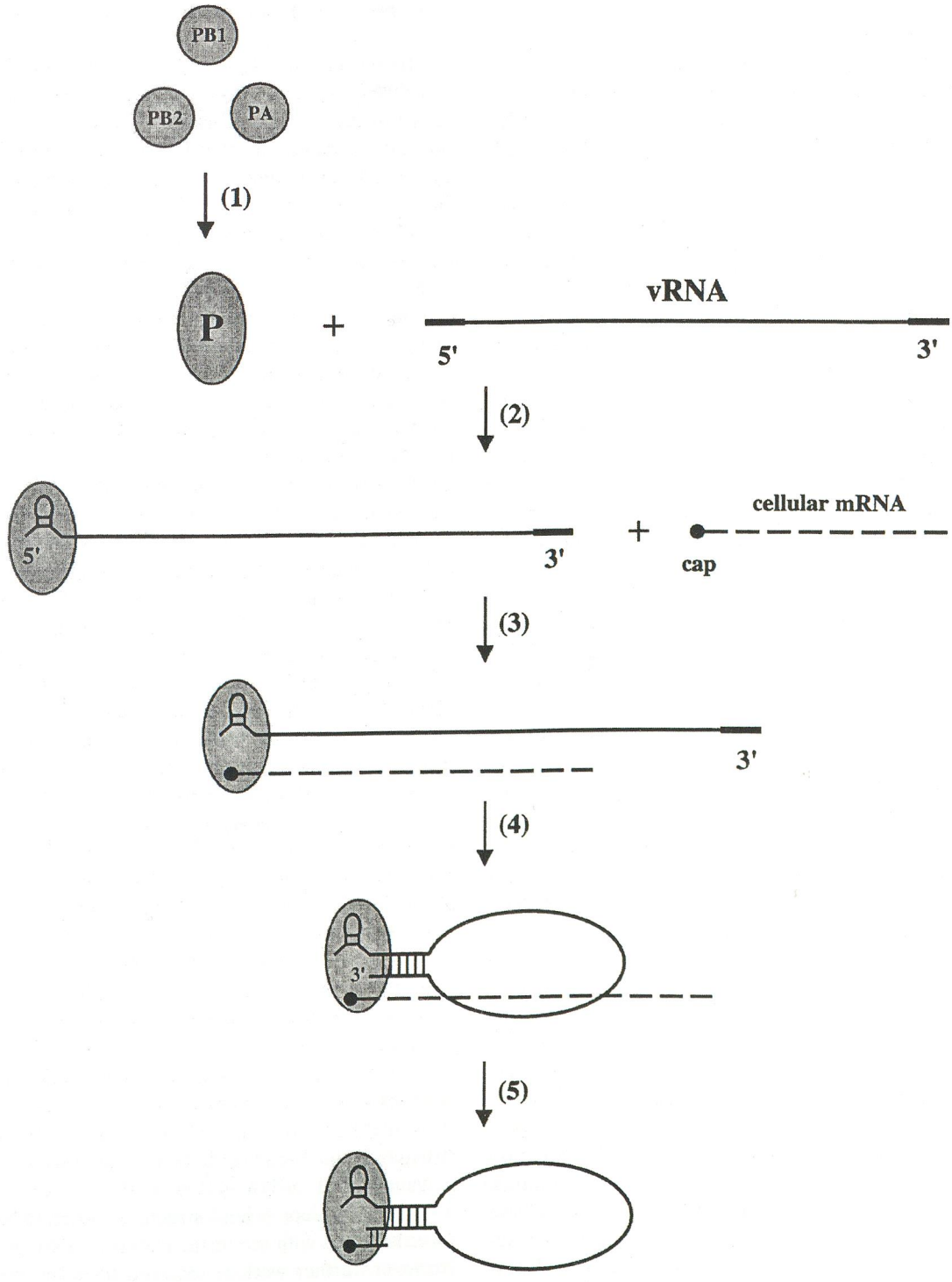

Fig. 4

Proposed mechanism of pre-initiation complex formation for mRNA synthesis (Cianci *et al.*, 1995; Li *et al.*, 1998)

The formation of the pre-initiation complex involves the following steps: (1) the three subunits of the RNA polymerase, PB1, PB2, and PA form a complex (P); (2) the polymerase complex binds to the 5′- end of vRNA; interactions of the polymerase with the stem-loop structure in the 5′-end might play a role in stabilizing polymerase binding; (3) as a result of the interaction of the polymerase with the 5′-end, the capped RNA binding activity of the polymerase is activated; (4) the 3′-end of vRNA binds to the complex formed from the RNA polymerase, the vRNA 5′-end, and cellular mRNA; (5) upon binding of the 3′-end of vRNA to the complex, the endonuclease activity of the polymerase is activated, the cellular mRNA is cleaved, and the capped primer for the initiation of mRNA synthesis is generated.

