Chapter 9
Orthomyxovirus Genome Transcription and Replication

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Viruses from the Orthomyxoviridae family infect a wide range of vertebrate hosts. Five genera are currently recognised: *Influenza A, B* and *C*, *Thogotovirus* (of which the homonymic viruses are the type species) and *Isavirus* (type species infectious salmon anaemia virus). Other so far unclassified and uncharacterised members of the family also exist (Da Silva et al., 2005). These viruses are typified by possessing a single-stranded, negative-sense RNA genome that is split into segments (between 6 and 8 depending on the genus), by replicating their genome in the nucleus and by an unusual mechanism for producing capped mRNAs (discussed in detail later); shared traits that make it likely they are descended from a common ancestor. Influenza A virus is by far the most important in terms of significance to human health and accordingly is the best studied. The World Health Organization estimates that between 5 and 15% of the population in temperate countries are infected each year, resulting in up to 500,000 deaths worldwide. In addition, periodic introduction of antigenically novel viruses from avian reservoirs into the human population causes worldwide pandemics with substantially higher attack rates and mortality levels. The current possibility that a highly pathogenic avian H5N1 strain of influenza A will make this species jump is of great concern (Peiris et al., 2007). Ongoing research has reinforced longstanding concepts that the viral RNA polymerase is an important determinant of host range and pathogenicity (Almond, 1977; Subbarao et al., 1993; Hatta et al., 2001; Tumpey et al., 2005), further justifying research into this area. This chapter provides an overview of orthomyxovirus RNA synthesis, focussing in detail on areas where recent progress has changed the consensus view of the molecular mechanisms involved. For in-depth treatments of other aspects, the reader is referred to other reviews (Amorim and Digard, 2006; Elton et al., 2006; Engelhardt and Fodor, 2006; Ortin and Parra, 2006).

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Overview of Orthomyxovirus RNA Synthesis

Each orthomyxovirus segment contains conserved sequences at the 3′ and 5′ termini that share partial sequence complementarity (Skehel and Hay, 1978; Robertson, 1979; Desselberger et al., 1980; Staunton et al., 1989) and can base-pair to form a panhandle structure (Hsu et al., 1987; shown schematically in Fig. 9.1a). The genome functions as ribonucleoproteins (RNPs), where each vRNA segment is separately encapsidated by the nucleoprotein (NP) and associated with one copy of the viral RNA-dependent RNA polymerase (Fig. 9.2a). NP plays an essential role in maintaining the structure of the RNPs. The viral polymerase complex is a heterotrimer composed of two basic proteins, PB1 and PB2, and the more acidic PA (Horisberger, 1980; Detjen et al., 1987; Honda et al., 1990).

Unlike most negative-sense RNA viruses, transcription of orthomyxovirus genomes takes place in the nucleus of infected cells (Herz et al., 1981; Siebler et al., 1996). During the infectious cycle, two types of positive-sense RNA molecules are transcribed from vRNA (Fig. 9.1b). Synthesis of capped and polyadenylated messenger RNAs (mRNAs) is primed by short capped oligonucleotides of around 10–12 nucleotides, which are scavenged from host cell pre-mRNAs by the combined cap-binding and endonuclease activities of the PB2 and PB1 components of the polymerase complex, respectively (Plotch et al., 1981; Li et al., 1998). Influenza mRNAs therefore contain non-templated host cell-derived sequences at their 5′ ends (Fig. 9.1a). Transcription terminates 15–17 nt before the 5′ end of the vRNA segment and the mRNA is polyadenylated by a process of stuttering on a poly (U) tract (Fig. 9.1a; Hay et al., 1977a; Robertson et al., 1981; Luo et al., 1991; Poon et al., 1998). In contrast, synthesis of the positive-sense cRNA involves unprimed

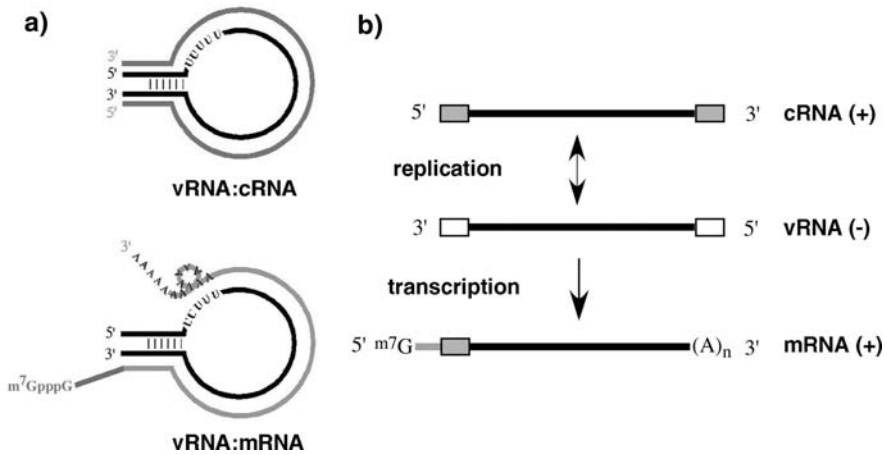

Fig. 9.1 Overview of influenza virus RNA synthesis. (**a**) Schematic depiction of the complementarity between m, c- and vRNA. *Black lines* indicate vRNA, *grey lines* positive-sense RNA as labelled. (**b**) Schematic depiction of the synthetic relationship between influenza virus RNA species. *Boxes* represent the conserved terminal promoter regions.

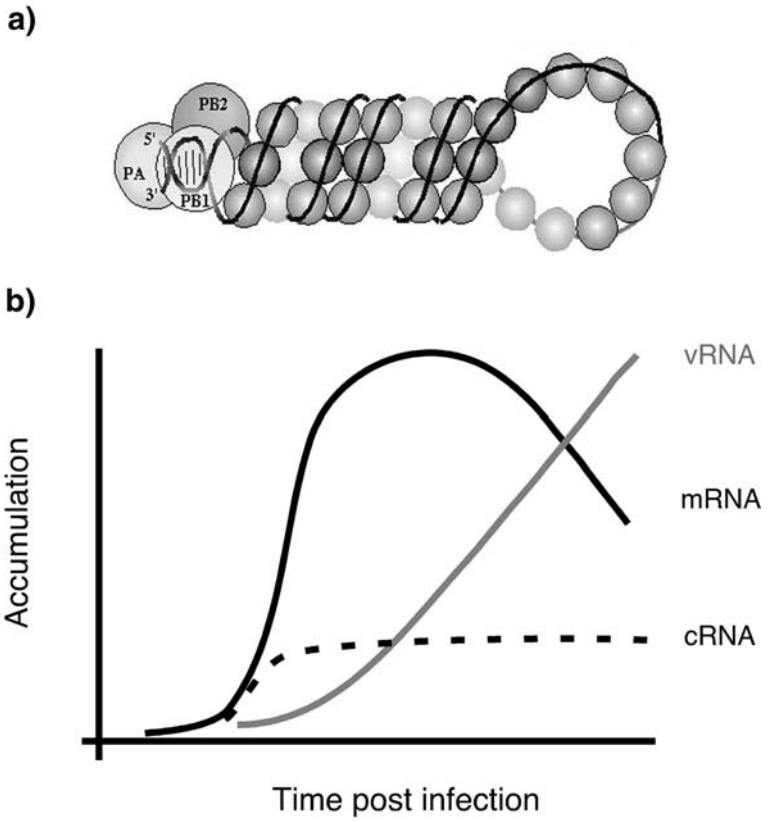

Fig. 9.2 (**a**) Cartoon depiction of an influenza virus RNP. *Small spheres* represent NP, *large spheres* the polymerase subunits and the *black line* genomic RNA. (**b**) Schematic depiction of the temporal regulation of viral RNA accumulation.

