The Role of Nucleoprotein in Transcription and Replication of the Influenza A Virus Genome

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Abstract

The eight vRNA genome segments of influenza A virus are bound by the viral RNA polymerase at the 5′ and 3′ ends, and associated with oligomeric nucleoprotein (NP) to form viral ribonucleoprotein (vRNP) complexes. These vRNP complexes carry out both viral transcription and replication within the nucleus of the host cell. The role of NP in viral transcription and replication, although essential, is not well understood. The findings presented in this thesis support a role for NP in the elongation phase of viral transcription and replication, but suggest that NP does not play a role in the regulation of initiation or termination of replication and transcription by the viral RNA polymerase. For NP to support elongation, it has to be recruited onto the viral RNA and oligomerise, to form a RNP complex. NP is likely to exist in a monomeric state, in the infected cell, prior to being recruited into the vRNP. The data presented here demonstrates that NP recruitment onto the viral RNA is mediated through NP-NP homo-oligomerisation and that oligomerisation of NP, within the infected cell, is regulated by reversible phosphorylation within the oligomerisation domain. In addition to this, compatibility between NP and the viral polymerase is essential for NP to support RNA polymerase activity. The N-terminus of NP is not required for this compatibility; however, evidence points towards loops within NP, responsible for RNA and polymerase binding, playing a role in the interplay between the RNA polymerase and NP within the RNP complex. Overall, this work provides new mechanistic insights into the assembly and regulation of viral RNP complexes.

Declaration

I declare that I carried out and completed the work presented in this thesis. I have acknowledged any contribution by others in the text of this thesis.

Lauren Turrell

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Abbreviations

293T	human embryonic kidney 293T
A	alanine
aa	amino acid
ANP	influenza A NP
APS	ammonium peroxodisulphate
BNP	influenza B NP
CAT	chloramphenicol acetyltransferase
CIP	calf intestinal phosphatase
cRNA	complementary RNA
D	aspartic acid
DMEM	dulbecco's modified eagle medium
E	glutamic acid
EM	electron microscopy
FCS	foetal calf serum
FRET	fluorescence resonance energy transfer
GFP	green fluorescence protein
HA	haemagglutinin
ISAV	infectious salmon anemia virus
L1	loop between alpha helices 2 and 3 in NP
L2	loop between alpha helices 8 and 9 in NP
LB	luria broth
M1	matrix protein 1
M2	matrix protein 2
MDBK	Madin-Darby bovine kidney
MEM	minimal essential medium
MOI	multiplicity of infection
mRNA	messenger RNA
N	N-terminus
NA	neuraminidase
NCR	non-coding region
NEP/NS2	nuclear export protein/ non-structural protein 2
NLS	nuclear localisation signal
NP	nucleoprotein
NPC	nuclear pore complex
NTP	nucleotide tri-phosphate
PA	polymerase acidic
PB1	polymerase basic 1
PB2	polymerase basic 2
PCR	polymerase chain reaction
PLB	protein loading buffer
PNK	T4 polynucleotide kinase
RanBP5	ran binding protein 5
S	serine
S.O.C	super optimal broth with catabolite repression
Sf	*spodoptera frugiperda*
SPR	surface plasmon resonance
TEV	tobacco etch virus
Vero	African green monkey kidney
vRNA	viral RNA
vRNP	viral ribonucleoprotein
wt	wildtype
YT	yeast extract tryptone

1 Introduction

Influenza virus causes yearly epidemics and in the 20th century influenza virus infection resulted in four worldwide pandemics. These four pandemics caused millions of deaths worldwide and led to both social and economic loss. A greater understanding into the mechanism of influenza virus transcription and replication and the interplay of these processes with the host cell is required to develop new ways in which to target the virus and reduce the impact it causes both in yearly epidemics and pandemics.

The presence and severity of influenza A virus disease during infection depends on the virus host and virus type. In humans virus infection occurs in the respiratory tract and replication of the virus within the respiratory tree peaks 48 hours after infection (Richman et al., 1976). During viral replication initially a cascade of innate immune reactions are activated. Cytokines are produced by epithelial and immune cells within the respiratory mucosa (Brydon et al., 2005) and neutrophil granulocytes are recruited (Mandelboim et al., 2001) leading to the onset of clinical symptoms. Influenza viruses encode virulence factors to evade this immune response including NS1, which sequesters dsRNA preventing it from triggering IFN α/β release in the innate immune response (Falcón et al., 2005;

García-Sastre et al., 1998). In addition to triggering the innate immune response, viral infection of host cells leads to a down regulation of host protein synthesis (Katze et al., 1986; Sanz-Ezquerro et al., 1995) and cell apoptosis (Ludwig et al., 2006; Wiley et al., 2001). The damage to the respiratory tract due to the activation of the immune response and epithelial cell death leads to a higher susceptibility to secondary infections including pneumonia causing morbidity and mortality, especially in elderly people (Rynda-Apple et al., 2015).

The three types of influenza virus, A, B and C, are all members of the *Orthomyxoviridae* family, characterised by their negative-sense segmented RNA genomes. The segmented nature of the viruses genomes enables genetic reassortment between influenza viruses; however, reassortment can only occur between influenza viruses of the same type. The influenza A virus is classified by serological subtypes with currently 17 haemagglutinin (HA) subtypes (H1-H17) and 9 neuraminidase (NA) subtypes (N1-N7) (Tong et al., 2012). Due to this large range of serological subtypes influenza A virus can infect a variety of mammals including pigs, horses, seals, whales, cats and bats. All influenza A virus serological subtypes circulate in aquatic birds whereas only some of the subtypes have been identified in humans. In contrast, influenza B and C virus do not have serological subtypes with the viruses primarily infecting humans, with the exception of influenza B infection in seals (Osterhaus et al., 2000). The different HAs, encoded by different virus types and subtypes, bind to sialic acid receptors on the cell surface and the type of sialic acid linkage expressed on the host cell surface determines viral tropism. In migratory waterfowl only α2,3-linked sialic acids are present on cells in the gastrointestinal tract whereas in humans α2,6-linked sialic acids are present on cells within the upper respiratory tract with α2,3-

linked sialic acids present on cells in the lower respiratory tract (Shinya et al., 2006). In swine both types of sialic acid linkages are found on the epithelial cells of the pig trachea (Trebbien et al., 2011).

The pathogenicity and infectivity of these different viruses strongly determines the effect of the virus on the population. H5N1 virus has a high death to infectivity ratio of 50% and this high virulence has been shown to be associated in part with the RNA polymerase in the viral ribonucleoprotein (vRNP) complex (Hatta et al., 2001; Hulse-Post et al., 2007; Salomon et al., 2006). The role of the RNA polymerase in virulence is demonstrated experimentally, as when the polymerase protein PB2 has a lysine in position 627 the H5N1 virus is virulent, whereas if there is a glycine at position 627 the H5N1 virus is non-virulent in mice (Hatta et al., 2001). It is therefore important to gain a greater understanding of the molecular mechanisms of transcription and replication carried out by the vRNP complex in order to begin to understand why different levels of pathogenicity are associated with particular mutations within the viral polymerase.

In the influenza virion the viral gene segments are packaged into vRNP complexes containing the viral RNA polymerase that carries out both transcription and replication. The genome of influenza virus consists of eight viral RNA (vRNA) gene segments used as templates for both viral replication and transcription, leading to the generation of progeny RNA segments and the production of up to 14 viral proteins (Chen et al., 2001; Jagger et al., 2012; Wise et al., 2009; Wise et al., 2012). This chapter will introduce the function of vRNP complexes within the virus life-cycle and in particular consider the role of nucleoprotein (NP), a major component of the vRNP complex, in viral transcription and replication.

1.1 Influenza virus life-cycle

The virus life-cycle is depicted in figure 1.1. During infection, the virus binds to a sialic acid receptor on the host cell surface via a viral cell surface glycoprotein, HA. The binding of the virus to the receptor triggers the internalisation of the virus through endocytosis. The low pH in the late endosome activates the matrix protein 2 (M2) ion channel and causes HA to undergo a conformational change. This conformational change in HA allows fusion of the viral membrane with the endosome membrane, leading to the formation of a pore between the interior of the virion and the cytoplasm (Stegmann, 2000). For the vRNP complexes to leave the virion through this pore, interactions between viral matrix protein 1 (M1) and vRNP complexes within the virion have to be weakened. This occurs via the influx of H^+ into the virus, through the activated M2 ion channel (Pinto et al., 1992). The vRNPs, once released into the cytoplasm, are imported into the nucleus to continue the viral life-cycle. Nuclear localisation signals (NLSs) are present on all RNP complex proteins; however, the NLSs on NP are thought to play a major role in the import of vRNPs into the nucleus (Cros et al., 2005; O'Neill et al., 1995; Wu et al., 2007). Importin α binds to NP, in the RNP complex, which is in turn bound by importin β allowing the RNP complexes to dock at the nuclear pore complex (NPC) and translocate to the nucleus. Once the vRNP complexes are in the nucleus, and dissociated from the NPC, they are responsible for carrying out both viral transcription (vRNA→mRNA) and replication (vRNA→cRNA→vRNA). These two processes will be discussed in detail later in this chapter. During transcription the viral messenger RNAs (mRNAs) are exported from the nucleus into the cytoplasm where they are

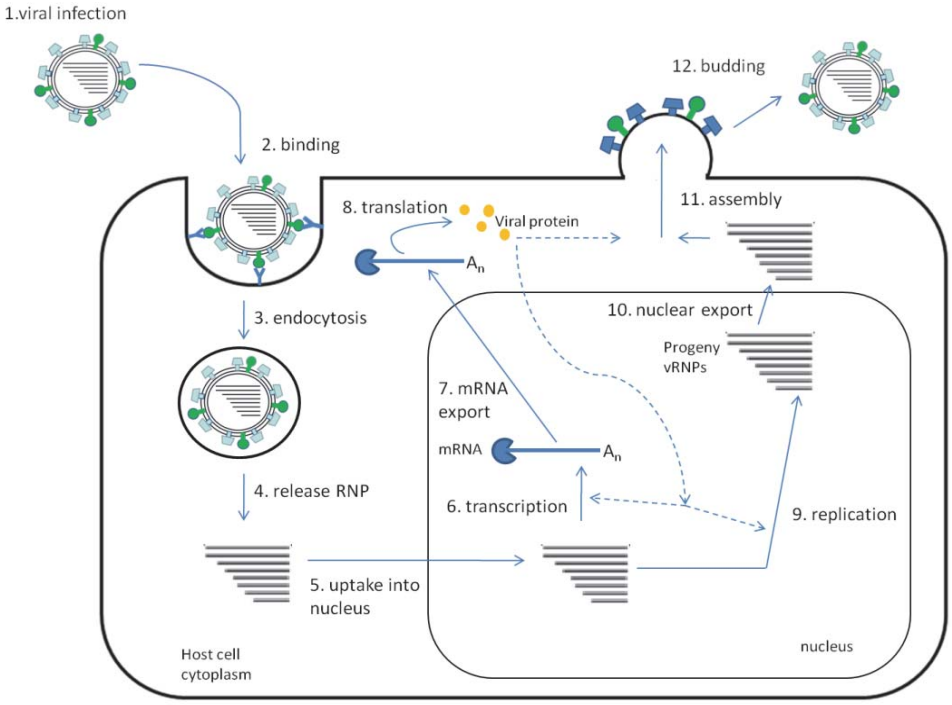


Figure 1.1. Schematic of the influenza virus life-cycle. The eight staggered lines represent the segmented single-stranded viral RNA genome. These RNA segments are encapsidated by the viral polymerase and nucleoprotein forming a vRNP complex.

translated, using the host translational machinery, to synthesise viral proteins. During replication, vRNA is replicated via a complementary RNA (cRNA) intermediate into progeny vRNA. Newly translated viral RNP complex proteins are imported into the nucleus and package the cRNA and vRNA into cRNP and vRNP complexes, respectively. This packaging protects the RNA from degradation and allows cRNA to be replicated to generate progeny vRNA (Vreede et al., 2004). The assembled progeny vRNPs can then carry out secondary transcription and replication. In addition, the progeny vRNPs are exported from the nucleus in a process involving M1 and nuclear export protein/ non-structural protein 2 (NEP/NS2) (Neumann et al., 2000; Paterson and Fodor, 2012). The viral surface glycoproteins are processed via the endoplasmic reticulum and Golgi apparatus and directed to the cell membrane, along with the progeny vRNPs, where viruses assemble. Newly assembled influenza viruses then bud from the apical membrane of polarised cells (Schmitt and Lamb, 2005).