Kim *et al.*, 1997). Unlike the panhandle model, the RNA fork model suggests that during initiation of transcription the extreme terminal sequences of the template vRNA are open like the prongs of a fork (Fodor *et al.*, 1994, 1995). This idea is based on *in vitro* transcription studies, which demonstrated that base-pairing between nt 1–9 at the 3′- and nt 1′–9′ at the 5′-end was not required for the promoter activity. Further *in vivo* studies of the vRNA promoter extended this model by proposing local secondary structures for the very ends of vRNA (Flick *et al.*, 1996). This RNA "corkscrew" model (Fig. 3C) suggested that nt 4–7 formed a tetraloop at the end of a 2 base pair stem formed by nt 2 and 3 and nt 8 and 9. This stem-loop structure was proposed to be present at both the 3′- and 5′-ends.

The following mechanism has been proposed for the formation of the pre-initiation complex (Fig. 4). Prior to the initiation of transcription, the polymerase complex recognizes and binds to the 5′-end of vRNA, which contains one or more binding sites for the subunits of the polymerase complex (Fodor *et al.*, 1994; Tiley *et al.*, 1994). The stem-loop structure in the 5′-end might be critical for polymerase binding (Pritlove *et al.*, 1999). Subsequently, the complex formed by the polymerase and the vRNA 5′-end recognizes and binds to the 3′-end of vRNA. This binding is facilitated by base pairing between the two vRNA termini but also by protein-RNA interactions. Though the 5′-end of vRNA contains the major polymerase binding site, it has been demonstrated that the polymerase can specifically bind, albeit at lower efficiency, to the 3′-end of vRNA (Fodor *et al.*, 1993; Tiley *et al.*, 1994). After binding to the 5′-end, the affinity of the polymerase for the 3′-end is enhanced because of the synergic effect caused by base pairing in the duplex region. Thus there are three events important for the formation of the pre-initiation complex, (i) binding of the RNA polymerase complex to the 5′-end of vRNA, (ii) base-pairing between the vRNA termini, and (iii) binding of the polymerase-5′-end complex to the 3′-end.

The ability of the polymerase complex to generate capped RNA primers for the initiation of mRNA synthesis is dependent on the interactions of the polymerase complex with vRNA. The cap-binding activity of the PB2 polymerase subunit is activated after binding of the polymerase complex to the 5′-end of vRNA. For the activation of the endonuclease associated with the polymerase complex, the polymerase-5′-end complex has to associate with the 3′-end of vRNA. Thus the polymerase complex is able to generate the capped RNA primers only if it interacts with both termini of vRNA (Fig. 4). Finally, generation of the capped RNA primer through cleavage of host the capped mRNA results in formation of a mature transcription initiation complex (Cianci *et al.*, 1995; Li *et al.*, 1998) allowing the initiation of transcription.

Termination of transcription–polyadenylation of mRNA

Transcription of mRNA is terminated at a sequence of 5-7 uridines near the 5′-end of vRNA, where polyadenylation occurs. Instead of transcribing the 5′-end of vRNA, the RNA polymerase pauses on this U sequence and reiteratively copies it. The U sequence acts directly as a template for the addition of a poly(A)-tail. If the U sequence is replaced by an A sequence, the influenza polymerase synthesizes a poly(U)-tail both *in vitro* and *in vivo* (Poon *et al.*, 1999, 2000).