initiation (Hay et al., 1982) and read-through of the poly (U) tract to produce unit length copies of the vRNA template (Fig. 9.1a). These cRNAs exist as low abundance RNPs and are the replicative intermediates for synthesis of new copies of vRNA (Fig. 9.1b), required for production of progeny virions. Production of these three species of viral RNA is temporally regulated in infected cells (Fig. 9.2b). Synthesis of mRNA occurs first, catalysed by the input vRNPs and independent of viral protein synthesis (Hay et al., 1977b; Taylor et al., 1977; Barrett et al., 1979). Maximum rates of mRNA synthesis occur around 2.5 h post-infection so the peak amounts of viral mRNA occur relatively early in infection after which levels decline (Hay et al., 1977b; Barrett et al., 1979; Mark et al., 1979; Smith and Hay, 1982; Shapiro et al., 1987; Mullin et al., 2004). Normally, cRNAs can only be detected after mRNA synthesis, consistent with their dependence upon viral protein synthesis (Hay et al., 1977b; Barrett et al., 1979), but their maximal rate of synthesis occurs before that of viral mRNA (Barrett et al., 1979; Shapiro et al.,

1987). Synthesis of vRNA follows cRNA and continues to increase even after synthesis of the other classes of RNA declines (Hay et al., 1977b; Barrett et al., 1979; Shapiro et al., 1987; Mullin et al., 2004). Differential expression of the viral gene products is achieved both transcriptionally and post-transcriptionally. Expression of the polymerase genes remains relatively low throughout infection and this correlates with the low abundance of their mRNAs (Hay et al., 1977b; Smith and Hay, 1982; Enami et al., 1985). NP and NS1 expression predominates at early times post-infection while synthesis of the major virion structural proteins M1 and HA lags until later times. It has been proposed that this reflects the kinetics of individual mRNA synthesis and that this is in turn regulated by differential synthesis of the vRNA templates from which the mRNAs are transcribed (Smith and Hay, 1982; Shapiro et al., 1987; reviewed by Elton et al., 2006). However, there is also the possibility of post-transcriptional regulation occurring via delayed nuclear export of the 'late gene' mRNAs (Hatada et al., 1989; Amorim et al., 2007). A further layer of post-transcriptional regulation undoubtedly occurs for the NS1/NS2 and M1/M2 genes which are expressed through differential splicing of primary transcripts and here the reader is referred to a prior review (Ortin and Parra, 2006).

Structure and Assembly of Viral RNPs

Early electron microscopy studies of virion RNPs showed helical ribbon structures with a terminal loop (Pons et al., 1969; Jennings et al., 1983; shown in cartoon form in Fig. 9.2a). The polymerase is present at one end of the structure, as shown by immunogold labelling (Murti et al., 1988). The polymerase maintains the association of the 5′ and 3′ ends of the RNA (Klumpp et al., 1997) forming the closed structure in the RNP. Artificial complexes generated in vitro with NP and RNA are structurally and biochemically similar to natural RNPs (Yamanaka et al., 1990). Furthermore, the helical form of the RNP is maintained when the RNA is replaced by negatively charged polymers, suggesting that NP determines RNP organisation rather than the viral RNA (Pons et al., 1969). This is supported by electron microscopy of purified RNA-free NP extracted from RNPs, which showed structures morphologically indistinguishable from intact RNPs (Ruigrok and Baudin, 1995). Deletion mutagenesis of NP suggests that two separate regions within the protein are capable of association with the full-length protein, of which a C-terminal sequence is more important for NP–NP oligomerisation (Elton et al., 1999a).

RNPs are flexible entities with variable length, depending on the RNA segment they contain, and are poor subjects for detailed structural analyses. However, much smaller recombinant RNPs have been generated by in vivo amplification in cells expressing the viral polymerase, NP and a model vRNA (Ortega et al., 2000). These more uniform populations of RNPs are amenable to analysis by electron microscopy and image processing and this system has provided valuable low-resolution information on the structure and organisation of the RNP. Initial studies revealed the presence of circular, elliptic or coiled particles, depending on the length of the

genomic RNA included (Ortega et al., 2000). From the length of the viral RNA present in these recombinant RNPs and the number of NP monomers observed, it could be calculated that around 24–25 nt are bound per NP molecule. Further analysis of one such recombinant RNP containing nine NP monomers showed a circular structure containing one copy of the polymerase complex (Martin-Benito et al., 2001). Two of the NP monomers are associated with the polymerase complex through non-identical contacts, which might reflect the NP-PB1 and NP-PB2 interactions identified biochemically (Biswas et al., 1998; Medcalf et al., 1999). The NP monomers have a banana-like structure with one main NP–NP contact. Viral RNA is included in these recombinant mini-RNPs and its termini are presumably bound by the polymerase complex, but the resolution of the reconstruction is so far insufficient to permit its localisation (Martin-Benito et al., 2001). Recently, a high-resolution crystal structure has been reported for NP (Ye et al., 2006). The protein crystallised as a trimer in which monomer interactions were primarily mediated by a flexible C-terminal loop, consistent with mutational data examining NP oligomerisation (Elton et al., 1999a). Monomers contain head and body domains made up of non-contiguous sequences arranged into a crescent shape reminiscent of the low-resolution EM pictures. The groove between domains is lined with multiple basic residues and therefore seems a plausible RNA-binding site (Ye et al., 2006). However, individual mutation of many of these residues did not affect NP–RNA interactions (Elton et al., 1999b), perhaps suggesting a degree of redundancy or conformational flexibility in how NP binds RNA, analogous to that proposed for how the vesicular stomatitis virus nucleoprotein binds RNA (Green et al., 2006).

Although there is no detailed structural data available for protein–RNA interactions within the RNP, there are solved structures for the terminal promoter elements of v- and cRNA. Consistent with early sequencing studies noting partial complementarity between the conserved 5′ and 3′ ends of the viral genomic segments (Skehel and Hay, 1978; Robertson, 1979; Desselberger et al., 1980), NMR analysis of short synthetic RNAs shows partial duplexes interrupted by bulge regions (Bae et al., 2001; Lee et al., 2003a; Park et al., 2003). However, not all aspects of the structure of these naked RNA ‘panhandles’ are compatible with a large body of mutagenesis data probing the function of the promoter elements, suggesting that perhaps an alternative conformation is adopted when the polymerase binds to the genome termini. For an in-depth discussion of this topic the reader is referred to other recent reviews (Elton et al., 2006; Ortin and Parra, 2006).