1.2 The ribonucleoprotein complex

During the virus life-cycle, the eight vRNA gene segments never exist as naked RNA; they are always found associated with the heterotrimeric viral RNA dependent RNA polymerase (RNA polymerase), comprising polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acidic (PA), and NP, in a vRNP complex (Murti et al., 1988; Palese and Shaw, 2007). The RNA polymerase, when in the context of a vRNP, carries out both viral transcription and replication within the nucleus of the host cell. The presence of the RNA polymerase within the

packaged vRNP complexes allows transcription and the generation of cRNA in the first stage of replication to occur without prior expression of viral proteins.

vRNP complexes isolated from virions have been visualised by negative-staining electron microscopy (EM), revealing a helical structure with NP monomers stacked in the helical section and the RNA loop most distal from the promoter containing non-stacked NP (Arranz et al., 2012; Compans and Chopin, 1975; Heggeness et al., 1982; Moeller et al., 2012; Noda and Kawaoka, 2010; Pons, 1971; Portela and Digard, 2002). The helical structure has been shown to be solely dependent on NP-NP interactions as in the absence of RNA, helical RNP-like complexes formed *in vitro* (Ruigrok and Baudin, 1995; Yamanaka et al., 1990). In the RNP complex the RNA polymerase is bound to the 5′ and 3′ RNA promoter sequences at the opposite end of the non-stacked NP loop, and this binding plays a role in maintaining the helical structure in the presence of viral RNA (Klumpp et al., 1997).

The three-dimensional structure of a mini-(248-nucleotide) RNP complex and a full-length RNP complex have been solved using cryo-EM (Arranz et al., 2012; Coloma et al., 2009; Martín-Benito et al., 2001; Moeller et al., 2012). The structure of the mini-RNP, at a resolution of approximately 12Å, showed a ring of 9 NP molecules (figure 1.2b) (Coloma et al., 2009). This is in agreement with NP binding approximately 24 nucleotides of RNA (Compans and Chopin, 1975; Hutchinson et al., 2014; Martín-Benito et al., 2001; Ortega et al., 2000). The structure showed the first and last NP molecules in the ring contacting the viral RNA polymerase. The structures of full-length RNP complexes showed a double-helical region flanked at opposite ends by the viral polymerase and a NP loop structure (Arranz et al., 2012; Moeller et al., 2012) (figure 1.2c-f). The central

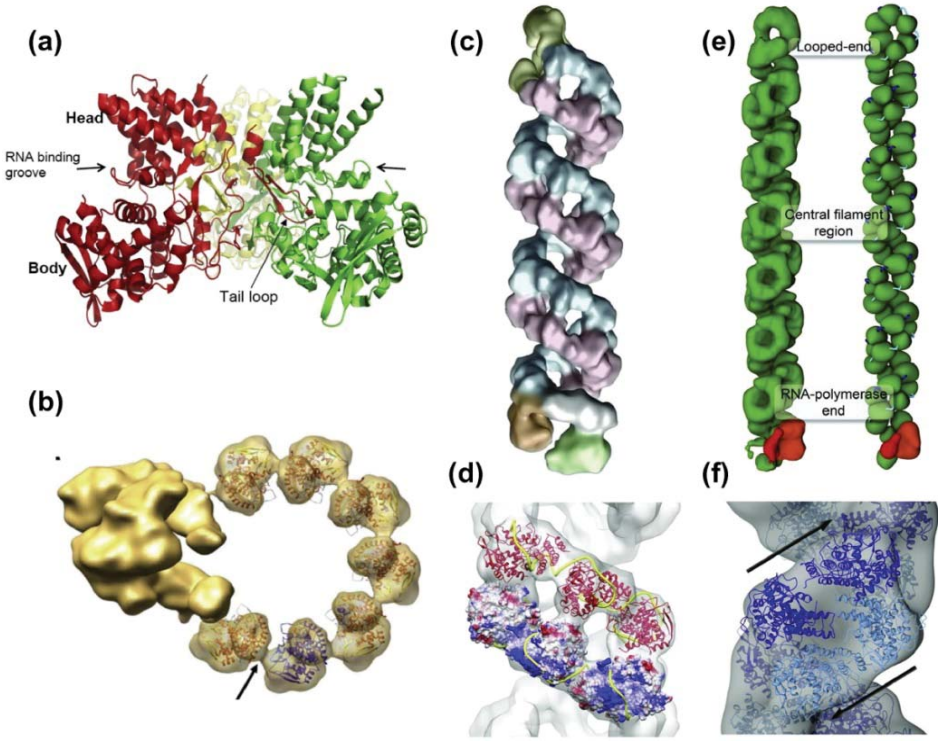


Figure 1.2. RNP complex. (A) Trimeric structure of NP with each NP monomer in a different colour. The structure shows the head and body domain, with the RNA binding groove between the two domains. This view shows the tail-loop of one NP monomer (red) interacting with the insertion-groove (green) of the neighbouring monomer (Ye et al., 2006). (B) Cryo-EM structure of the mini-RNP with the NP structure from A fitted into the density. The arrow indicates electron density, that, presumably, contains vRNA and the tail-loop insertion-groove interaction (Martin-Benito et al., 2001, Coloma et al., 2009). Cryo-EM structure of full-length RNP complexes from (C) Arranz et al., 2012 and (E) Moeller et al., 2012. (D) Double helical region from (C) and (E) double helical region from (F) with the structure of NP fitted into the density. Arrows indicate the RNA polarity. Figure adapted from Zheng et al., 2013.

double helical structure forms a major and minor groove and is stabilised by two major contacts between NP. One contact occurs between neighbouring NP monomers on the same RNA strand (intra-stand interaction) and the second between two NP monomers on opposite strands of the RNA (inter-stand interaction). The helical parameters in the double-helical region were very similar in both structures, with approximately five or six NP monomers per turn, both predicting that each NP binds approximately 24 nucleotides of RNA, in agreement with other studies (Compans and Chopin, 1975; Hutchinson et al., 2014; Martín-Benito et al., 2001; Ortega et al., 2000). However, loops of RNA within the RNP complex have been shown to be accessible for protein binding (York and Fodor, 2013) and have been proposed to play a role in RNP complex virion packaging (Fournier et al., 2012), suggesting that not all RNA is bound by NP. NP from infectious salmon anemia virus (ISAV) was shown to bind 12 nucleotides of RNA (Zheng et al., 2013). It is unclear how RNA secondary structures form when in the context of a RNP complex.

The overall architectures of the two RNP complex structures were very similar; however, the RNP helix in one study is left-handed (Arranz et al., 2012) whereas in the other study it is right-handed (Moeller et al., 2012). It is unclear why the two structures have different helix handedness: it could be due to the modest resolution, data processing or the source of the RNP complex (Zheng et al., 2013). In Arranz et al., the RNP complexes were isolated from virions, whereas in Moeller et al., the RNP complexes were reconstituted via plasmid driven expression of the RNP complex components. The opposite handedness of the two RNP complex structures led to differences in the docking of the NP monomer, derived from the trimer structure (figure 1.2a), into the electron density (figure

1.2d,f). In Arranz et al., the NP structure is positioned so that the positively-charged RNA binding surface of NP is accessible on the surface of the RNP complex, which is in agreement with findings that vRNA within the RNP complex is sensitive to RNase treatment (Duesberg, 1969; Ruigrok et al., 2010). This docking suggests that the N-terminus of NP is involved in the inter-stand interaction of NP monomers; however, the N-terminus contains a NLS and this positioning would prevent this NLS functioning in the import of the RNP complex (Wang et al., 1997). In Moeller et al., the NP docking positions the RNA binding surface (Ng et al., 2008; Ye et al., 2006) and the NP-RNA polymerase binding residues (Marklund et al., 2012) at the interface of the NP-NP inter-strand interaction. This buried position would require local disassembly of the vRNP complex, in order for the RNA polymerase to access the RNA for transcription and replication. In addition, this docking positions the bi-partite NLS (Weber et al., 1998) in a buried position; however, the N-terminus NLS is positioned on the surface of the RNP complex (Wang et al., 1997). Neither of the docking positions seem to fit with previous data suggesting that both NLSs are required for import of vRNP complexes into the nucleus (Wu et al., 2007).

The loop of NPs at the distal end of the RNP complex is composed of 3 NP molecules in the Arranz et al., structure and 5 to 8 NP molecules in the Moeller et al., structure. The structures of these loops show NP interacting with its neighbouring NP monomer, even in this tight turn, demonstrating the flexibility of the NP oligomerisation domain. The viral RNA polymerase structure is very different in both of the structures and these differences may be due to the different sources of vRNP complexes as discussed above. In Arranz et al., the RNA polymerase was found in two different conformations. In Moeller et al., one

polymerase conformation was observed, attributing some of the density to an NP that is not part of the helical region of the RNP complex. None of these structures of the RNA polymerase fully corresponded to the conformation of the polymerase observed in the mini-RNP complex at higher resolution (Coloma et al., 2009). The viral polymerase structure will be discussed below.

The isolation of cRNP complexes and visualisation by negative-staining EM revealed that the overall architecture of the cRNP complex was indistinguishable from a vRNP complex at this moderate resolution (York et al., 2013). The cRNP complex contained a central double helical NP region, with the NP loop and RNA polymerase at opposite ends of the NP double helix.

1.2.1 RNA dependent RNA polymerase

The three polymerase subunits, PB1, PB2 and PA, bind to the partially complementary 5′ and 3′ ends of the vRNA and make up the functional heterotrimeric RNA dependent RNA polymerase complex (Braam et al., 1983). Very recently the first structures of the trimeric polymerase complex have been published (Pflug et al., 2014; Reich et al., 2014). Previous to this, structures of domains of the RNA polymerase have been published which include the PA endonuclease N-terminal domain (Dias et al., 2009; Yuan et al., 2009), the PA C-terminal domain (He et al., 2008; Obayashi et al., 2008), the cap-binding domain of PB2 (Guilligay et al., 2008), the PB2 domain involved in host adaptation (Tarendeau et al., 2008), the PB2 C-terminal NLS domain (Tarendeau et al., 2007), the N-terminal fragment of PB1 interacting with PA (He et al., 2008; Obayashi et al., 2008) and the C-terminal fragment of PB1 interacting with PB2 (Sugiyama et al., 2009). The complete RNA polymerase structure provides insight

into how these domains interact and carry out their functions (Pflug et al., 2014; Reich et al., 2014). The structures show that the three viral proteins form a U-shaped complex in the pre-initiation state, with an intricate intertwining of subunits. One structure of the influenza A polymerase (fluA) and two structures of the influenza B polymerase (fluB1 and fluB2) were solved. The inter-subunit interactions and mode of RNA binding was conserved between the structures, but the orientation of the PB2 cap-binding domain was very different (figure 1.3). The publication of these structures revealed new information about each of the polymerase subunits, as discussed below.

The PA subunit can be divided into two domains; the endonuclease domain, which makes up one of the arms of the U shaped complex, and the C-terminal domain, forming the base of the U, interacting with PB1. These two domains are on opposite sides of the molecule and are connected by a PA linker that passes over PB1. The structure of the PA endonuclease and the PA C-terminus within the complex are very similar to the structures obtained of the individual domains (Dias et al., 2009; He et al., 2008; Obayashi et al., 2008; Yuan et al., 2009).

The PB2 N-terminus is on the opposite side of the PA linker and this region contacts both PA and PB1. The C-terminus of PB2 contains the cap-binding and host-species determination domains. The PA endonuclease and the PB2 cap-binding domains, making up the two arms in the U-shape, face each other in the fluA structure. In the fluB1 structure, the PB2 cap-binding domain is rotated by 70° when compared to the domain position in the fluA structure. The functional implications for this will be discussed below.