Initially, the base-paired structure of the panhandle was thought to cause a premature termination of transcription and stuttering of the polymerase complex resulting in polyadenylation of viral mRNAs. According to this model the panhandle acts as a physical barrier, which prevents the polymerase to complete the transcription (Robertson *et al.*, 1981). Early *in vivo* studies supported this idea because they showed the importance of the proposed panhandle structure for gene expression. However, after the discovery of the polymerase binding site at the 5′-end of vRNA (Fodor *et al.*, 1994; Tiley *et al.*, 1994) another model of polyadenylation was proposed. According to this model the polyadenylation is performed by a *cis*-acting polymerase, which is attached to the 5′-end of the template vRNA molecule (Fig. 5). If the polymerase remains bound to the 5′-end of the template vRNA during transcription, when it reaches the site to which it is bound, further transcription is blocked. Polyadenylation of mRNA proceeds by reiterative copying of the U_{5-7} sequence. For the polyadenylation to occur, it is required that a functional polymerase binding site is present in the 5′-end of vRNA and that the 5′-end is able to interact with the 3′-end of the same vRNA molecule through base pairing (Poon *et al.*, 1998; Pritlove *et al.*, 1998). The nucleotides at the 5′-end, which have been shown to be important for polymerase binding are also important for the synthesis of polyadenylated mRNA transcripts.

In addition, it was also shown that the stem-loop structure in the 5′-vRNA end (as suggested by the corkscrew model) is necessary for the synthesis of polyadenylated mRNA molecules (Pritlove *et al.*, 1999). In contrast, the analogous structure at the 3′-end could be disrupted without a loss of polyadenylated mRNA synthesis. It was concluded that a 5′-vRNA stem-loop or hook structure is likely to be essential for interaction with the influenza virus RNA polymerase. However, further work is required to define the precise interaction of the 5′-vRNA hook with the individual components of the RNA polymerase complex.

Replication

Viral replication leading to the synthesis of cRNA and vRNA molecules is different from mRNA transcription. It

most likely occurs by a different mechanism of RNA synthesis which requires both primer-independent initiation and chain completion without premature termination and without poly(A)-addition (Hay *et al.*, 1982; Smith and Hay, 1980).

The first stage in replication is the synthesis of complementary copies of vRNA. The synthesis of cRNA requires (i) a switch from the capped RNA-primed initiation to unprimed initiation, and (ii) anti-termination at the U_{5-7} sequence, 16–17 nt from the 5′-end of vRNA (Hay *et al.*, 1982). It is not clear whether cRNA synthesis is initiated from a panhandle structure like mRNA transcription, i.e. whether the 5′-end is part of the promoter for replication. It is tempting to speculate that replication occurs from a single 3′-terminus without the involvement of the 5′-end (Fodor *et al.*, 1994; Tiley *et al.*, 1994). In the absence of the 5′-end binding, the polymerase would be prevented from generating capped RNA primers, which then could allow primer-independent initiation on a single 3′-terminus. Moreover, the absence of the RNA polymerase from the 5′-end would result in the synthesis of full-length cRNA product.

Genetic and biochemical studies suggest that NP is required for switching RNA synthesis from transcription to replication, but the mechanism of this switch remains unclear (Beaton and Krug, 1986). NP may somehow hinder polymerase binding to the 5′-end and prevent stuttering of the polymerase complex at the U sequence of the vRNA template facilitating anti-termination leading to completion of cRNA synthesis. However, transcripts initiated with capped primers are not anti-terminated in the presence of soluble NP. This suggests that inititation and termination are coordinated.

This coordination can be explained by the hypothesis that NP directly interacts with the polymerase protein complex and thereby modifies its activity and causes primer independent initiation of cRNA molecules and their anti-termination without poly(A)-tail addition. Recent experiments showed that NP independently interacts with the PB1 and PB2 subunits of the polymerase complex (Biswas *et al.*, 1998). Binding of NP protein to PB2 protein might change the cap recognition or cap binding function of PB2 protein. This would reduce the availability of 5′-capped primers required for transcription initiation. Furthermore, the binding of NP to PB1 may facilitate efficient cap-independent initiation and elongation, and the anti-termination property of NP would permit chain completion of cRNA molecules. However, a more detailed analysis of NP-PB1 as well as NP-PB2 interactions is needed to better understand the precise function of NP in this critical regulatory step of RNA synthesis.