The enzyme responsible for RNA synthesis in the RNP is the virus polymerase complex, a heterotrimer in which the PB1 subunit constitutes the core to which both PB2 and PA subunits are bound (Digard et al., 1989). Several laboratories have examined the regions of these subunits involved in complex formation (reviewed in Elton et al., 2006). These studies suggest an N-terminal to C-terminal tandem arrangement of the subunits in the order PA-PB1-PB2, but with a further degree of interlinking between PB1 and PB2. However, the first three-dimensional EM reconstruction models reported for the polymerase present in recombinant RNPs show a compact, roughly globular structure in which the location of individual subunits is not apparent (Martin-Benito et al., 2001; Area et al., 2004). The position

of specific domains of PB1, PB2 and PA proteins within the polymerase was determined by imaging of RNP-monoclonal antibody complexes or tagged RNPs (Area et al., 2004). Both the N-terminal region of PB2 and the C-terminus of PB1 are close to the areas of the polymerase that contact the adjacent NP monomers in the RNP, in agreement with the reports of in vitro interactions (Biswas et al., 1998; Medcalf et al., 1999). On the other hand, the C-terminal region of PA is opposite to the NP-polymerase contacts. The three-dimensional model reported for the polymerase corresponds to the enzyme present in a mature RNP, which can be activated for transcription in vitro and can be rescued into infectious virus in vivo. It represents the enzyme present in virion RNPs, poised for transcription but still not activated in the absence of a capped primer or nucleotides. Much biochemical evidence indicates that transcriptional activation of the polymerase involves allosteric cross-talk between the various RNAs and subunits (reviewed by Elton et al., 2006) and the latest EM imaging study from the Ortin laboratory provides elegant structural confirmation of this. Image processing analysis of non-RNP-associated polymerase complexes revealed a more open but still globular complex in which several regions (in particular density thought to be PB2) showed conformational changes when compared to RNP-associated polymerase (Torreira et al., 2007).

Limited amounts of other structural data are available for the influenza A virus polymerase complex. Partial proteolysis and functional mapping experiments suggest that the N-terminal 200 or so amino acids of PA form a discrete domain (Sanz-Ezquerro et al., 1996; Hara et al., 2006). Circular dichroism and structural prediction analysis suggests that the C-terminal 75 amino acids of PB1 (with PB2-binding function) form an α-helical domain (Poole et al., submitted for publication). In addition, the first high-resolution structural information for the polymerase has just been reported. NMR and crystal structures of the C-terminal 80 amino acids of PB2 show a compact α–β domain that contains a nuclear localisation signal (NLS) (Tarendeau et al., 2007).

It has long been known that the polymerase is a heterotrimer that exists even when not bound to an RNP (Braam et al., 1983; Detjen et al., 1987) and that PB1 forms the backbone of the complex (Digard et al., 1989). However, much recent work has concerned the mechanism by which the polymerase trimer is assembled, in large part prompted by further elucidation of how the P proteins are trafficked to the nucleus. NLSs have been identified in each of the individual P proteins (Nath and Nayak, 1990; Mukaigawa and Nayak, 1991; Nieto et al., 1994; Tarendeau et al., 2007). However, more recent analysis has confirmed an earlier suggestion that PB1 and PA are not efficiently imported into the nucleus unless in the form of a heterodimer (Nieto et al., 1992; Fodor and Smith, 2004). Further work identified the cellular Ran binding protein 5 (RanBP5, also known as importin ß3) as a binding partner of PB1 or PB1-PA but not the full trimer (Deng et al., 2006a; Mayer et al., 2007). SiRNA-mediated depletion of RanBP5 reduced nuclear import of PB1 and PA, suggesting that this interaction is indeed functionally important for nuclear trafficking of part of the polymerase complex (Deng et al., 2006a). PB2 undergoes efficient nuclear import in the absence of other influenza virus proteins and this is likely mediated by interactions with the

canonical cellular import machinery. A bipartite NLS has been identified in the C-terminus of the protein and the structure of this region co-crystallised in complex with importin α5 has been solved (Tarendeau et al., 2007). PB2 has also been shown to interact with cellular hsp90 and to relocalise it to the nucleus (Momose et al., 2002; Naito et al., 2007). As with the PB1-PA dimer and RanBP5, the interaction of PB2 and hsp90 is lost on formation of a full PB1-PB2-PA trimer (Naito et al., 2007). Overall, these data suggest that the trimeric polymerase complex may only form in the nucleus after separate import of individual or subcomplexes of the P proteins along with accessory cellular proteins that perform import and/or chaperone functions (Deng et al., 2006a; Naito et al., 2007). Consistent with this hypothesis, a functional polymerase complex could be assembled in vitro by the addition of separately expressed PB2 to a PB1-PA dimer (Deng et al., 2005). Whether the subcomplexes of P proteins have any functional significance prior to their assembly into a trimer remains controversial (reviewed by Elton et al., 2006).

Mechanism of Viral mRNA Synthesis

Initiation

The influenza virus polymerase complex is essentially inactive for any of its enzymatic functions in the absence of viral RNA. A popular model for the mechanism of mRNA synthesis involves sequential binding of the polymerase to the 5′ and 3′ termini of vRNA, with each interaction causing allosteric changes to the proteins that result in activation of the cap-binding, endonuclease and nucleotide polymerisation functions in a regulated manner. In this model, the polymerase complex binds to the 5′ end of a vRNA segment (Fodor et al., 1994; Tiley et al., 1994), primarily through PB1–RNA interactions (Li et al., 1998; Gonzalez and Ortin, 1999a). This induces a conformational change in the polymerase that activates the cap-binding activity of PB2 (Cianci et al., 1995; Li et al., 1998) and allows the polymerase to bind a cellular pre-mRNA. The 3′ end of the vRNA template then enters the complex through a combination of protein–RNA interactions (again, primarily through PB1; Li et al., 1998; Gonzalez and Ortin, 1999b) and base-pairing between the 5′ and 3′ sequences. This event stimulates endonuclease activity (Cianci et al., 1995; Hagen et al., 1995; Li et al., 1998) and the cellular 5′ cap structure, together with 9–15 nucleotides, is endonucleolytically cleaved by PB1 (Plotch et al., 1981; Li et al., 1998). The 3′ end of the truncated mRNA is then used to prime transcription initiation by PB1 (Braam et al., 1983; Biswas and Nayak, 1994; Asano et al., 1995), with addition of a guanosine residue directed by the second residue of the vRNA template (Plotch et al., 1981). The nascent viral mRNA chain is then elongated by sequential addition of ribonucleotides, as directed by the vRNA template. Characterisation of the polymerase's Km for ATP suggests a transition from an initiation mode to a processive transcription mode between positions 4 and 5 (Klumpp et al., 1998). PB2 releases the cap structure after the first 11–15 nucleotides have been added (Braam et al.,

1983) but the mechanism that triggers this is not known. However, there is evidence that prior to this, binding of the cap structure to PB2 increases the transcriptional activity of PB1 (Penn and Mahy, 1984; Kawakami et al., 1985). Insofar as is known, other orthomyxoviruses (as well as the bunya, arena and tenuiviruses) possess similar 'cap-snatching' mechanisms for transcription initiation. However, thogotovirus generates a shorter, more homogeneous cellular-derived primer containing only the cap structure and one additional nucleotide (Albo et al., 1996; Weber et al., 1996).

The key features of the sequential addition model for transcription initiation are that the polymerase assembles on the 5′ end of vRNA, cap-binding is activated, the polymerase then binds to the 3′ end of vRNA and this results in endonuclease activation. Although much support for the sequential model has been generated, recent studies suggest that this sequence of events is not obligatory. Using an assay where vRNA was added to recombinant polymerase either as a pre-annealed duplex of 5′ and 3′ vRNA or as sequential components, it was shown that the sequence of assembly had a marked effect on the stability of cap-binding but not endonuclease activity (Lee et al., 2003b). Polymerase bound to pre-annealed vRNA template showed high-level capped primer binding and endonuclease activity which resulted in enhanced levels of mRNA transcription activity, compared to that from polymerase bound initially to just 5′ vRNA. However, the low levels of capped-RNA substrate bound by polymerase associated with only the 5′ end of vRNA were cleaved efficiently, indicating that the 3′ end was not required for activation of the endonuclease, and that the original enhancement of endonuclease activity attributed to the presence of the 3′ end actually resulted from increased levels of cap-binding (Lee et al., 2003b). In addition, much of the early in vitro data on influenza virus transcription was gained using rabbit ß-globin mRNA or latterly, a synthetic capped RNA made by in vitro transcription. Although these mRNAs are good substrates for the endonuclease, they are primarily cleaved after a G residue (Plotch et al., 1981) and are not used efficiently as primers for transcription initiation (Rao et al., 2003). However, in vivo the polymerase complex shows a preference for cleavage of host mRNAs after an A, or in around 20% of the available cloned sequences (for influenza A virus), CA residues (reviewed in Elton et al., 2006). Furthermore, when a capped substrate with a CA sequence upstream of the cleavage site was used as a primer the 3′ end of vRNA was not required for full endonuclease activity (Rao et al., 2003). Overall therefore, while many of the details of the sequential model of influenza virus mRNA transcription are intact, some aspects are still not fully defined.