PB1 is the catalytic domain of the RNA polymerase and forms the central scaffold in the protein complex, with a right-handed RNA dependent RNA polymerase

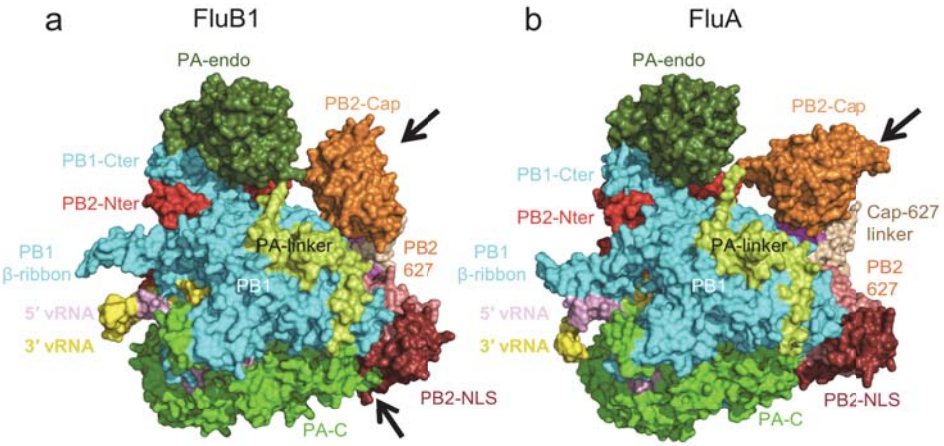


Figure 1.3. Structure of the influenza viral RNA polymerase. Structure of RNA polymerase from influenza B (FluB1) (A) and influenza A (FluA) (B) with the different polymerase domains highlighted. The top arrows highlight the rotation of the cap domains between the two structures and the bottom arrow in (A) shows extra electron density that is not observed in the fluA structure. Adapted from Reich et al. 2014.

fold (Biswas and Nayak, 1994; Kobayashi et al., 1996; Poch et al., 1989). The priming loop to initiate *de novo* RNA synthesis during viral replication is present in close proximity to the active site (Pflug et al., 2014; Reich et al., 2014). The structure also shows a positively charged narrow channel leading to the priming loop, proposed to attract and channel nucleotide tri-phosphates (NTPs) to the active site. The priming loop was not observed in the fluB structures due to it being disordered. Superimposition of the primer-template complex of poliovirus polymerase on PB1 suggested that the priming loop would have to be displaced once elongation starts, as it would clash with the emerging template-product duplex (Reich et al., 2014). The PB1 structure also reveals a long-hinged ribbon (amino acid (aa) 177-214) that is solvent exposed and this region of PB1 contains the NLS that binds to Ran binding protein 5 (RanBP5) (Deng et al., 2006a; Hutchinson et al., 2011). The ribbon plays a role in interacting with the RNA in all three structures. In the fluA structure it is bent towards the polymerase and contacts the duplex region of the promoter, in fluB1 it points away from the polymerase and is solvent exposed with the tip interacting with the crystal symmetry related RNA and in fluB2 the ribbon is bent interacting with the proximal 3′ end of the RNA. When the polymerase structure is fitted into the density attributed to the viral polymerase in the mini-RNP complex (Coloma et al., 2009), PB1-PA fits well but PB2 would have to undergo a large conformational change (figure 1.4a,b). If the fit of PB1-PA and the docking of NP are correct, then the PB1 ribbon may play a role in the NP-RNA polymerase interaction (aa139-157). In addition residues 1-24 in PB2, 679-751 in PB1 and 1-23 in PA may interact with NP (aa 92-112 and/or aa 253-378) (figure 1.4c).

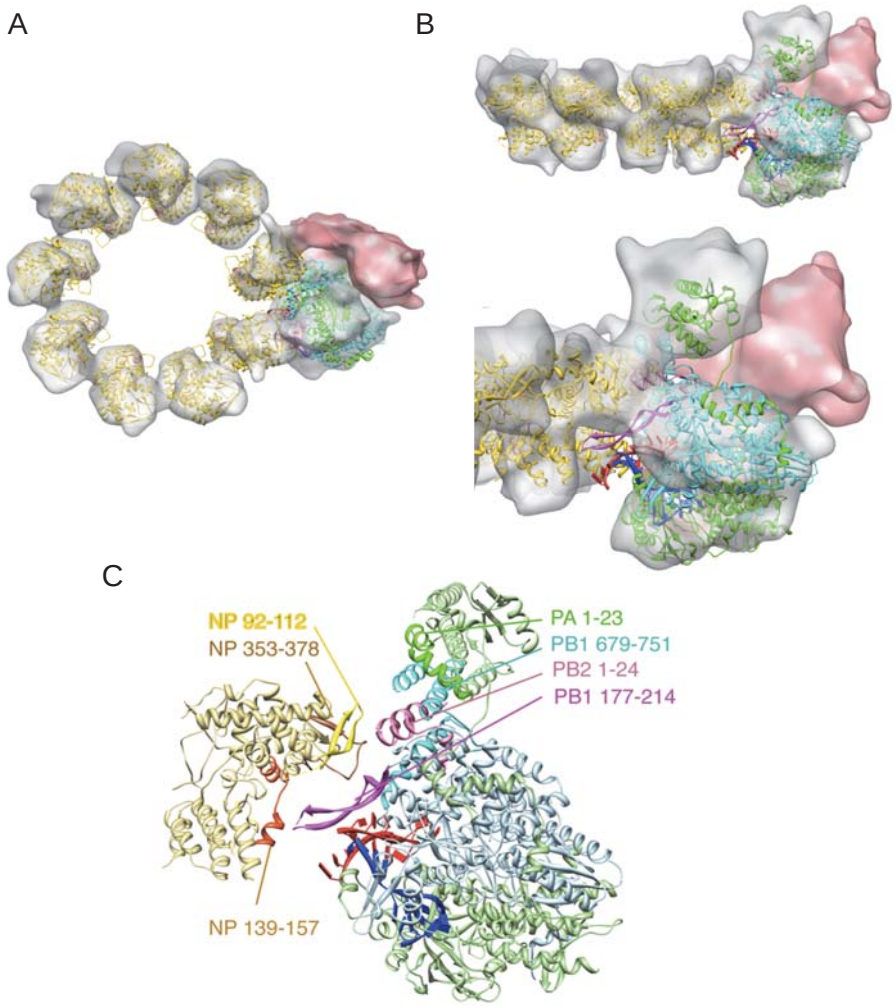


Figure 1.4. Proposal of NP polymerase interactions through docking. Mini-RNP complex from the top (A) and from the side (B) with the structure of the viral polymerase docked into the electron density thought to be the viral polymerase. PA (green), PB1 (cyan) and PB2 aa1-32 (rose). From this docking residues within NP and the viral polymerase that are involved in the NP-polymerase interaction can be proposed (C). The vRNA 5′ and 3′ are shown in dark red and dark blue, respectively. Figure adapted from Reich et al., 2014.

1.2.2 Viral RNA

Each of the influenza virus gene segments contains non-coding regions (NCRs) at the extreme 5′ and 3′ ends of the vRNA flanking the coding region (Baudin et al., 1994; Hay et al., 1982). The NCR is specific to each viral gene segment, yet they all contain conserved nucleotide sequences that come together to form the promoter, bound by the viral polymerase and necessary for activity (Fodor et al., 1994; Tiley et al., 1994). Various structures of the promoter region have been proposed; the panhandle (Cheong et al., 1999; Hsu et al., 1987), the fork (Fodor et al., 1995), the corkscrew (Flick and Hobom, 1999; Flick et al., 1996; Tomescu et al., 2014) and the structure observed in the crystal structures (Pflug et al., 2014; Reich et al., 2014). In the recent crystal structures the 5′ promoter region adopts a hook structure (Pflug et al., 2014; Reich et al., 2014). The nucleotides in the 5′ promoter region can be visualised in all 3 structures, whereas for the 3′ promoter region nucleotides 1-18 can be visualised only in the fluB2 structure, as in the other two structures the 3′ terminal nucleotides are missing. The terminal end of the 5′ promoter forms a hook structure sandwiched by PA and PB1. The part of the 5′ promoter RNA that is not in the hook structure is duplexed with the distal part of the 3′ promoter. Although in the fluB2 structure the 3′ vRNA extremity is present, it is not in the active site but instead bound on the surface of the polymerase making contact with the PB1. It is unclear how the 3′ end would relocate to the active site of PB1 for initiation.

1.2.3 NP

NP is a major component of the RNP complex and it is found bound along the length of the vRNA that is not bound by the viral RNA polymerase. Several structures of NP in different oligomeric forms have been published; trimeric NP from influenza A virus H1N1 (Ye et al., 2006) (figure1.2a and 1.5b) and H5N1 (Ng et al., 2008) (figure 1.5c), monomeric NP R416A mutant from influenza A virus H1N1 (Chenavas et al., 2013) (figure 1.5a), dimeric NP obtained following deletion of the tail-loop from influenza A virus H1N1 (Ye et al., 2012), tetrameric NP from influenza B virus (Ng et al., 2012) and dimeric NP from ISAV (Zheng et al., 2013). These structures of NP have similar overall architectures, comprising a head and a body domain. To form the head and body domains, the polypeptide chain of NP goes back and forth, leading to a constraint in the movement of the two domains in relation to each other. The head and body domains are made up of 19 α-helices, 8 β-strands and interconnecting loops. The head domain is more structurally conserved, compared to the body domain, and contains a tail-loop structure required for oligomerisation (Chan et al., 2010). The tail-loop structure is made up of a helix-loop-helix motif protruding from the head domain and connected by two flexible linker regions. The flexibility of these linker regions allows NP to oligomerise in different constrained orientations i.e. in the double helical region of the RNP and the tight NP loop at the extremity of the RNP (Arranz et al., 2012; Moeller et al., 2012). The different orientations of the tail-loop, observed in the different structures, are shown in figure 1.5b, c and 1.6. The tail-loop structure is maintained by intra-molecular interactions between the two alpha helices and within the loop structure (Ng et al., 2012; Ng et al., 2008). Oligomerisation of NP in the published structures occurs via the tail-loop of one

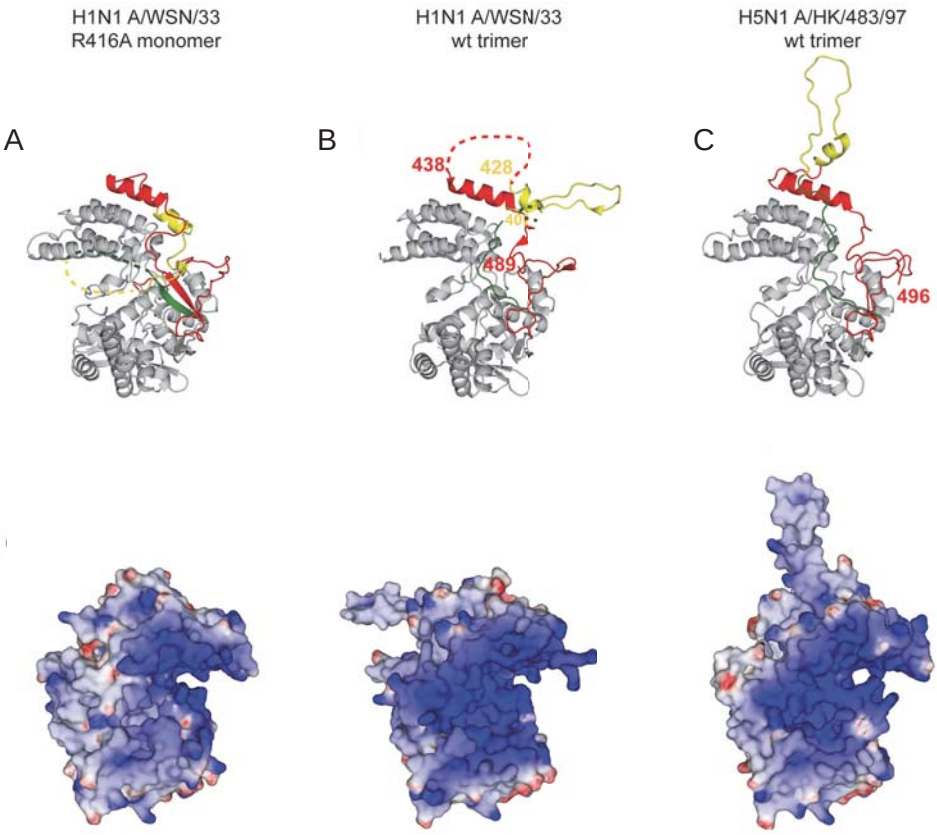


Figure 1.5. Comparison of structures of NP. Top panel shows the ribbon view of different structures of NP from (A) H1N1 mutant R416A (Chenavas et al., 2013) (B) H1N1 wildtype NP (Ye et al., 2006) (C) H5N1 wildtype NP (Ng et al., 2008). The bottom panel shows the electrostatic charge on the surface of NP, with blue representing positive charge, red representing negative charge and white representing neutral charge. Image adapted from Chenavas et al., 2013.

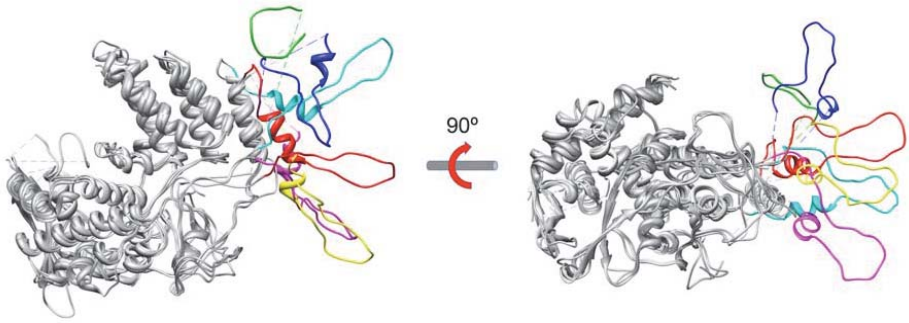


Figure 1.6. Different conformations of NP tail-loop from different NP structures. Alignment of the head and body domains of the different NP structures highlights the flexible nature of the linker regions to enable different conformations of the tail-loop structure. Loops are coloured according to the structure from where it has been derived; yellow Ye et al., 2012, pink PBD code 3GT6, cyan Ng et al., 2012, dark blue NP docked into RNP from Moeller et al., 2012 and green NP docked into RNP from Arranz et al., 2012. Figure adapted from Arranz et al., 2012.