In the second stage of replication, the positive-strand cRNAs produced in the first stage of replication act as templates for the production of faithful copies of the genome segments. This step is also dependent on viral protein

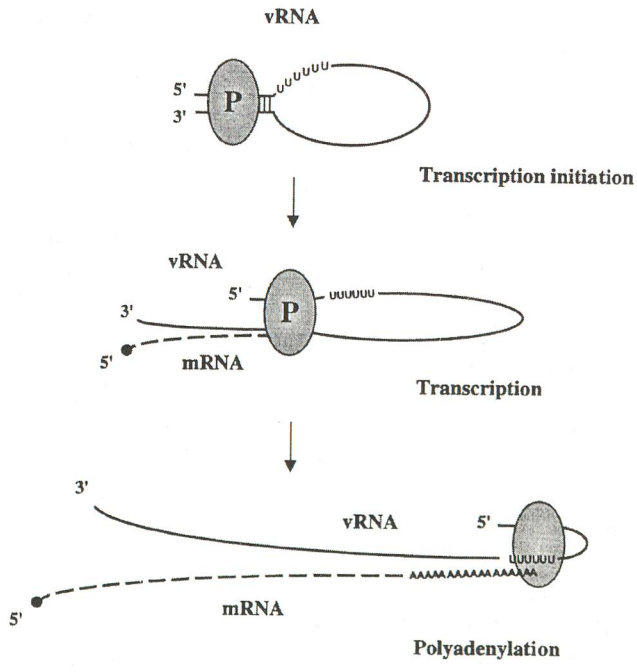

Fig. 5

Proposed mechanism for polyadenylation of influenza virus mRNA molecules (Fodor *et al.*, 1994; Hagen *et al.*, 1994)

Transcription is initiated by RNA polymerase bound to both the 5′- and 3′-ends of the vRNA template. Throughout transcription, the polymerase remains attached to the 5′-end of the template. As a result, the polymerase is unable to transcribe the site to which it is bound. Instead, the polyadenylation of mRNA occurs by reiterative copying of the U_{5-7} sequence (Poon *et al.*, 1999).

synthesis. The roles of each of the subunits of the RNA polymerase complex in replication have not been defined, but studies with temperature-sensitive mutants indicate that both PB1 and PA proteins are involved (Krug *et al.*, 1975; Mahy *et al.*, 1981). The role of PB2 protein, if any, in replication is ambiguous as neither a cap nor a primer is known to be involved in viral RNA replication. The study of replication is hampered by the fact that with none of the *in vitro* systems presently available is it possible to direct efficient synthesis of primer-independent transcripts from added RNA templates.

Concluding remarks

Although the application of the techniques of molecular biology have led to a significant advance in our understanding of molecular mechanisms of transcription and replication of the influenza vRNA genome, many questions remain to be answered. As shown above, little is known about the molecular mechanism of the mRNA to cRNA switch

and replication in general. In addition, it is not understood how the synthesis of the three types of influenza virus RNA (mRNA, cRNA and vRNA) and their relative abundance are controlled throughout the replication cycle of the virus. The replication cycle can be divided into an early and a late stage, which differ in the pattern of gene replication and transcription (Shapiro *et al.*, 1987). Neither of the current models for transcription and replication is able to provide an explanation for the temporal regulation of genome transcription and replication, and protein expression. Likewise, it is not understood, how the amounts of expressed proteins are regulated.

It is also not clear how the eight individual vRNA segments encapsidated in NP are assembled together and packaged into progeny virions. Clearly, the terminal sequences of vRNA segments contain all essential RNA signals required for packaging (Luytjes *et al.*, 1989), but the actual "packaging signal" has not yet been characterised. The influenza virion must have a copy of each of the eight vRNA segments to be infectious, but how this is achieved is not known. The observation that the ratio of different vRNA segments in infected cells differs from that found in virions (Smith and Hay, 1982) supports a selective mechanism, but there is also evidence which suggests that a random packaging mechanism could be feasible (Enami *et al.*, 1991).

A detailed study of the function of viral and cellular proteins involved in viral genome transcription and replication might help to answer the above questions. Currently, little is known about the role of the individual polymerase subunits in these processes. Although there is evidence that all subunits might be involved in recognizing promoter structures (Fodor *et al.*, 1993, 1994), it is not known how the different subunits cooperate in promoter recognition. Moreover, understanding of the detailed mechanism of interaction of NP with PB1 and PB2 proteins might help to elucidate the role of NP in transcription and replication. Practically, very little is known about the interactions of viral proteins with cellular factors, and which cellular factors participate in influenza replication. Recently, new evidence emerged that actin filaments might play a significant role in NP localization (Digard *et al.*, 1999). It is hoped that the recent development of techniques to generate fully recombinant influenza viruses from DNA (Fodor *et al.*, 1999; Neumann *et al.*, 1999; Hoffmann *et al.*, 2000) will help to answer many of the remaining questions about gene expression in influenza A and related viruses.

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