Polyadenylation

Processive synthesis of mRNA halts at a stretch of 5–7 uridine residues ~17 nt from the 5′ end of the vRNA template (Robertson et al., 1981), adjacent to the base-paired region of the panhandle structure (Hsu et al., 1987; Fig. 9.1a). Polymerase stuttering at this site to reiteratively copy the U(5–7) track produces a poly A tail ranging from 60 to 350 residues for mRNA isolated from virus-infected cells (Plotch and Krug,

1977), and up to 120–150 in vitro (Perales et al., 1996; Pritlove et al., 1998). Direct evidence for this being a non-processive, template-directed process rather than a poly A polymerase activity came from a study where the U track was mutated to A_6 leading to synthesis of positive-sense capped RNAs with poly (U) tails (Poon et al., 1999). Initially it was proposed that base-pairing of the vRNA panhandle was a physical block to polymerase processivity, resulting in stuttering on the adjacent U track (Robertson, 1979; Luo et al., 1991). The discovery that the polymerase binds tightly to the 5′ end of vRNA suggested the hypothesis that the polymerase itself prevented processive transcription through the poly (U) stretch by remaining bound to the 5′ end of its template (Fodor et al., 1994; Tiley et al., 1994). In this model, the continued association of the polymerase with the 5′ end of vRNA creates a loop of untranscribed template that becomes progressively shorter until the polymerase is arrested with its active site over the poly (U) stretch immediately adjacent to its 5′ binding site. This steric block forces the polymerase to stutter and polyadenylate the transcript. Experimental evidence supporting this hypothesis comes from the observation that nucleotides required for polymerase binding to the 5′ end of vRNA (Fodor et al., 1994; Tiley et al., 1994) are also essential for polyadenylation (Poon et al., 1998; Pritlove et al., 1998).

It is generally believed that there is a mechanism to couple the mode of initiation by the polymerase complex to that of termination. Hay et al. (1982) observed that most full-length transcripts of the vRNA templates were uncapped, while in vivo and in vitro studies have found that most polyadenylated viral RNAs have host sequences at their 5′ ends (Shaw and Lamb, 1984; Vreede and Brownlee, 2007). In support of a mechanism to couple initiation and termination, transcripts initiated in vitro with a capped primer are also polyadenylated, even in the presence of free NP (Beaton and Krug, 1986). The finding that binding of the polymerase to duplex-form genome termini promotes high levels of cap-primed transcription initiation suggests a mechanism for achieving this coupling, as it is reasonable to suppose that any interaction of the RNA termini in the absence of the polymerase is more likely to happen in *cis* than in *trans* (Lee et al., 2003b). However, this hypothesis is yet to be tested in vivo.

Interactions Between Viral and Cellular Transcription Machinery

One facet of influenza virus transcription where there has been significant recent progress concerns the cell biological aspects of the interaction between host and viral transcription machinery. The point in the cellular transcription cycle at which viral RNPs capture host cell cap structures has not been defined as in theory it could occur at any point before the cellular mRNA is exported to the cytoplasm. The pre-mRNA cap structure normally becomes associated with the cap-binding complex (CBC) soon after transcription initiation (Howe, 2002). The CBC remains bound to the cap up until nuclear export of the mature mRNA so it is plausible that the CBC and the influenza polymerase compete for the mRNA cap. Recent work has

shown that the influenza polymerase interacts with the host RNA Pol II (Engelhardt et al., 2005; Mayer et al., 2007) mediated through the C-terminal repeat domain (CTD) of RNA Pol II with a preference for the form phosphorylated on serine 5 (Engelhardt et al., 2005). Serine 5 phosphorylation occurs during initiation of the Pol II transcriptional cycle and is thought to activate the cellular cap synthesis complex (Howe, 2002). Potentially therefore, the influenza polymerase targets Pol II at the initiation stage to compete for newly synthesised cap structures for use as primers (Engelhardt et al., 2005).

A physical association between host and viral transcription machinery may also serve to direct nuclear export of viral mRNAs. The maturation of a cellular mRNA leading up to and including its export to the cytoplasm is thought to be coupled to RNA Pol II transcription through a suite of accessory proteins that are loaded onto the nascent transcript in a sequential fashion (Howe, 2002). Excluding the first dozen or so nucleotides captured from host mRNAs, influenza virus mRNAs are made by the viral polymerase but still need to access cellular machinery for nuclear export. The pathways and mechanisms are currently poorly defined but it has been observed that certain viral transcripts (notably those encoding HA and M1) are retained in the nucleus by drugs that affect the phosphorylation of the RNA Pol II CTD (Vogel et al., 1994; Amorim et al., 2007). The latter study used a variety of chemically and mechanistically distinct Pol II inhibitors to infer that the block to nuclear export of the viral mRNAs was reversible and depended on Pol II transcription (Amorim et al., 2007), a result consistent with recent work regarding the nuclear export of microinjected cellular mRNAs (Tokunaga et al., 2006). Two recent studies concluded that influenza infection somehow downregulates Pol II transcription (Chan et al., 2006; Rodriguez et al., 2007). This occurs late in infection when viral mRNA synthesis is diminishing and thus is consistent with a role for RNA Pol II in viral mRNA expression.

Mechanism of Genome Replication

Synthesis of cRNA

The process of genome replication is less well characterised in comparison to that of mRNA synthesis. Incoming vRNPs are the templates for synthesis of cRNAs which are then used to make more vRNA (Fig. 9.1b). cRNA constitutes only 5–10% of the total plus-sense viral RNA present in infected cells (Hay et al., 1977a; Barrett et al., 1979; Herz et al., 1981; Mullin et al., 2004). Viral mRNAs cannot serve as replicative intermediates because of the host-derived sequences at their 5′ ends and because they are truncated at their 3′ ends when polyadenylated (Fig. 9.1a). cRNAs are uncapped, 5′ triphosphorylated (Hay et al., 1982), full-length copies that are not polyadenylated. They cannot be generated through the endonucleolytic processing of a cap-primed intermediate, as this would leave a monophosphate terminus (Olsen et al., 1996). To generate a full-length copy, the polymerase must read through

the polyadenylation signal towards the 5′ end of the vRNA template. Unlike viral mRNA, cRNA is encapsidated by NP to form RNP structures, in much the same way as vRNA (Pons, 1971; Dalton et al., 2006). Thus, cRNA synthesis is mechanistically distinct from viral transcription.

Early studies indicated that RNPs from purified influenza virions are able to synthesise mRNA but not cRNA in vitro (Plotch and Krug, 1977; Skorko et al., 1991), indicating a requirement for other factors besides transcriptionally active RNPs. However, a recent study re-examining this question reached the opposite conclusion and found that virion RNPs were fully competent with no extra factors necessary (Vreede and Brownlee, 2007). Differing methodologies for the detection of cRNA may underlie the discrepancy.