NP monomer binding to the insertion-groove of a neighbouring NP monomer (Ng et al., 2012; Ng et al., 2008; Ye et al., 2006; Zheng et al., 2013). The insertion-groove is located in the body domain of NP and contains a glutamic acid (E339 in influenza A NP (ANP) and E395 in influenza B (BNP)) that forms a salt-bridge with an arginine residue located on the tip of the tail-loop (R416 in ANP and R472 in BNP). NP can no longer oligomerise when either of these residues, involved in the salt-bridge bond, are mutated to alanine (Chan et al., 2010; Ng et al., 2012; Ng et al., 2008). In the monomeric-R416A mutant structure the tail-loop is folded back and is binding to its own insertion-groove through hydrogen bonds between E339 within the insertion-groove and R461 in the tail-loop (Chenavas et al., 2013) (figure 1.5a). It is unlikely that this structure would form in wildtype NP, as there would be a clash between R416 and R461 (Chenavas et al., 2013). When the tail-loop of NP was deleted, NP formed a dimer (Ye et al., 2012); however, this dimer interface overlaps with the tail-loop insertion-groove interface.

On the opposite side of the tail-loop is a positively charged concave groove, responsible for NP-RNA binding (figure 1.2a). 11 out of 16 basic residues at the RNA binding surface are conserved among influenza A, B and C viruses (A/WSN/33, B/AnnArbor/1/66, C/California/78) (Ye et al., 2006). NP binds viral RNA with high affinity but no sequence specificity (Baudin et al., 1994; Elton et al., 1999; Yamanaka et al., 1990). There are two main clusters of positively charged residues within the concave groove, proposed to play a role in RNA binding (Ng et al., 2008; Ye et al., 2006). The residues in the first cluster (G1) in ANP are R74, R75, R174, R175, and R221 and in BNP are K125, K126, K131, K132, and K133. The residues in the second cluster (G2) in ANP are R150, R152,

R156, and R162 and in BNP are R211, K213, and R217. Mutation of each of these residues to alanine resulted in a reduction in NP-RNA binding affinity to different degrees (Ng et al., 2012; Ng et al., 2008). When four residues within G1 of ANP were mutated (G1(4)- R74A, R75A, R174A and R175A), no NP-RNA binding could be detected by surface plasmon resonance (SPR) (Vreede et al., 2011). The G1 cluster contains a flexible loop, between alpha helices 2 and 3 (L1- aa 72-91 in ANP and aa 125-149 in BNP), and a protruding element (aa 167-186 in ANP and 225-247 in BNP) (Ng et al., 2012; Ng et al., 2008). The flexible L1 loop has been deleted in both ANP and BNP, and resulted in a 6 and a 14-fold reduction in RNA binding affinity compared to wildtype NP, respectively (Ng et al., 2012; Ng et al., 2008). This flexible loop has been proposed to sample the environment and capture RNA, delivering it to the concave groove where the RNA may be clamped by the protruding element. A second flexible loop, between alpha helices 8 and 9 (L2- aa 202-212 in ANP and aa 267-270 in BNP (Ng et al., 2012; Ye et al., 2006)), is in close proximity to L1. Residues in ANP, D88 within L1 (Gui et al., 2014) and R204, W207 and R208 within L2 (Marklund et al., 2012) have been proposed to play a role in NP-polymerase binding.

A noticeable difference in NP from different influenza types is the length of the N-terminus. The N-terminus is not visible in the crystal structures of ANP (aa1-23) (Chenavas et al., 2013; Ng et al., 2008; Ye et al., 2006) or BNP (aa1-71) (Ng et al., 2012), implying a flexible and unstructured nature. However, the N-terminus was visible in the crystal structure of NP ISAV (aa 1-111) and consisted of 3 antiparallel β-sheets and two α-helices, organised in an extended shape interacting with the rest of NP through a long α-helix partially covering the RNA binding groove (Zheng et al., 2013). The deletion of the N-terminus in ISAV did

not affect NP-RNA binding, although in ANP the N-terminus has been proposed to play a role in RNA binding (Avalos et al., 1997). The N-terminus of ANP has also been shown to contain a non-conventional NLS involved in the import of vRNP complexes into the nucleus, as discussed above (Wang et al., 1997). Two additional NLSs have been identified in ANP: the bipartite NLS between residues 198-216, also discussed above, (Neumann et al., 1997) and a NLS between residues 320-400 (Bullido et al., 2000). Nuclear localisation of ANP has also been proposed to be regulated by phosphorylation of S9 and Y10 within the N-terminus (Zheng et al., 2015). NLSs in BNP are still not clearly defined. It has been recently proposed that the N-terminus of BNP contains a NLS (Sherry et al., 2014; Wanitchang et al., 2012; Wanitchang et al., 2013); however, when green fluorescence protein (GFP) was fused to the N-terminus amino acid sequence of BNP, no GFP localised to the nucleus (Stevens and Barclay, 1998). Unlike the N-terminus, the C-terminus of NP is structurally ordered in the monomeric-R416A structure and bound to the NP-RNA binding groove (Chenavas et al., 2013). In agreement with this, the deletion of the C-terminus of NP resulted in an increase in NP-RNA binding affinity (Chenavas et al., 2013; Ye et al., 2012). In contrast, the C-terminus in the trimeric H5N1 NP structure is pointing away from the RNA binding groove (Ng et al., 2008).

1.3 Assembly of the RNP complex

During the viral life-cycle, newly translated NP and polymerase proteins have to be transported into the nucleus to bind to the replication products cRNA and vRNA forming progeny cRNPs and vRNPs, respectively. NP binds to viral RNA

in a non-sequence specific manner and self-polymerises in the absence of RNA. *In vitro* studies have shown that when NP is maintained as a monomer it can bind along the length of RNA, in a chain forming a NP polymer (Chenavas et al., 2013; Tarus et al., 2012a), whereas when NP is oligomeric, prior to RNA binding, it cannot form a chain of NP polymers along the RNA. Taken together this suggests that NP is assembled onto the RNA as a monomer and, therefore, there must be a mechanism within the host-cell that maintains NP in a monomeric RNP complex compatible form, prior to assembly. Regulation of NP-oligomerisation and RNA binding has been proposed to occur via different mechanisms: phosphorylation of NP in the oligomerisation domain (Hutchinson et al., 2012), NP binding to importin α5 (Boulo et al., 2011) or NP binding to other host factors (Kawaguchi et al., 2011; Momose et al., 2001; Momose et al., 1996; Momose et al., 2002; Naito et al., 2007). Currently it is still unclear if NP uses one, or a combination of these mechanisms to regulate oligomerisation and RNA binding within the host.

RNP complexes assemble when the RNA polymerase proteins, NP and a viral RNA genome segment are expressed in a host cell, which suggests that no other viral proteins are required for RNP complex assembly (Moeller et al., 2012). However, when these components are mixed with a RNA template *in vitro* they do not form a double helical rod structure like the structure formed in the presence of NP alone (Kingsbury et al., 1987; Murti et al., 1988; Ruigrok and Baudin, 1995; Yamanaka et al., 1990). NP has been shown to directly interact with the viral polymerase subunits PB1 and PB2 (Biswas et al., 1998; Newcomb et al., 2009). It has been suggested that this direct interaction between the viral polymerase and NP could facilitate the recruitment of NP onto the progeny RNA (Crescenzo-Chaigne et al., 1999). The co-operative oligomerisation of NP is also thought to

play a role in RNP complex assembly (Chan et al., 2010). The N-terminus has also been proposed to play a role in RNP complex assembly via the regulation of RNA binding (Avalos et al., 1997; Li et al., 2009; Sanchez et al., 2014).

Currently, it is unclear how the RNP complex forms its double-helical structure. A mini-RNP containing a 248-nucleotide template formed a circular ring of 9 NPs (Coloma et al., 2009), this therefore suggests that the RNA template has to be of a certain length before the double helical region forms.

1.4 RNA synthesis

Even though the vRNP complex is responsible for both viral transcription and replication, they occur via two independent mechanisms and they are differentially regulated. During viral infection, differential phases of transcription and replication occur; predominantly mRNA accumulates early in infection, peaking at 4-6 hours post-infection and then declining, while vRNA continues to accumulate throughout viral infection. The level of cRNA during infection is a lot lower than the levels of mRNA and vRNA. cRNA accumulation plateaus approximately 4-6 hours post-infection and then the levels remain constant (Hay et al., 1982; Scholtissek and Rott, 1970; Taylor et al., 1977). A schematic of RNA synthesis carried out by the influenza RNP complex is shown in figure 1.7.

1.4.1 Transcription

During transcription, the negative-sense vRNA is transcribed into positive sense mRNA using a primer dependent mechanism. A 9-13 nucleotide capped primer is generated by cleavage of cellular mRNA by the RNA polymerase, using the cap-

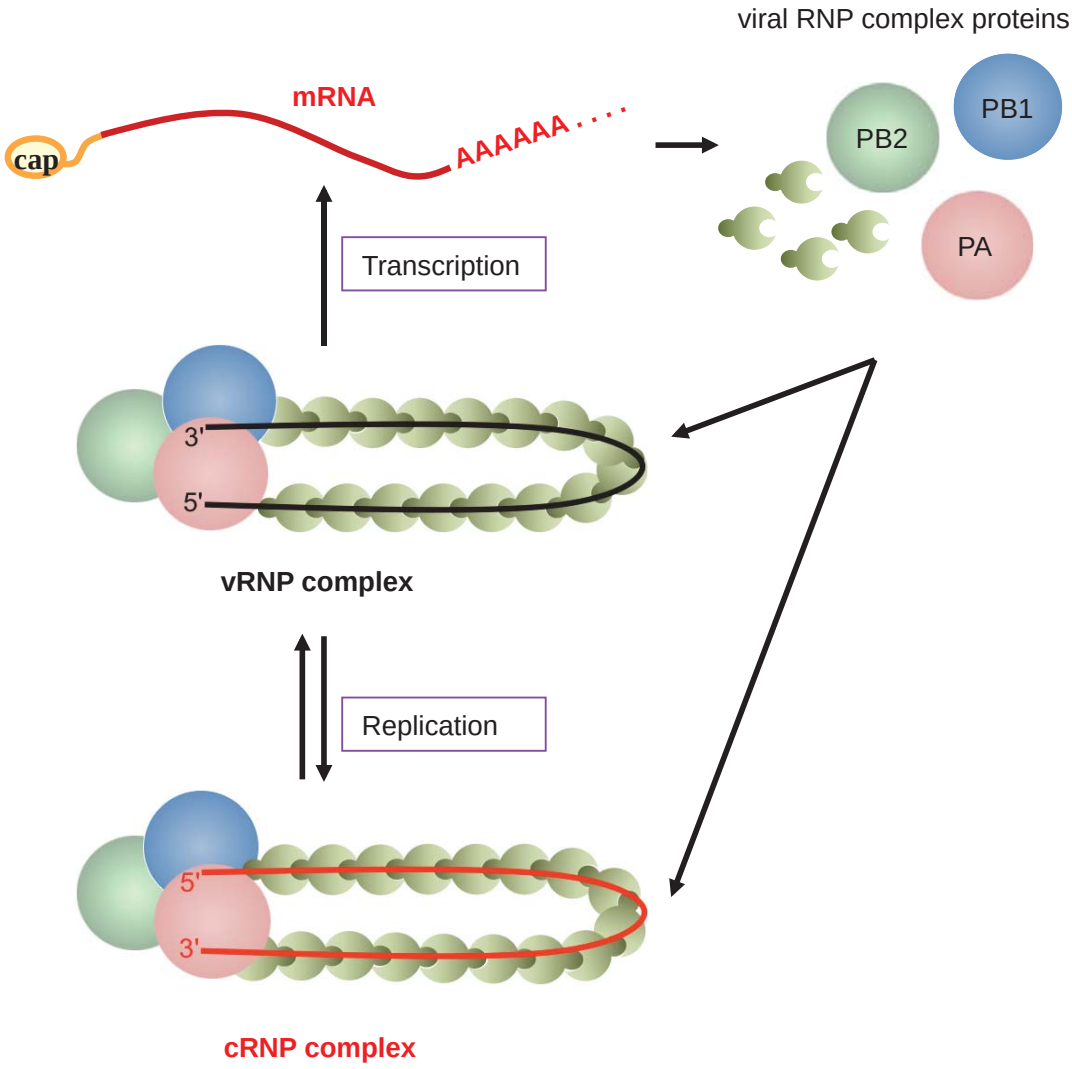


Figure 1.7. Schematic of vRNP complex RNA synthesis.

binding activity of PB2 and the endonuclease activity of PA (Dias et al., 2009; Yuan et al., 2009). The distance between the PA endonuclease domain and PB2 cap-binding domain in the fluA structure is compatible with cleavage of the host RNA 10-15 nucleotides downstream of the cap (Dias et al., 2009; Krug, 1981; Pflug et al., 2014; Yuan et al., 2009). The variability in the capped primer length may reflect the flexibility of the PB2 cap-binding domain. The 3′ end of the cleaved capped primer would then need to relocate to the active site in PB1, which carries out RNA synthesis. It has been proposed that the rotation of the cap-binding domain, observed between the fluA and fluB1 structures, would allow the re-positioning of the RNA capped primer into the active site (Reich et al., 2014). Rotation of the PB2 cap domain would allow the cap to remain bound to PB2 while the first 11-15 nucleotides of mRNA are synthesised. The release of the transcript in this PB2 conformation would cause it to emerge into a basic channel, avoiding the endonuclease domain (Reich et al., 2014). Modelling of the elongation phase, suggests that there would be a clash between the newly synthesised duplex RNA and both the PB2 N-element and the priming loop; therefore, a conformational change would be required to displace these elements for elongation to occur. Polyadenylation of the transcript is carried out by the viral polymerase and is dependent on the presence of a stretch of 5-7 uracil residues, approximately 16 nucleotides from the 5′ end of the vRNA template (Li and Palese, 1994; Luo et al., 1991). The polymerase remains bound to the 5′ end of the vRNA, leading to steric hindrance, causing the polymerase to stutter on the uracil residues and generate a poly(A) tail (Poon et al., 1999; Zheng et al., 1999). The relative positions of the 5′ promoter hook structure and the PB1 active site, in the RNA polymerase structure, is compatible with this model for the generation of a

poly(A) tail (Reich et al., 2014). The poly(A) signal is critical for gene expression as when the stretch of uracil residues was replaced with adenine residues, resulting in the generation of a poly(U) tail, this caused a reduction in the export of mRNA from the nucleus (Poon et al., 2000).