Unquestionably, the same RNPs introduced into a cell by infection act as templates for both mRNA and cRNA synthesis. Nuclear extracts prepared from normal cells infected with influenza virus supported the synthesis of both types of positive-sense RNA (Beaton and Krug, 1984; del Rio et al., 1985; Beaton and Krug, 1986; Takeuchi et al., 1987; Shapiro and Krug, 1988). Early experiments showed that cRNA accumulation was dependent upon synthesis of viral and/or cellular proteins whereas mRNA synthesis was not (Hay et al., 1977b). Isolated RNP complexes recovered from the infected nuclear extracts by centrifugation lost the ability to make cRNA, but this could be restored by addition of the supernatant fraction unless it was immuno-depleted of NP (Beaton and Krug, 1986; Shapiro and Krug, 1988). This early data led to the concept of a ‘switch’ mechanism operating in infected cells to divert a minor fraction of polymerase activity from transcription to cRNA synthesis. Various hypotheses concerning how such a control mechanism might operate, mostly centred around NP, but also concerning the viral polymerase or putative cellular factors have been proposed. The reader is referred to other reviews where this work is considered in detail (Elton et al., 2006; Ortin and Parra, 2006). Instead, this chapter will focus on recent data suggesting alternative models for how influenza A virus replicates its genome.

The Stabilisation Model for cRNA Synthesis

NP is the prime candidate for a regulatory factor in the active ‘switching’ hypothesis, from the evidence described above and by analogy with non-segmented negative-sense viruses, where the intracellular concentration of the equivalent N protein is thought to regulate the balance between transcription and replication (Blumberg et al., 1981; Arnheiter et al., 1985). However, experimental manipulation of NP levels showed a slight negative rather than any positive correlation with levels of genome replication in cells (Mullin et al., 2004). In addition, NP is not necessary in vitro for the polymerase to initiate unprimed (replication mode) RNA synthesis (Lee et al., 2002; Deng et al., 2006b; Vreede and Brownlee, 2007) although it may function as a processivity factor (Beaton and Krug, 1986; Shapiro and Krug, 1988). A recent key study has shown that cRNA synthesis can occur in the absence of protein synthesis if a supply of pre-existing polymerase is available (Vreede et al.,

2004). This is dependent on the promoter-binding activity but not on the catalytic activity of the pre-expressed polymerase as a polymerisation defective mutant PB1 can fulfil this role whereas RNA-binding mutants cannot. Pre-existing NP was neither necessary nor sufficient to permit cRNA accumulation. However, its presence substantially increased the levels of cRNA accumulation. Thus a new model for the first step in genome replication proposes that cRNA synthesis is an intrinsic property of negative-sense RNPs but its accumulation is dependent on stabilisation resulting from polymerase binding (and enhanced by NP) rather than by an active switch mechanism (Vreede et al., 2004). Because cRNA molecules are not capped or polyadenylated they are quickly degraded by cellular nucleases unless there is a source of viral RNP polypeptides, particularly the polymerase components, present to encapsidate and stabilise them (Fig. 9.3). Paradoxically, a PB1-PA dimer possessing high levels of promoter-binding activity (Lee et al., 2002; Deng et al., 2005) does not suffice for this purpose (Vreede et al., 2004). This current model suggests that the first event in influenza A virus genome replication is not actively regulated, but is instead a stochastic process based on the probability of the viral polymerase initiating cap-primed or unprimed transcription. Nevertheless, evidence for regulated cRNA synthesis at some level remains. For instance, maximum rates of synthesis and levels of cRNA accumulation are reached early in infection and are not substantially amplified by the subsequent rise in vRNA levels (Barrett et al., 1979; Shapiro et al., 1987; Mullin et al., 2004; Dalton et al., 2006), despite the large pool of non-RNP-associated polymerase that remains in the nucleus until the end of the

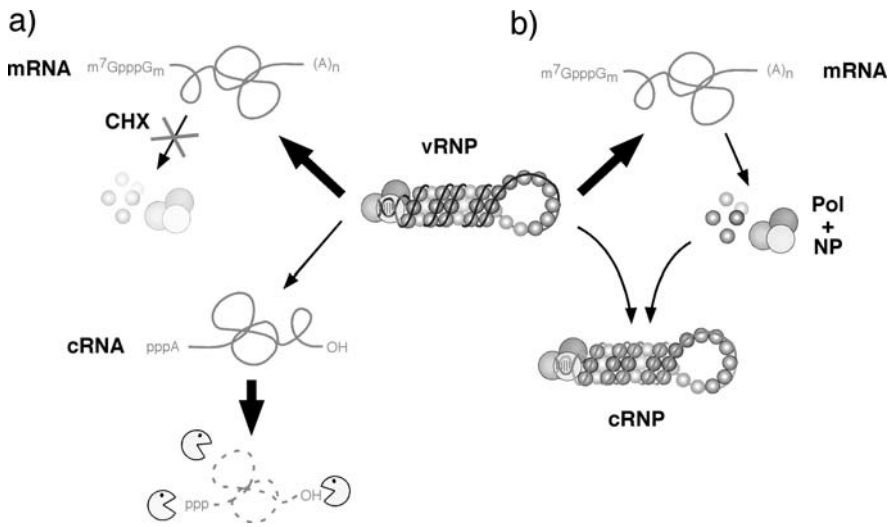

Fig. 9.3 Stabilisation model for influenza virus cRNA synthesis. (**a**) When protein synthesis is blocked by cycloheximide (CHX), RNPs synthesise mRNA and cRNA but the unencapsidated cRNA is quickly degraded. (**b**) In untreated cells, viral mRNAs are translated to produce new polymerase and NP which co-transcriptionally encapsidates nascent cRNA strands and protects them from degradation. After Vreede et al. (2004).

viral lifecycle (Detjen et al., 1987; Carrasco et al., 2004). This perhaps implies that only input (and not newly replicated) vRNA templates are used for cRNA synthesis, but this remains to be determined.

The Mechanism of vRNA Synthesis

The synthesis of vRNA from a cRNA template can be viewed as a simpler process than the transcription or replication of positive-sense RNA, since it is the only type of RNA transcribed from a cRNA template. Like cRNA, initiation of vRNA synthesis is unprimed and the products have 5′ triphosphorylated ends (Young and Content, 1971; Hay et al., 1982). Nevertheless, recent work suggests a significant difference in their modes of transcription initiation. Based on the results of experiments examining the precise origins of the first 2–3 nucleotides polymerised on WT and mutant cRNA and vRNA templates (Deng et al., 2006c) concluded that initiation with ATP occurs at the very 3′ end of the vRNA template and leads to synthesis of pppApG that is subsequently elongated to a full-length cRNA transcript (Fig. 9.4a). vRNA synthesis also initiates with ATP to produce a pppApG dinucleotide, but surprisingly, this is templated by positions 4 and 5 of the 3′ cRNA promoter. The pppApG dinucleotide is then postulated to translocate back to the very 3′ end of the template and there act as a primer for initiation of a nascent full-length vRNA molecule (Fig. 9.4b). Theoretically, the internally templated ApG could also be released by the polymerase to prime transcription in *trans* on other cRNA or vRNA templates (Deng et al., 2006c). Ironically, ApG dinucleotides have been used by influenza scientists as a tool to stimulate in vitro transcription by the viral polymerase for more than 30 years (McGeoch and Kitron, 1975).

Differential activation of the polymerase complex has been observed with the vRNA and cRNA promoters. This may be due to their binding to different sequences within the PB1 subunit, although there is some disagreement on the PB1 sequences involved (Li et al., 1998; Jung and Brownlee, 2006). Binding to the 5′ end of vRNA or cRNA stimulates cap-binding activity of the polymerase (Cianci et al., 1995), which may increase overall levels of transcription through allosteric upregulation of PB1 activity (Penn and Mahy, 1984; Kawakami et al., 1985). However, only binding to vRNA templates triggers cap-primed transcription activity of the polymerase. Until recently, this was thought to be due to a failure of cRNA templates to activate the endonuclease activity of the complex (Cianci et al., 1995; Honda et al., 2001), but as discussed earlier, this may only hold true for certain cap-donor RNAs. Addition of a CA cleavage site-containing cap donor to a reconstituted polymerase complex bound to 5′cRNA stimulated endonuclease activity to levels approaching those achieved with 5′vRNA. However, as these products are not subsequently extended (Rao et al., 2003), the synthesis of non-functional capped vRNA is prevented. Although the cRNA template does not contain a full polyadenylation signal (as it lacks the poly U tract), the polymerase still binds to the 5′ arm of cRNA (Tiley et al., 1994; Cianci et al., 1995; Gonzalez and Ortin, 1999b) and evidence indicates that the cRNA promoter can exist as a panhandle (Elton et al., 2006; Ortin and Parra,

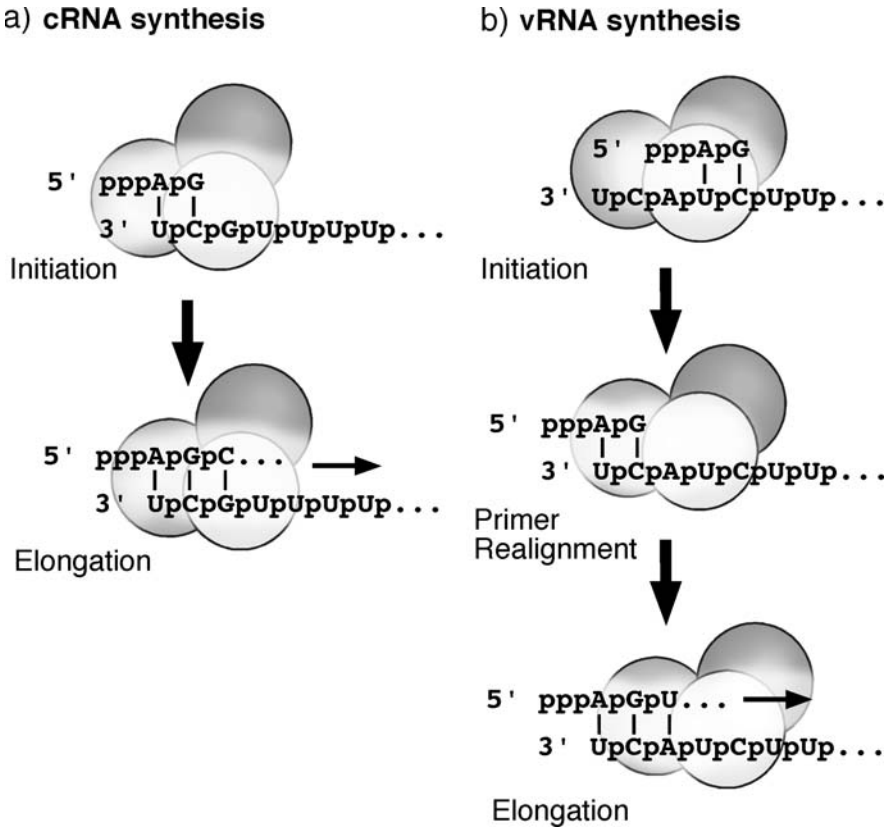

Fig. 9.4 Initiation modes for viral genome replication. (**a**) cRNA synthesis initiates by synthesis of an ApG dinucleotide templated by the 3′ end of vRNA that is then processively extended. (**b**) For vRNA synthesis, ApG is synthesised internally using residues 4 and 5 of the cRNA template, then translocated back to the 3′ end (primer realignment) to prime processive elongation. After Deng et al. (2006).

2006). This raises the question of how the steric block proposed for vRNA-directed polyadenylation is avoided in the case of cRNA to allow synthesis of a full-length vRNA transcript. Estimates of the dissociation constants for the interaction of PB1 with the 5′ ends of vRNA and cRNA are similar (Gonzalez and Ortin, 1999a, b). Nevertheless, the overall interaction of the polymerase with the cRNA promoter is more labile than with the vRNA promoter and significantly more temperature sensitive (Dalton et al., 2006).

The genetics of vRNA synthesis are similar to that of cRNA, with early experiments on *ts* mutants providing evidence that both PA and NP are important (Elton et al., 2006). However, the two polarities of genome replication are separable, since mutants have been isolated that can synthesise positive-sense RNA but appear to be specifically deficient for synthesis of vRNA (Thierry and Danos, 1982; Markushin

and Ghendon, 1984) or vice versa (Mena et al., 1999). In addition, mutations in the NS1 gene show a partial deficiency in the accumulation of vRNA, but not in that of cRNA, suggesting that NS1 acts as a cofactor in the second step of viral RNA replication (Falcon et al., 2004). This may be related to the observed association of NS1 with viral RNPs (Marion et al., 1997). Analysis of in vitro transcription reactions carried out with infected cell extracts has shown that, as with cRNA synthesis, a supply of non-RNP-associated NP is required to support vRNA synthesis (Shapiro and Krug, 1988). However, it is notable that prior expression of the polymerase and NP in cells before infection in the presence of a cycloheximide block does not support vRNA synthesis even though cRNA is made (Vreede et al., 2004).

Conclusions

Orthomyxoviral RNA synthesis has been a topic of continual research for over 40 years now and yet despite this, even after significant recent advances, much remains to be discovered. The molecular mechanisms of viral mRNA transcription are still incomplete, especially with regard to the cell biology of the process. New models have been formulated for how influenza virus replicates its genome but these require further testing. The longstanding lack of structural information on the viral RNA synthesis machinery is beginning to be rectified but there is still much work to be done. These remain worthwhile areas of study and may eventually lead to the design of novel antivirals targeted against an enzymatic complex that occupies the coding capacity of well over half of the influenza virus genome.

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References

Albo, C., Martin, J. and Portela, A. 1996. J Virol 70: 9013–9017.
Almond, J. W. 1977. Nature 270: 617–618.
Amorim, M. J. and Digard, P. 2006. Vaccine 24: 6651–6655.
Amorim, M. J., Read, E. K., Dalton, R. M., Medcalf, L. and Digard, P. 2007. Traffic 8: 1–11.
Area, E., Martin-Benito, J., Gastaminza, P., Torreira, E., Valpuesta, J. M., Carrascosa, J. L. and Ortin, J. 2004. Proc Natl Acad Sci U S A 101: 308–313.
Arnheiter, H., Davis, N. L., Wertz, G., Schubert, M. and Lazzarini, R. A. 1985. Cell 41: 259–267.
Asano, Y., Mizumoto, K., Maruyama, T. and Ishihama, A. 1995. J Biochem (Tokyo) 117: 677–682.
Bae, S. H., Cheong, H. K., Lee, J. H., Cheong, C., Kainosho, M. and Choi, B. S. 2001. Proc Natl Acad Sci U S A 98: 10602–10607.
Barrett, T., Wolstenholme, A. J. and Mahy, B. W. 1979. Virology 98: 211–225.
Beaton, A. R. and Krug, R. M. 1984. Proc Natl Acad Sci U S A 81: 4682–4686.
Beaton, A. R. and Krug, R. M. 1986. Proc Natl Acad Sci U S A 83: 6282–6286.
Biswas, S. K., Boutz, P. L. and Nayak, D. P. 1998. J Virol 72: 5493–5501.
Biswas, S. K. and Nayak, D. P. 1994. J Virol 68: 1819–1826.
Blumberg, B. M., Leppert, M. and Kolakofsky, D. 1981. Cell 23: 837–845.