Two of the viral gene segments produce spliced transcripts. Splicing of viral mRNA is performed by the cellular splicing machinery (Lamb and Horvath, 1991) in a highly regulated process that produces approximately 90% un-spliced and 10% spliced transcripts during infection (Lamb and Choppin, 1981; Lamb and Horvath, 1991). However, the exact mechanism and regulation of splicing in the case of viral mRNA is unclear. The spliced and un-spliced viral transcription products are packaged into mRNP complexes (York et al., 2013). These mRNP complexes are composed of several host-proteins, such as the cap-binding complex, and are thought to be indistinguishable from host mRNP complexes. These mRNP complexes are exported from the nucleus, and once in the cytoplasm, are translated into viral proteins using the cellular translation machinery. Viral proteins that function within the nucleus, such as NP, are imported into the nucleus via their NLSs. These viral proteins are required to encapsidate progeny RNA and have been proposed to play a role in the regulation of the RNA polymerase in transcription versus replication of vRNA, discussed below in section 1.4.3.

1.4.2 Replication

Viral replication occurs via a two-step primer-independent process. The template vRNA is first replicated into cRNA, which is a positive-sense full-length copy of the vRNA. As the cRNA is synthesised it is assembled with polymerase and NP

into a RNP complex (cRNP). The cRNA is then used as a template to generate progeny vRNA. The newly synthesised vRNA associates with PB1, PB2, PA and NP to form progeny vRNP complexes, which carry out secondary transcription and replication and are exported from the nucleus by a mechanism involving M1 and NEP/NS2 (Baudin et al., 2001; Chase et al., 2011; Noton et al., 2007; Shimizu et al., 2011; Ye et al., 1999).

Replication occurs via a primer independent mechanism. For the synthesis of cRNA two models have been proposed for the generation of pppApG in *de novo* initiation. In the first model the penultimate 3′ C residue directs the binding of GTP followed by the recruitment of ATP, directed by the residue at position 1 of the 3′ end (Vreede et al., 2008). In the second model a non-templated residue (preferentially a purine) is first added to the 3'-end of the vRNA template by a cellular ribonucleotidyltransferase, leading to the initiation of replication directed by the penultimate 3′ residue and the synthesis of pppApG (Zhang et al., 2010). For the synthesis of vRNA, using the cRNA template, pppApG is thought to be synthesised on positions 4 and 5 of the 3′ end and then realigned to the 3′ terminal residues for nucleotide addition during elongation (Deng et al., 2006b). The different mechanisms of initiation for the replication of vRNA and cRNA suggest that the 3′ end of the promoter RNA may have different positions in the active site (Azzeh et al., 2001; Crow et al., 2004).

Currently there are conflicting opinions in the literature as to whether both stages of replication, the generation of cRNA and then progeny vRNA, are carried out by the resident polymerase within the RNP complex, or if a trans-acting polymerase carries out the second stage or both stages of replication. The synthesis of RNA by a trans-acting polymerase, distinct from the resident polymerase within the RNP

complex, would occur via the binding of the trans-acting polymerase to the 3′ end of the RNA and copying of the template (Jorba et al., 2009). The 3′ vRNA promoter was bound on the surface of the RNA polymerase in the crystal structure of the RNA polymerase in the pre-initiation state; this position may facilitate the binding of the trans-acting polymerase to the promoter RNA (Pflug et al., 2014; Reich et al., 2014). Branched vRNPs were observed when images of replicating vRNPs were taken, with a second polymerase located at the branch position, suggesting that a trans-acting polymerase may carry out the first stage of replication (Moeller et al., 2012). The synthesis of vRNA from cRNA, in the second stage of replication, may also be carried out by a trans-acting polymerase (Jorba et al., 2009; York et al., 2013). When cRNP complexes were isolated, they were only active in the presence of additional, active or inactive, RNA polymerase (York et al., 2013). This suggests that the trans-acting polymerase may not be required for the catalytic synthesis of the RNA, but may act as a platform for synthesis by the resident RNA polymerase, as a trans-activating polymerase.

1.4.3 Determining the initiation mode of the viral polymerase

The viral RNA polymerase carries out both transcription and replication, which occur by two very different mechanisms and are differentially regulated during the virus life-cycle. However, it is still unclear what determines whether the viral polymerase carries out transcription versus replication. NP and other viral and cellular factors have been suggested to play a role in this regulation between transcription and replication (reviewed in (Resa-Infante et al., 2011)). Models of how these two essential viral processes are regulated include a trans-acting polymerase carrying out replication (as discussed above (Jorba et al., 2009)), the

concentrations of vRNA 5′ end, the mRNA cap and polymerase source impacting which process is carried out (Olson et al., 2010), the viral NEP/NS2 protein causing a preference for replication (Robb et al., 2009) and the presence of small vRNA impacting the transition from transcription to replication in the viral life-cycle (Perez et al., 2010; Scull and Rice, 2010; Umbach et al., 2010).

The availability of viral NP has been proposed to play a role in determining the initiation mode of the polymerase; cRNA but not mRNA synthesis was affected in the presence of NP temperature-sensitive mutants at non-permissive temperatures (Krug et al., 1975; Medcalf et al., 1999; Shapiro and Krug, 1988). Models suggesting that NP plays a role in the transition of the viral polymerase from transcription to replication include the encapsidation model where cRNA synthesis requires NP to bind to the nascent RNA (Beaton and Krug, 1986; Shapiro and Krug, 1988). In the stabilisation model, infecting vRNP complexes are able to synthesise both mRNA and cRNA, but until there is a sufficient pool of polymerase and NP to encapsidate the cRNA, it is degraded; therefore, at early times post-infection there is a bias towards mRNA accumulation (Vreede et al., 2004). Another two models proposed that the binding of NP to the viral polymerase (Biswas et al., 1998; Newcomb et al., 2009) or the viral RNA (Klumpp et al., 1997) causes a switch in polymerase function from transcription to replication. However, evidence in the literature has been building that NP does not play a role in determining the mode of the viral polymerase. Replication of a short vRNA-like template, 46-nucleotides in length, was shown to occur in the absence of NP *in vivo* (Resa-Infante et al., 2010), suggesting that NP is not required for replication, neither through interaction with the polymerase or encapsidation of the transcript. In addition increasing concentrations of NP had no effect on

altering the ratio of transcription to replication (Mullin et al., 2004). It is, therefore, still unclear whether NP does play a role in determining the mode of initiation of the viral polymerase.

1.5 The role of NP within the RNP complex

NP forms a major component of the RNP complex, and even though we know that it is essential for viral polymerase activity on a full-length vRNA template, the exact role it plays in supporting polymerase activity is unclear. It is essential for NP to bind the template vRNA and cRNA to form RNP complexes, in order for the polymerase to produce mRNA, cRNA and vRNA (Ng et al., 2008; Ye et al., 2006). The viral polymerase can initiate, in both transcription and replication, in the absence of NP *in vitro,* carrying out endonucleolytic cleavage of capped RNA and *de-novo* replication (Honda et al., 1988). The addition of NP allowed the generation of longer length-transcripts, suggesting that NP was functioning as an elongation factor. Whether NP is involved in elongation directly, via melting of RNA secondary structure (Baudin et al., 1994; Yamanaka et al., 1990), or whether it recruits host-cellular factors, that are required for elongation, is unclear. NP interacts with several host-factors including the minichromosome maintenance replicative helicase complex; this host-factor stimulates elongation of cRNA synthesis *in vitro* and is required for cRNA synthesis *in vivo* (Kawaguchi et al., 2011; Kawaguchi and Nagata, 2007). NP also interacts with UAP56, a host-helicase, and this interaction causes an increase in RNA synthesis (Momose et al., 2001; Naito et al., 2007; Wisskirchen et al., 2011).

For NP to support viral transcription and replication it has to both bind to the RNA template and oligomerise. Oligomerisation mutants, R416A and E339A, can

bind to the cRNA product stabilising it; however, the RNP-like complex was inactive (Vreede et al., 2011). These oligomerisation mutants are also unable to support the production of mRNA and cRNA by the viral RNA polymerase when in the context of a RNP reconstitution on a full-length template (Chan et al., 2010). The maintenance of the oligomerisation tail-loop structure, through intra-molecular interactions, has also been shown to be essential for NP to support RNA polymerase activity (Chan et al., 2010).

The structures of RNP complexes show a clear interaction between the RNA polymerase and NP (Arranz et al., 2012; Moeller et al., 2012). When RNA polymerase from influenza A virus was combined with NP from influenza B virus (B/Panama/45/90 or B/Lee/40), the RNP complex was unable to carry out transcription (Iwatsuki-Horimoto et al., 2008). This suggests that there has to be compatibility between the RNA polymerase and NP in order for the RNP complex to be active. The mutation of NP residues R204, W207 and R208, within the loop between alpha helices 2 and 3 (L2), inhibited both NP-polymerase binding and RNA polymerase activity (Marklund et al., 2012). In addition, D88, within the loop between alpha helices 8 and 9 (L2), has been shown to interact with PB2 of the RNA polymerase (Gui et al., 2014). Molecular dynamics simulations showed that a salt-bridge could form between R208 in L2 and E80 in L1 (Tarus et al., 2012b). Formation of this salt-bridge would reduce access to the RNA binding groove, possibly stabilising the RNA within the groove. This is supported by the mutation of E80A/E81A and R204A/R207A resulting in a decrease in NP-RNA binding affinity (Tarus et al., 2012b).

1.6 Thesis outlooks

Despite NP being the major component of the RNP complex the role NP plays in transcription and replication is not fully understood, nor how it is assembled onto the RNA to form a RNP complex or how this assembly is regulated. The aim of this thesis is to gain a greater understanding of NP in these processes.

2 Materials and methods

2.1 Materials

2.1.1 General reagents

Chemical reagents were purchased from Roche, GE healthcare, Millipore, Invitrogen Life technologies, Sigma Aldrich, New England Biolabs, QIAGEN, Bio-Rad, Thermo Scientific, Promega and Agilent Technologies. The source of reagents used will be indicated in the text. [γ-^{32}P]ATP [6000 Ci/mmol (10 mCi/ml)] was purchased from Perkin Elmer, U.S.A.

2.1.2 Enzymes

Restriction endonucleases were purchased from New England Biolabs, Thermo Scientific or Roche. Tobacco Etch Virus (TEV) protease was supplied by Invitrogen (AcTEV), T4 polynucleotide kinase (PNK) by Thermo Scientific, RNase A by Sigma, calf intestinal phosphatase (CIP) by Promega. Pfu turbo DNA

polymerase was purchased from Stratagene, Phusion DNA polymerase from New England Biolabs, and GoTaq polymerase from Promega.

2.1.3 Oligonucleotides

DNA oligonucleotides for use in PCR, DNA sequencing and primer extension were purchased from Sigma Aldrich or Invitrogen Life Technologies.

2.1.4 Antibodies

The primary and secondary antibodies used and their sources are detailed in table 2.1.

2.1.5 Tissue culture media

Dulbecco's modified eagle medium (DMEM), minimal essential medium (MEM) and foetal calf serum (FCS) were all purchased from Sigma. TrypLE Express was supplied by Invitrogen. Insect cell medium Insect-XPRESS protein-free was purchased from Lonza Group Ltd.