Braam, J., Ulmanen, I. and Krug, R. M. 1983. Cell 34: 609–618.
Carrasco, M., Amorim, M. J. and Digard, P. 2004. Traffic 5: 979–992.
Chan, A. Y., Vreede, F. T., Smith, M., Engelhardt, O. G. and Fodor, E. 2006. Virology 351: 210–217.
Cianci, C., Tiley, L. and Krystal, M. 1995. J Virol 69: 3995–3999.
Da Silva, E. V., Da Rosa, A. P., Nunes, M. R., Diniz, J. A., Tesh, R. B., Cruz, A. C., Vieira, C. M. and Vasconcelos, P. F. 2005. Am J Trop Med Hyg 73: 1050–1058.
Dalton, R. M., Mullin, A. E., Amorim, M. J., Medcalf, E., Tiley, L. S. and Digard, P. 2006. Virol J 3: 58.
del Rio, L., Martinez, C., Domingo, E. and Ortin, J. 1985. Embo J 4: 243–247.
Deng, T., Engelhardt, O. G., Thomas, B., Akoulitchev, A. V., Brownlee, G. G. and Fodor, E. 2006a. J Virol 80: 11911–11919.
Deng, T., Sharps, J. L. and Brownlee, G. G. 2006b. J Gen Virol 87: 3373–3377.
Deng, T., Sharps, J., Fodor, E. and Brownlee, G. G. 2005. J Virol 79: 8669–8674.
Deng, T., Vreede, F. T. and Brownlee, G. G. 2006c. J Virol 80: 2337–2348.
Desselberger, U., Racaniello, V. R., Zazra, J. J. and Palese, P. 1980. Gene 8: 315–328.
Detjen, B. M., St Angelo, C., Katze, M. G. and Krug, R. M. 1987. J Virol 61: 16–22.
Digard, P., Blok, V. C. and Inglis, S. C. 1989. Virology 171: 162–169.
Elton, D., Digard, P., Tiley, L. and Ortin, J. (2006). Structure and function of the influenza virus RNP. *Influenza virology; current topics*. Wymondham, Caister Academic Press: 1–36.
Elton, D., Medcalf, E., Bishop, K. and Digard, P. 1999a. Virology 260: 190–200.
Elton, D., Medcalf, L., Bishop, K., Harrison, D. and Digard, P. 1999b. J Virol 73: 7357–7367.
Enami, M., Fukuda, R. and Ishihama, A. 1985. Virology 142: 68–77.
Engelhardt, O. G. and Fodor, E. 2006. Rev Med Virol 16: 329–345.
Engelhardt, O. G., Smith, M. and Fodor, E. 2005. J Virol 79: 5812–5818.
Falcon, A. M., Marion, R. M., Zurcher, T., Gomez, P., Portela, A., Nieto, A. and Ortin, J. 2004. J Virol 78: 3880–3888.
Fodor, E., Pritlove, D. C. and Brownlee, G. G. 1994. J Virol 68: 4092–4096.
Fodor, E. and Smith, M. 2004. J Virol 78: 9144–9153.
Gonzalez, S. and Ortin, J. 1999a. J Virol 73: 631–637.
Gonzalez, S. and Ortin, J. 1999b. Embo J 18: 3767–3775.
Green, T. J., Zhang, X., Wertz, G. W. and Luo, M. 2006. Science 313: 357–360.
Hagen, M., Tiley, L., Chung, T. D. and Krystal, M. 1995. J Gen Virol 76 (Pt 3): 603–611.
Hara, K., Schmidt, F. I., Crow, M. and Brownlee, G. G. 2006. J Virol 80: 7789–7798.
Hatada, E., Hasegawa, M., Mukaigawa, J., Shimizu, K. and Fukuda, R. 1989. J Biochem (Tokyo) 105: 537–546.
Hatta, M., Gao, P., Halfmann, P. and Kawaoka, Y. 2001. Science 293: 1840–1842.
Hay, A. J., Abraham, G., Skehel, J. J., Smith, J. C. and Fellner, P. 1977a. Nucleic Acids Res 4: 4197–4209.
Hay, A. J., Lomniczi, B., Bellamy, A. R. and Skehel, J. J. 1977b. Virology 83: 337–355.
Hay, A. J., Skehel, J. J. and McCauley, J. 1982. Virology 116: 517–522.
Herz, C., Stavnezer, E., Krug, R. and Gurney, T., Jr. 1981. Cell 26: 391–400.
Honda, A., Endo, A., Mizumoto, K. and Ishihama, A. 2001. J Biol Chem 276: 31179–31185.
Honda, A., Mukaigawa, J., Yokoiyama, A., Kato, A., Ueda, S., Nagata, K., Krystal, M., Nayak, D. P. and Ishihama, A. 1990. J Biochem (Tokyo) 107: 624–628.
Horisberger, M. A. 1980. Virology 107: 302–305.
Howe, K. J. 2002. Biochim Biophys Acta 1577: 308–324.
Hsu, M. T., Parvin, J. D., Gupta, S., Krystal, M. and Palese, P. 1987. Proc Natl Acad Sci U S A 84: 8140–8144.
Jennings, P. A., Finch, J. T., Winter, G. and Robertson, J. S. 1983. Cell 34: 619–627.
Jung, T. E. and Brownlee, G. G. 2006. J Gen Virol 87: 679–688.
Kawakami, K., Mizumoto, K., Ishihama, A., Shinozaki-Yamaguchi, K. and Miura, K. 1985. J Biochem (Tokyo) 97: 655–661.