2.1.6 Cell lines

Human embryonic kidney 293T (293T) cells, Madin-Darby bovine kidney epithelial (MDBK) cells, African green monkey kidney (Vero) cells and *spodoptera frugiperda* (Sf9) cells were obtained from the cell bank of the Sir William Dunn school of pathology.

Target protein	Figure	Primary antibody		Secondary antibody	
		Source	Dilution	Source	Dilution
Western Blotting					
PB1	3.10	Goat poly-clonal Santa Cruz 17601	1:500	Rabbit anti goat IgG HRP Sigma A5420	1:10000
ANP	3.7, 3.10 4.4	Rabbit poly-clonal Gift from Otto Haller, Freiburg	1:10000	Goat anti rabbit IgG HRP Sigma A0545	1:10000
ANP	3.11 5.5	Rabbit poly-clonal Gift from Paul Digard, Glasgow	1:1000		
RanBP5	3.7, 3.10, 3.11 4.4 5.5, 5.7	Rabbit poly-clonal Santa Cruz 11369	1:500		
BNP	5.7	Mouse mono-clonal AbD serotech® B017	1:500	Goat anti mouse IgG HRP Sigma A4416	1:10000
Immunofluorescence					
ANP	3.8 4.5 6.3, 6.5	Mouse mono-clonal Abcam 128193	1:500	Donkey anti mouse IgG Cy3 Jackson Immunoresearch 715-165-150	1:500
BNP	6.3, 6.5	Mouse mono-clonal AbD serotech® B017	1:500	Donkey anti mouse IgG Cy3 Jackson Immunoresearch 715-165-150	1:500
Immunoprecipitation					
c-Myc	3.11	Mouse mono-clonal Sigma M4439	1:10000	N/A	N/A

Table 2.1. Antibodies used in western blot and immunofluorescence.

2.1.7 Plasmids

The protein expression plasmids pcDNA-PB1, pcDNA-PB1a (D445A/D446A), pcDNA-PB2, pcDNA-PA, pcDNA-PA-His6, pcDNA-NP and pcDNA-3A have been described previously (Fodor et al., 2002; Vreede et al., 2004), as have NP mutants pcDNA-NP 4R16A, pcDNA-NP E339A and pcDNA-NP G1(4) (R74A/R75A/R174A/R175A) (Vreede et al., 2011). Plasmids for polymerase I-driven transcription of vRNA: pPOLI-PB1, pPOLI-NP, pPOLI-NA and pPOLI-NS have been described previously (Fodor et al., 1999).

Plasmids used to rescue recombinant influenza virus have also been described previously (Hoffmann and Webster, 2000). The eight pHW2000 plasmids with polymerase I- polymerase II promoters were a kind gift from Gülsah Gabriel (University of Hamburg, Germany).

The protein expression plasmids encoding influenza B virus components were kindly provided by Wendy Barclay (Imperial College, UK) and have been described previously (Jackson et al., 2002). pCI-PB1, pCI-PB2, pCI-PA, and pCI-NP sequences are derived from B/Panama/45/90 virus and the polymerase I-driven plasmid expresses the vRNA NA template from influenza B/Beijing/1/87 virus (pPR-NA).

2.2 Mutagenesis and cloning

2.2.1 PCR-mutagenesis and cloning

The plasmids pPOLI-NP-47nt, pPOLI-NP-76nt, pPOLI-NP-80nt, pPOLI-NP-85nt, pPOLI-NP-91nt, pPOLI-NP-95nt, pPOLI-NP-101nt, pPOLI-NP-125nt, pPOLI-NP-149nt, pPOLI-NP-173nt, pPOLI-NP-197nt, pPOLI-NP-221nt, pPOLI-

NP-246, pPOLI-NP-287nt, pPOLI-NP-583nt, pPOLI-NS-60nt and pPOLI-NS-172nt were constructed by PCR amplification using outward facing primers (table 2.2) with a SpeI restriction site at the 5′ end, resulting in an internal deletion of the open reading frame to different degrees. The polymerase chain reaction (PCR) reaction was carried out using 10 ng of template plasmid, 250 nmol of each primer (table 2.2), 200 µM of dNTP and 1.25 U Pfu Turbo (Stratagene) in 1X Pfu turbo reaction buffer, incubated in a thermocycler at 95°C for 30 sec, 55°C for 30 sec and 72°C for 5 minutes for a total of 30 cycles. The products of the PCR reaction were analysed using a 1% agarose gel. The bands on the gel corresponding to the expected product size were cut out and the DNA was extracted using QIAGEN gel extraction kit, according to manufacturer's instructions. The DNA was eluted in 30 µl of sterile nuclease-free water. Digestion of the DNA was carried out using 28 µl of the eluted DNA and 5 U SpeI (Roche) in 1X buffer H (Roche) at 37°C for 2 hours. The DNA was purified with QIAGEN PCR purification kit, according to manufacturer's instructions, and eluted in 10 µl of sterile nuclease-free water. Ligation of the DNA was carried out using 1.5 µl of the eluted DNA and 1.5 µl of Ligation High (Toyobo) at 16°C for 30 minutes. The ligation reaction was transformed into bacterial cells (see section 2.2.2).

The pPOLI-NP plasmid (encoding the full-length 1565-nucleotides NP vRNA) was modified by site directed mutagenesis to eliminate the start codon (M1S) and to introduce a stop codon (M238STOP), preventing the translation of NP from the transcribed mRNA template. Site directed mutagenesis was also used to modify pPOLI-NP and pcDNA-NP to generate pcDNA-NP S165A, pcDNA-NP S165E, pcDNA-NP S165D, pPOLI-NP S165A, pPOLI-NP S165E, pPOLI-NP S165D.

pPOLI-NP-47nt	vNP 5′ PROMOTER-SpeI: 5′ CCAACTAGTCTACCCTGCTTTTGCT 3′ (25) vNP 3′ NCR-SpeI: 5′ ACGACTAGTAAAGAAAAATACCCTTG 3′ (26)
pPOLI-NP-76nt	vNP 5′ NCR-SpeI: 5′ GGTACTAGTGATTTCGATGTCACTC 3′ (25) vNP 3′ NCR-SpeI: 5′ ACGACTAGTAAAGAAAAATACCCTTG 3′ (26)
pPOLI-NP-80nt	vNP 5′ NCR-SpeI: 5′ GGTACTAGTGATTTCGATGTCACTC 3′ (25) vNP 3′ NCR-SpeI: 5′ ACGACTAGTAATTAAAGAAAAATACCCTTG 3′ (30)
pPOLI-NP-85nt	vNP 5′ NCR-SpeI: 5′ GGTACTAGTGATTTCGATGTCACTC 3′ (25) vNP 3′ 85nt-SpeI: 5′ ACGACTAGTACGACAATTAAAGAAAAATACCC 3′ (32)
pPOLI-NP-91nt	vNP 5′ 91-SpeI: 5′ GGTACTAGTCGCCATGATTTCGATG 3′ (25) vNP 3′ 85nt-SpeI: 5′ ACGACTAGTACGACAATTAAAGAAAAATACCC 3′ (30)
pPOLI-NP-95nt	vNP 5′ 91-SpeI: 5′ GGTACTAGTCGCCATGATTTCGATG 3′ (25) vNP 3′ 100nt-SpeI: 5′ CAGACTAGTGAGTACGACAATTAAAGAAAAATACCC 3′ (36)
pPOLI-NP-101nt	vNP 5′ 101nt-SpeI: 5′ GTCACTAGTTTTGGTCGCCATGATTTCG 3′ (28) vNP 3′ 101nt-SpeI: 5′ CAGACTAGTGAGTACGACAATTAAAGAAAAATACCC 3′ (36)
pPOLI-NP-125nt	vNP 5′ 125nt-SpeI: 5′ GTAACTAGTTCGTTTGGTGCCTTTGG 3′ (26) vNP 3′ 125nt-SpeI: 5′ CAGACTAGTGACAATGCAGAGGAGTACG 3′ (28)
pPOLI-NP-149nt	vNP 5′ 149nt-SpeI: 5′ CTGACTAGTCTGTTCGTAAGATCGTTTGG 3′ (29) vNP 3′ 149nt-SpeI: 5′ CAGACTAGTTATTTCTTCGGAGACAATGC 3′ (29)
pPOLI-NP-173nt	vNP 5′ 149nt-SpeI: 5′ CTGACTAGTCTGTTCGTAAGATCGTTTGG 3′ (29) vNP 3′ 173nt-SpeI: 5′ GAGACTAGTTTTGACATGAGTAATGAAGGAT 3′ (31)
pPOLI-NP-197nt	vNP 5′ 149nt-SpeI: 5′ CTGACTAGTCTGTTCGTAAGATCGTTTGG 3′ (29) vNP 3′ 197nt-SpeI: 5′ GAGACTAGTGCAACGAGCCCGATCGTG 3′ (27)
pPOLI-NP-221nt	vNP 5′ 149nt-SpeI: 5′ CTGACTAGTCTGTTCGTAAGATCGTTTGG 3′ (29) vNP 3′ 221nt-SpeI: 5′ CTGACTAGTGTCTTCGAGCTCTCGGACG 3′ (28)
pPOLI-NP-246nt	vNP 5′ PACKAGING-SpeI: 5′ TCCACTAGTCTCCATCTGTTCG 3′ (22) vNP 3′ PACKAGING-SpeI: 5′ TGAACTAGTGTCTTTCCAGGGGCG 3′ (24)
pPOLI-NP-287nt	vNP 5′ 287nt-SpeI: 5′ CTGACTAGTGGCATTCTGGCGTTCTCC 3′ (27) vNP 3′ 287nt-SpeI: 5′ CTGACTAGTGCAAGACCAGAAGATGTG 3′ (27)
pPOLI-NP-583nt	vNP 5′ 538nt-SpeI: 5′ CTGACTAGTTTAAGTTCGGTGCACATTTG 3′ (29) vNP 3′ 461nt-SpeI: 5′ CTGACTAGTGGAGGGAACACCAATCAAC 3′ (28)
pPOLI-NS-60nt	vNS 5′ NCR-SpeI: 5′ CTGACTAGTTATGTCTTTGTCACCCTGC 3′ (28) vNS 3′ NCR-SpeI: 5′ ACGACTAGTAATAATAAAAAACACCCTTGTTTC 3′ (33)
pPOLI-NS-172nt	vNS 5′ PACKAGING-SpeI: 5′ GTCACTAGTGCGGACATGCCAAAGAAAGC 3′ (29) vNS 3′ PACKAGING-SpeI: 5′ AGTACTCAACTATTGCTTGAAGTGGAGC 3′ (28)

Table 2.2. Sequences of primers used for the construction of plasmids to express short vRNA-like templates.

Primers were designed using QuickChange Primer Design (Agilent). The PCR site-directed mutagenesis reactions were carried out using 20 ng of template plasmid, 250 nmol of each primer, 200 μM of each dNTP and 1 U Phusion (New England Biolabs) in 1X Phusion high fidelity buffer, and incubated in a thermocycler at 98°C for 30 sec, 52-57°C (dependent on the primer) for 45 sec and 72°C for 12 minutes, for a total of 24 cycles. The PCR reaction was digested with 10 U DpnI (Fermentas) at 37°C for 3 hours and a small proportion was analysed by agarose gel electrophoresis. A proportion of the PCR product was transformed into bacterial cells.

pcDNA-NP E339A-myc-his construct was made by PCR amplification of the NP open reading frame from a pcDNA-NP-E339A template with a 5′ end primer that included a NotI site and a 3′ end primer that included an ApaI site and introduced a mutation in the stop codon of the open reading frame. The PCR conditions were as above using Pfu turbo. The PCR product and pcDNA ™3.1/myc-His A MCS (Invitrogen) vector were digested with 1 U of ApaI (New England Biolabs) restriction enzyme in 1X CutSmart™ Buffer with 0.1 μg BSA in a total reaction volume of 10 μl at 25°C for 3 hours. The digested products were analysed on a 1% agarose gel and extracted. The eluted products were digested with 1 U of NotI (New England Biolabs) restriction enzyme in 1X NEBuffer 3.1 with 0.1 μg BSA in a total reaction volume of 10 μl at 37°C for 3 hours. The digested products were analysed and extracted as before. The insert and the vector were ligated at a molar ratio of 4:1 respectively, using Ligation High (Toyobo) in a total reaction volume of 3 μl at 16°C for 30 minutes. All 3 μl of the ligation reaction was used for transformation into bacterial cells.