Klumpp, K., Ford, M. J. and Ruigrok, R. W. 1998. J Gen Virol 79 (Pt 5): 1033–1045.
Klumpp, K., Ruigrok, R. W. and Baudin, F. 1997. Embo J 16: 1248–1257.
Lee, M. K., Bae, S. H., Park, C. J., Cheong, H. K., Cheong, C. and Choi, B. S. 2003a. Nucleic Acids Res 31: 1216–1223.
Lee, M. T., Bishop, K., Medcalf, L., Elton, D., Digard, P. and Tiley, L. 2002. Nucleic Acids Res 30: 429–438.
Lee, M. T., Klumpp, K., Digard, P. and Tiley, L. 2003b. Nucleic Acids Res 31: 1624–1632.
Li, M. L., Ramirez, B. C. and Krug, R. M. 1998. Embo J 17: 5844–5852.
Luo, G. X., Luytjes, W., Enami, M. and Palese, P. 1991. J Virol 65: 2861–2867.
Marion, R. M., Zurcher, T., de la Luna, S. and Ortin, J. 1997. J Gen Virol 78 (Pt 10): 2447–2451.
Mark, G. E., Taylor, J. M., Broni, B. and Krug, R. M. 1979. J Virol 29: 744–752.
Markushin, S. G. and Ghendon, Y. Z. 1984. J Gen Virol 65 (Pt 3): 559–575.
Martin-Benito, J., Area, E., Ortega, J., Llorca, O., Valpuesta, J. M., Carrascosa, J. L. and Ortin, J. 2001. EMBO Rep 2: 313–317.
Mayer, D., Molawi, K., Martinez-Sobrido, L., Ghanem, A., Thomas, S., Baginsky, S., Grossmann, J., Garcia-Sastre, A. and Schwemmle, M. 2007. J Proteome Res 6: 672–682.
McGeoch, D. and Kitron, N. 1975. J Virol 15: 686–695.
Medcalf, L., Poole, E., Elton, D. and Digard, P. 1999. J Virol 73: 7349–7356.
Mena, I., Jambrina, E., Albo, C., Perales, B., Ortin, J., Arrese, M., Vallejo, D. and Portela, A. 1999. J Virol 73: 1186–1194.
Momose, F., Naito, T., Yano, K., Sugimoto, S., Morikawa, Y. and Nagata, K. 2002. J Biol Chem 277: 45306–45314.
Mukaigawa, J. and Nayak, D. P. 1991. J Virol 65: 245–253.
Mullin, A. E., Dalton, R. M., Amorim, M. J., Elton, D. and Digard, P. 2004. J Gen Virol 85: 3689–3698.
Murti, K. G., Webster, R. G. and Jones, I. M. 1988. Virology 164: 562–566.
Naito, T., Momose, F., Kawaguchi, A. and Nagata, K. 2007. J Virol 81: 1339–1349.
Nath, S. T. and Nayak, D. P. 1990. Mol Cell Biol 10: 4139–4145.
Nieto, A., de la Luna, S., Barcena, J., Portela, A. and Ortin, J. 1994. J Gen Virol 75 (Pt 1): 29–36.
Nieto, A., de la Luna, S., Barcena, J., Portela, A., Valcarcel, J., Melero, J. A. and Ortin, J. 1992. Virus Res 24: 65–75.
Olsen, D. B., Benseler, F., Cole, J. L., Stahlhut, M. W., Dempski, R. E., Darke, P. L. and Kuo, L. C. 1996. J Biol Chem 271: 7435–7439.
Ortega, J., Martin-Benito, J., Zurcher, T., Valpuesta, J. M., Carrascosa, J. L. and Ortin, J. 2000. J Virol 74: 156–163.
Ortin, J. and Parra, F. 2006. Annu Rev Microbiol 60: 305–326.
Park, C. J., Bae, S. H., Lee, M. K., Varani, G. and Choi, B. S. 2003. Nucleic Acids Res 31: 2824–2832.
Peiris, J. S., de Jong, M. D. and Guan, Y. 2007. Clin Microbiol Rev 20: 243–267.
Penn, C. R. and Mahy, B. W. 1984. Virus Res 1: 1–13.
Perales, B., de la Luna, S., Palacios, I. and Ortin, J. 1996. J Virol 70: 1678–1686.
Plotch, S. J., Bouloy, M., Ulmanen, I. and Krug, R. M. 1981. Cell 23: 847–858.
Plotch, S. J. and Krug, R. M. 1977. J Virol 21: 24–34.
Pons, M. W. 1971. Virology 46: 149–160.
Pons, M. W., Schulze, I. T., Hirst, G. K. and Hauser, R. 1969. Virology 39: 250–259.
Poon, L. L., Pritlove, D. C., Fodor, E. and Brownlee, G. G. 1999. J Virol 73: 3473–3476.
Poon, L. L., Pritlove, D. C., Sharps, J. and Brownlee, G. G. 1998. J Virol 72: 8214–8219.
Pritlove, D. C., Poon, L. L., Fodor, E., Sharps, J. and Brownlee, G. G. 1998. J Virol 72: 1280–1286.
Rao, P., Yuan, W. and Krug, R. M. 2003. Embo J 22: 1188–1198.
Robertson, J. S. 1979. Nucleic Acids Res 6: 3745–3757.
Robertson, J. S., Schubert, M. and Lazzarini, R. A. 1981. J Virol 38: 157–163.
Rodriguez, A., Perez-Gonzalez, A. and Nieto, A. 2007. J Virol 81: 5315–5324.
Ruigrok, R. W. and Baudin, F. 1995. J Gen Virol 76 (Pt 4): 1009–1014.

Sanz-Ezquerro, J. J., Zurcher, T., de la Luna, S., Ortin, J. and Nieto, A. 1996. J Virol 70: 1905–1911.
Shapiro, G. I., Gurney, T., Jr. and Krug, R. M. 1987. J Virol 61: 764–773.
Shapiro, G. I. and Krug, R. M. 1988. J Virol 62: 2285–2290.
Shaw, M. W. and Lamb, R. A. 1984. Virus Res 1: 455–467.
Siebler, J., Haller, O. and Kochs, G. 1996. Arch Virol 141: 1587–1594.
Skehel, J. J. and Hay, A. J. 1978. Nucleic Acids Res 5: 1207–1219.
Skorko, R., Summers, D. F. and Galarza, J. M. 1991. Virology 180: 668–677.
Smith, G. L. and Hay, A. J. 1982. Virology 118: 96–108.
Staunton, D., Nuttall, P. A. and Bishop, D. H. 1989. J Gen Virol 70 (Pt 10): 2811–2817.
Subbarao, E. K., London, W. and Murphy, B. R. 1993. J Virol 67: 1761–1764.
Takeuchi, K., Nagata, K. and Ishihama, A. 1987. J Biochem (Tokyo) 101: 837–845.
Tarendeau, F., Boudet, J., Guilligay, D., Mas, P. J., Bougault, C. M., Boulo, S., Baudin, F., Ruigrok, R. W., Daigle, N., Ellenberg, J., Cusack, S., Simorre, J. P. and Hart, D. J. 2007. Nat Struct Mol Biol 14: 229–233.
Taylor, J. M., Illmensee, R., Litwin, S., Herring, L., Broni, B. and Krug, R. M. 1977. J Virol 21: 530–540.
Thierry, F. and Danos, O. 1982. Nucleic Acids Res 10: 2925–2938.
Tiley, L. S., Hagen, M., Matthews, J. T. and Krystal, M. 1994. J Virol 68: 5108–5116.
Tokunaga, K., Shibuya, T., Ishihama, Y., Tadakuma, H., Ide, M., Yoshida, M., Funatsu, T., Ohshima, Y. and Tani, T. 2006. Genes Cells 11: 305–317.
Torreira, E., Schoehn, G., Fernandez, Y., Jorba, N., Ruigrok, R. W., Cusack, S., Ortin, J. and Llorca, O. 2007. Nucleic Acids Res 35: 3774–3783.
Tumpey, T. M., Basler, C. F., Aguilar, P. V., Zeng, H., Solorzano, A., Swayne, D. E., Cox, N. J., Katz, J. M., Taubenberger, J. K., Palese, P. and Garcia-Sastre, A. 2005. Science 310: 77–80.
Vogel, U., Kunerl, M. and Scholtissek, C. 1994. Virology 198: 227–233.
Vreede, F. T. and Brownlee, G. G. 2007. J Virol 81: 2196–2204.
Vreede, F. T., Jung, T. E. and Brownlee, G. G. 2004. J Virol 78: 9568–9572.
Weber, F., Haller, O. and Kochs, G. 1996. J Virol 70: 8361–8367.
Yamanaka, K., Ishihama, A. and Nagata, K. 1990. J Biol Chem 265: 11151–11155.
Ye, Q., Krug, R. M. and Tao, Y. J. 2006. Nature 444: 1078–1082.
Young, R. J. and Content, J. 1971. Nat New Biol 230: 140–142.