For the expression of NP in insect cells a pFL-TAP-NP plasmid was constructed by Frank Vreede, from the Dunn School of Pathology, through the insertion of the synthetic NP gene with an N-terminal 2X protein A tag (Influenza A/NT/60/68 NP (H3N2) codon-optimised (GeneArt-Invitrogen)) into the pFL vector. The pFL vector was a kind gift from Dr Imre Berger (EMBL, Grenoble). The construct and vector were digested with 1 U of XhoI (Roche) restriction enzyme in 1X Buffer H with 0.1 μg BSA in a total reaction volume of 10 μl at 37°C for 3 hours. The digested products were analysed on a 1% agarose gel and extracted. The eluted products were digested with 1 U of NheI (New England Biolabs) restriction enzyme in 1X NEBuffer 2.1 with 0.1 μg BSA in a total reaction volume of 10 μl at 37°C for 3 hours. The digested products were analysed and extracted as before. The insert and the vector were ligated at a molar ratio of 4:1 respectively, using Ligation High (Toyobo) in a total reaction volume of 3 μl at 16°C for 30 minutes. All 3 μl of the ligation reaction was transformed into bacterial cells. The pFL-TAP NP plasmid was mutated using site directed mutagenesis, as above, to generate six mutants: pFL-TAP-NP R416A, pFL-TAP-NP E339A, pFL-TAP-NP G1(4), pFL-TAP-NP S165A, pFL-TAP-NP S165E, and pFL-TAP-NP S165D.

Plasmids expressing NP with mutations in both the oligomerisation and/or RNA-binding domains (pcDNA-NP E339A/R416A, pcDNA-NP G1(4)/R416A and pcDNA-NP G1(4)/E339A) were constructed by Frank Vreede at the Sir William Dunn School of Pathology. The NP constructs with multiple mutations were made from the individual mutants by restriction enzyme mediated subcloning. For pcDNA-NP E339A/R416A, plasmids pcDNA-NP E339A (vector) and pcDNA-NP R416A (insert) were both digested with 1 U of XcmI (New England Biolabs) restriction enzyme in 1X NEBuffer 2.1 with 0.1 μg BSA in a total reaction volume

of 10 µl at 37°C for 3 hours. For pcDNA-NP G1(4)/R416A and pcDNA-NP G1(4)/E339A, plasmids pcDNA-NP G1(4) (vector) and pcDNA-NP R416A and pcDNA-NP E339A (inserts) respectively were all digested with 1 U of BstBI (New England Biolabs) restriction enzyme in 1X CutSmart™ Buffer with 0.1 µg BSA in a total reaction volume of 10 µl at 65°C for 3 hours. 2 µl of CIP (New England Biolabs) was then added to the vector reaction, which was incubated at 37°C for a further hour. The digested products were analysed on a 1% agarose gel and extracted. The insert and the vector were ligated at a molar ratio of 4:1 respectively, using Ligation High (Toyobo) in a total reaction volume of 3 µl at 16°C for 30 minutes. All 3 µl of the ligation reaction was used for transformation into bacterial cells.

Brontë Paice constructed influenza A and B virus NP chimeras during her Biochemistry Part II project at the Sir William Dunn School of Pathology. Primers were designed where the nucleotide sequence encoded the heterologous element flanked by sequence complementary to the regions either side of the element in the homologous system (table 2.3). The PCR reaction was carried out as above in site-directed mutagenesis with Phusion, and either pcDNA-NP or pCI-NP were used as templates. The PCR reaction was digested with 10 U DpnI (Fermentas) at 37°C for 3 hours and a small proportion was analysed by agarose gel electrophoresis. A proportion of the PCR product was transformed into bacterial cells. To generate pcDNA-ANP+BN the pcDNA-NP plasmid excluding the N-terminus and the pCI-NP N-terminus were amplified by PCR (Phusion) with primers that encoded a BsmI site. The PCR products were digested with 1 U of BsmI (New England Biolabs) restriction enzyme in 1X CutSmart™ Buffer with 0.1 µg BSA in a total reaction volume of 10 µl at 65°C for 3 hours. 2 µl of CIP

pcDNA-NP ΔN	5′ GAGTGACATCGAAATCATGGAAATCAGAGCATCTGTCG 3′ (38) 5′ CGACAGATGCTCTGATTTCCATGATTTCGATGTCACTC 3′ (38)	
pcDNA-NP + BNP N	pcDNA-NP	5′ GACCGTCTCGGATTTCGATGTCACTCTGTGAGTGATTATC 3′ (41) 5′ GACCGTCTCGGAAATCAGAGCATCTGTCGGAAAAATGATTG 3′ (41)
	pCI-NP	5′ GACCGTCTCGAATCATGTCCAACATGGATATTGACGG 3′ (37) 5′ GACCGTCTCGTTTCCTGTTTCTTTTGGGTTTTCCTTCC 3′ (38)
pcDNA-NP + BNP L1	5′ GACCGTCTCGGGCATTCTTTTTCCTCTGGAATTCAGTTTTAAAAGCAGAGAGCACCATTCTC 3′ (62) 5′ GACCGTCTCGTGCCAGAGATGTCAAAGAAGGAAAAGAAGAAATAGACCACGGAGGACC TATATACAGGAG 3′ (70)	
pPOLI-NP + BNP L2	5′ GACCGTCTCGTCTCTCAAATTGATCCCACGTTTGATCATTCTG 3′ (43) 5′ GACCGTCTCGGAGACATCCGGAGAACAAGGATTGCTTATG 3′ (40)	
pCI-NP ΔN	5′ TCTCTGTCGGGGTCATTTTGCCCGGGGTAC 3′ (30) 5′ GTACCCCGGGCAAAATGACCCCGACAGAGA 3′ (30)	
pCI-NP + ANP N	5′ GACCGTCTCGGTTCGTAAGATCGTTTGGTGCCTTTGGTCGCCATTTTGCCCGGGGTACCACG 3′ (62) 5′ GACCGTCTCGGAACAGATGGAGACTGATGGAGAACGCCAGAATGCCACTACCCCGACAGAGATA AAGAAG 3′ (68)	
pCI-NP + ANP L1	5′ GACCGTCTCGGGGATGTTCTTCTAGATATTTATTCCTCCTCTCGTCCTTGTCATCAGTGGCAGCC 3′ (65) 5′ GACCGTCTCGTCCCAGTGCGGGAAAGATCCTAAGAAAACTAACAAAACAGGAGGCACCTTTTAC 3′ (64)	
pCI-NP + ANP L2	5′ CGAGACAGAGGGCTAGATCGGAACTTCTGGAGGGGTGAGAATGGAAAAGCCAAGACTGCG 3′ (60) 5′ GGCAGTCTTGGCTTTTCCATTCTCACCCCTCCAGAAGTTCCGATCTAGCCCTCTGTCTGC 3′ (60)	

Table 2.3. Primers used to construct NP chimeras.

(New England Biolabs) was added to the PCR reaction where pcDNA-NP was used as a template and incubated at 37°C for a further hour. The digested products were analysed on a 1% agarose gel and extracted. The insert and the vector were ligated at a molar ratio of 4:1 respectively, using Ligation High (Toyobo) in a total reaction volume of 3 µl at 16°C for 30 minutes. All 3 µl of the ligation reaction was used for transformation into bacterial cells.

2.2.2 Transformation of competent cells

Chemically competent *E.coli* DH5-α cells (Invitrogen) were thawed on ice and cells were added to the DNA at a 1:10 volume ratio. Cells were incubated on ice for 30 minutes and then heated to 42°C for 45 seconds, before being incubated on ice for a further 2 minutes. 100 µl of super optimal broth with catabolite repression (S.O.C) medium (Invitrogen) was added to the cells and they were incubated with shaking at 37°C for 1 hour. The cells were plated onto 100 µg/ml ampicillin plates and incubated overnight at 37°C.

2.2.3 Plasmid preparation and sequencing

For small scale plasmid preparation, 2 ml cultures of luria broth (LB) medium (10 g bacto-tryptone, 5 g yeast extract, and 10 g NaCl per litre) containing 100 µg/ml ampicillin were inoculated with a bacterial colony and incubated with shaking at 37°C overnight. The following day 2 ml of the culture was pelleted and the plasmid DNA was extracted using a QIAprep spin miniprep kit (QIAGEN), according to the manufacturer's instructions.

For large scale plasmid preparation 100 ml cultures of LB medium, containing 100 µg/ml ampicillin, were inoculated with 200 µl of the small scale culture

described above and incubated with shaking at 37°C overnight. The plasmid DNA was extracted using a QIAfilter maxiprep kit (QIAGEN), according to the manufacturer's instructions.

1µg of plasmid DNA was digested with 10 U XbaI (Roche) in 1X H buffer (Roche) at 37°C for 2 hours and analysed on 1% agarose gels. Plasmids were sequenced by Geneservice, Oxford (Source Bioscience Plc). Plasmids were sent for sequencing at concentrations of 100 ng/µl with primers at 3.2 pmol/µl.

2.3 Eukaryotic cell culture

293T cells and Vero cells were cultured in DMEM with 10% FCS at 37°C with 5% CO_2. MDBK cells were cultured in MEM with 10% FCS at 37°C with 5% CO_2. The cells were trypsinised by TrypLE express and passaged at a one in ten dilution. Sf9 cells were cultivated in a suspension culture with Insect-XPRESS media supplemented with 1% Penicillin-Streptomycin, incubated at 27°C with shaking at 90 rpm. Cells were counted every 48 hours using a hemocytometer and light microscope and split to ~0.5-0.8 x 10^6 cells/ml to maintain the culture.

2.4 Influenza virus handling

2.4.1 Reverse genetics

Wildtype and recombinant influenza A/WSN/33 (H1N1) viruses were rescued using the eight plasmid rescue system (Hoffmann and Webster, 2000). For the rescue of both wildtype and recombinant viruses 293T cells were transfected with pcDNA-NP, pPOLI-NP, pHW2000-PB1, pHW2000-PB2, pHW2000-PA, pHW2000-HA, pHW2000-NA, pHW2000-NS and pHW2000-M, using

Lipofectamine-2000 transfection reagent (Invitrogen), according to the manufacturer's instructions. For the negative control pcDNA-NP was omitted. Cells in DMEM (10% FCS) were incubated at 37°C with 5% CO_2 for 24 hours and then the media was replaced with DMEM (0.5% FCS). After a further 48 hour incubation, the media was collected and transferred onto MDBK cell monolayer (80% confluent) and incubated at room temperature for 1 hour. Following the 1 hour incubation, MEM (0.5% FCS) was added to the cell monolayer and the cells were incubated at 37°C with 5% CO_2 for 72 hours. The media was then collected, centrifuged to remove cell debris, and aliquoted for storage at -80°C. Recombinant viruses were generated by introducing mutations into the pPOLI-NP plasmid. Viral titre of the media collected was determined by plaque assay.

2.4.2 Plaque assay

Wildtype and recombinant A/WSN/33 virus was serially diluted in MEM (0.5% FCS). MDBK cells were infected as monolayers in 35 mm dishes with 200 µl of virus for 1 hour at room temperature. After aspiration of the virus inoculum, 2 ml of agarose solution (2% agarose in PBS plus an equal volume of MEM with 0.5% FCS) was overlayed. The dishes were incubated at 37°C with 5% CO_2. At 48 hours post-infection the agarose was removed and the plaques were stained using 2 ml of Coomassie Brilliant Blue dye in 7.5% glacial acetic acid and 50% ethanol.

2.4.3 Growth curve analysis

Virus stocks were diluted in MEM (0.5% FCS) to produce a multiplicity of infection (MOI) of 0.001. Monolayers of 293T were infected with virus for 1 hour at room temperature. After this the virus was aspirated and MEM (0.5% FCS) was

added to the cells which were incubated at 37°C with 5% CO_2. A fraction of the medium was harvested at each time point, as indicated, clarified by centrifugation and aliquoted for storage at -80°C. Viral titres were determined by plaque assay.

2.5 RNA analysis

2.5.1 RNP reconstitution assay

Transfections for RNP reconstitution assays were performed in human kidney 293T cells in 35mm dishes (approximately 10^6 cells) by using a 1:1 ratio of Lipofectamine 2000 (Invitrogen) to DNA. 1 μg of each of the relevant plasmids were used, except where indicated otherwise, with 500μl of opti-MEM (Invitrogen) in 1.5 ml of DMEM (10% FCS). Cells were harvested at 28-40 hours post-transfection.

2.5.2 TRIzol extraction

RNA from transfected cells in 35mm dishes was isolated using 500 μl of TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. 100 μl of chloroform was added, followed by centrifugation at 17,000g for 15 minutes at 4°C. The clear supernatant was then removed (400 μl) and added to a clean microcentrifuge tube with 350 μl of isopropanol and centrifuged at 17,000g for 15 minutes at 4°C. The pelleted RNA was washed in 70% ethanol, dissolved in 30 μl of nuclease-free water and stored at -20°C.

2.5.3 Labelling of primers

Primers (10 pmol/µl), to detect positive and negative sense viral RNA (table 2.4), were radiolabelled using 2.5 U of PNK (Roche) with Adenosine 5'-Triphosphate [γ-^{32}P] ([γ-^{32}P]ATP ~6000 Ci/mmol (10 mCi/ml) Perkin Elmer, U.S.A). Residual γ-^{32}P-ATP was removed using a QIAGEN nucleotide removal kit according to the manufacturer's instructions and the primers were eluted in 30 µl nuclease-free water and stored at -20°C. A primer detecting cellular 5S rRNA-100 (5'-TCCCAGGCGGTCTCCCATCC-3'), used as a loading control in the primer extension assay, was also radiolabelled and was eluted in 50 µl nuclease-free water.

2.5.4 Primer extension analysis

1/60th of each of the RNA samples isolated by TRIzol extraction was analysed by primer extension (see figure 2.1). RNA was mixed with 0.25µl of each ^{32}P-labelled DNA primers in 5 µl and denatured by heating at 95°C for 3 minutes. The mixture was cooled on ice and transferred to 50°C for 2 minutes prior to the addition of 5 µl of a 2X transcription mix (2X First Strand Buffer (Invitrogen), 20 mM DTT, 1 mM dNTP mix and 50 units SuperScript III RNase H- reverse transcriptase (Invitrogen)) also heated to 50°C. The reaction was stopped after 1 hour at 50°C and then raised to 70°C for 10 minutes to heat-inactivate the transcriptase. The samples were then held at 4°C while 8 µl of formamide mix (90% formamide (Sigma), 10 mM EDTA, bromophenol blue and xylene cyanol) was added to each sample. The samples were then heated at 95°C for 3 minutes.

Construct	Name	vRNA primer	Name	mRNA primer
pPOLI-NP-47nt	NP 47-	5'AAGCAGGGTAGACTAGTAAAGAAA 3'	NP 47+	5' TTACTAGTCTACCCTGCTTTTGC 3'
pPOLI-NP-76nt	NP 76-	5' GCAGGGTAGATAATCACTCACAGA 3'	NP 76+	5' TGATTTCGATGTCACTCTGTGAG 3'
pPOLI-NP-80nt				
pPOLI-NP-85nt				
pPOLI-NP-91nt				
pPOLI-NP-95nt				
pPOLI-NP-101nt				
pPOLI-NP-125nt				
pPOLI-NP-149nt	NP 149-	5' ATTTCTTCGGAGACAATGCAG 3'	NP 149+	5' TAAGATCGTTTGGTGCCTTTG 3'
pPOLI-NP-172nt				
pPOLI-NP-197nt				
pPOLI-NP-221nt				
pPOLI-NP-246nt				
pPOLI-NP-287nt				
pPOLI-NP-583nt				
pPOLI-NS-60nt	NS 60-	5' GCAGGGTGACAAAGACATAA 3'	NS 60+	5' CTTTGTCACCCTGCTTTTG 3'
pPOLI-NS-172nt	NS 172-	5' GCTTGAAGTGGAGCAAGAGA 3'	NS 172+	5' AGCTTGACACAGTGTTTGGA 3'
pPOLI-NS	NS-734	5' TGATTGAAGAAGTGAGACACAG 3'	NS1-226	5' CGCTCCACTATTTGCTTTCC 3'
pPOLI-NA	NA-1280	5' TGGACTAGTGGGAGCATCAT 3'	NA-160	5' TCCAGTATGGTTTTGATTTCCG 3'
pPOLI-PB1	PB1-2208	5' TGATTTCGAATCTGGAAGGA 3'	PB1-146	5' TCCATGGTGTATCCTGTTCC 3'
pPR-NA	NA-1405	5' ACAATTGCTGTGGGACACTG 3'	NA-110	5' TAACACTCCCCCTGATGTGAG 3'

Table 2.4. Primers used in primer extension assays to analyse vRNA and mRNA.

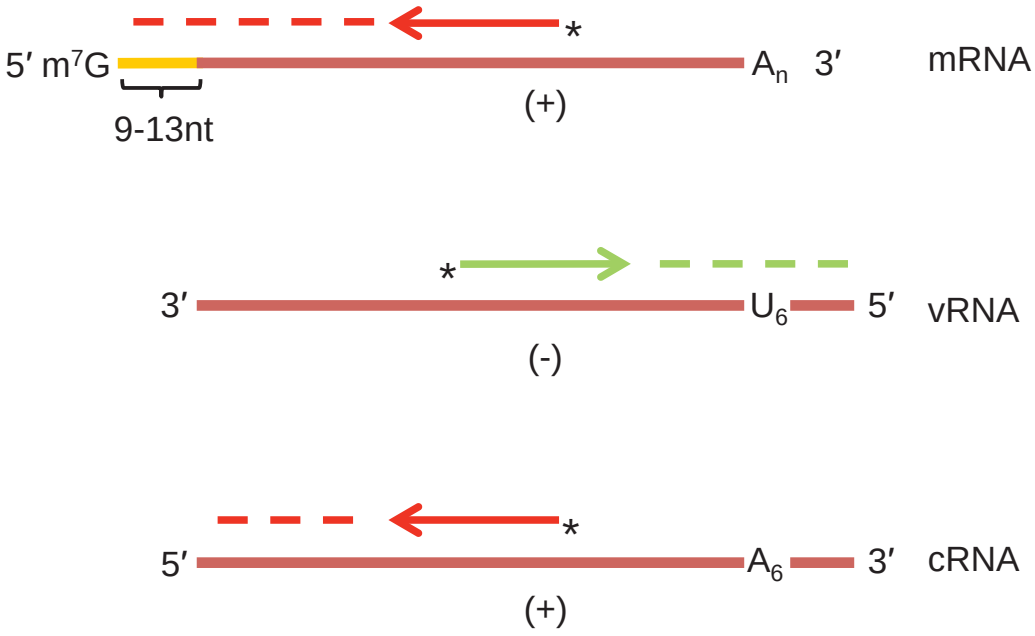


Figure 2.1. Primer extension method. Annealing positions of primers for the detection of mRNA, cRNA and vRNA by primer extension. * denotes radiolabel.

2.5.5 Polyacrylamide electrophoresis

Transcription products from the primer extension assay were analysed on polyacrylamide gels containing either 6% or 12% acrylamide/bisacrylamide (19:1) (National Diagnostics, ultra-pure) in 7 M urea and 1X TBE (90 mM Tris base, 90 mM orthoboric acid and 2.5 mM EDTA). The gel was polymerized by the addition of a final concentration of 0.1% ammonium peroxodisulphate (APS) and 0.01% TEMED. 10 µl of each sample was loaded onto the gel, directly from 95°C, and run at 1250 volts for 1.5-3 hours in 1X TBE buffer. Bands were detected by autoradiography, using Kodak Biomax intensifying screens. Quantitation was performed using Fujifilm imaging plates and a Fujifilm FLA-5000 fluorescent image analyser. Data was analysed using AIDA software (Raytest). A one-sample, two tailed statistical t-test was used to compare accumulation of mRNA and vRNA in the presence or absence of NP and in the presence of different NP mutants compared to wildtype NP from three independent experimental-repeats.

2.5.6 Poly(A)-containing RNA separation

Transfection was carried out in 60 mm dishes (approximately $3x10^6$ cells) with 3 µg each of the corresponding plasmids, 15 µl of Lipofectamine 2000 (Invitrogen) and 500 µl of opti-MEM (Invitrogen) in 3 ml of DMEM (10% FCS). 48 hours post-transfection RNA extraction was carried out using 1 ml of TRIzol reagent (Invitrogen) as before and the RNA was dissolved in 50 µl of nuclease-free water prior to separation on oligo(dT)-cellulose. The protocol for a

commercial kit for mRNA isolation (Micro-Fast Track, Invitrogen) was modified so that both poly(A)- and poly(A)+ RNA fractions were retained, as described previously (Pritlove et al., 1999). The total RNA sample was denatured, by heating to 95°C for 10 minutes, before being added to the oligo(dT)-cellulose. One fourth of an oligo(dT) cellulose tablet was mixed with 40 µl RNA in 200 µl of binding buffer (0.5 M NaCl, 10 mM Tris-HCl pH 7.5) and incubated with shaking at room temperature for 1 hour. The oligo(dT)-cellulose mixture was pelleted in a desktop microcentrifuge at 13,000 rpm for 10 seconds. The supernatant was collected as the poly(A)- fraction. The pellet was washed twice in binding buffer (1 ml) at room temperature, followed by incubation in 200 µl of pre-warmed low-salt buffer (0.25 M NaCl, 10 mM Tris-HCl pH 7.5) at 37°C for 15 minutes and then repelleted. After washing the pellet with an additional 200 µl of low-salt buffer for 15 minutes at room temperature, the bound poly(A)+ RNA was eluted with 200 µl pre-warmed RNase-free water by incubating at 50°C for 15 minutes, followed by repelleting and collection of the supernatant (poly(A)+ fraction). The poly(A)+ and poly(A)- fractions were then treated with TRIzol, as per the manufacturer's instructions, to extract the RNA which was then analysed by primer extension.

2.6 Protein purification and analysis

2.6.1 Expression of NP in insect cells

2.6.1.1 Integration of NP gene into the MultiBac Baculoviral genome

Electrocompetent DH10MultiBac cells were produced by the protocol detailed in Bieniossek et al., 2008, by Narin Hengrung at the Sir William Dunn School of Pathology. 10 ng of PFL-NP, wildtype or mutant, was incubated with 50 µl of electrocompetent DH10MultiBac cells and electroporated at 2.5 kV pulse (Bieniossek et al., 2012; Bieniossek et al., 2008). 1 ml of 2X Yeast extract tryptone (YT) media (16 g bacto-tryptone, 10 g yeast extract, and 5 g NaCl per litre) was added to the cells and they were incubated at 37°C overnight. This overnight cell suspension was then plated out onto 2X YT plates supplemented with ampicillin (100 µl/ml) and gentamicin (10 µl/ml) and incubated at 37°C for 30 hours. Integration of the NP gene into the MultiBac genome was confirmed by colony PCR with a pFL reverse primer and NP specific forward primer (table 2.5). Colonies were picked from the 2X YT plates and added to a 10 µl reaction mix (1X GoTaq buffer (Promega), 0.25 µl GoTaq polymerase (Promega), 0.2 µM dNTP mix (Fermentas) and 250 nmol each primer). The reaction was incubated in a thermocycler at 95°C for 30 sec, 57°C for 45 sec and 72°C for 1 minutes, for a total of 24 cycles. The positive colonies were picked and used to inoculate 5 ml 2X YT cultures with ampicillin (100 µl/ml) and gentamicin (10 µl/ml) which were incubated overnight at 37°C. To extract the Bacmid DNA the cells from the overnight culture were pelleted and lysed following the protocol provided in the

pFL-MCS2-rev	5' CTCGACGAAGACTTGATCACC3'
NP-R74A, R75A-for	5' CGCTTTCGACGACGAGGGTGCTAACAAGTAC CTGGAAGAACACCC 3'

Table 2.5. Primers used in colony PCR to identify the integration of the NP gene into the MultiBac genome.

QIAGEN miniprep kit using solution I, II and III. Adding an equal volume of isopropanol to the supernatant precipitated the Bacmid DNA, which was pelleted by centrifugation (17,000 g, 20 minutes, 4°C). The pellet was then washed twice in 70% ethanol and dissolved in 10 mM Tris-HCl, pH 8.

2.6.1.2 Transfection of insect cells with Bacmid genome and generation of virus

Freshly seeded Sf9 cells, at 0.5 x10^6, in a 6-well tissue culture plate were left to adhere for 15 minutes at 27°C in Insect-XPRESS antibiotic-free medium. For each transfection 4 µg of Bacmid DNA and 6 µl of Lipofectin (Life Technologies) was mixed with 100 µl of Insect-XPRESS antibiotic-free medium, incubated at room temperature for 20 minutes, and then added to the adhered cells. The plates were then sealed with parafilm and incubated at 27°C for four days, when the media (V_0) was harvested and stored at 4°C in the dark. The titre of these viruses was not determined.

2.6.1.3 Virus amplification

For the generation of V_1 virus, 3 ml of V_0 supernatant was added to 50 ml of Sf9 cells at a density of 0.5 x10^6 cells/ml. The virus was harvested 48 hours after cell proliferation arrest by centrifugation (800g, 5min) and stored in the dark at 4°C. The cell pellet was analysed by SDS-PAGE and SimplyBlue staining and/or Western blotting for the expression of recombinant NP. Once NP expression was confirmed V_2 stocks were generated by adding 5 ml of V_1 to 400 ml of Sf9 cells at

a density of 0.5 x10^6 cells/ml. The virus (V_2) was harvested 48 hours after cell proliferation arrest by centrifugation (800g, 5min) and stored in the dark at 4°C.

2.6.1.4 Virus infection and protein production

The day before infection Sf9 cells were split at 0.6x10^6 cells/ml with different volumes of starting culture due to the different expression levels of the different mutants. The cells were infected with the corresponding amount of V_2 virus stock to achieve an MOI of approximately 1, causing inhibition of proliferation after 48 hours. The cells were incubated at 27°C at 120 rpm for 72 hours. After 72 hours the cells were lysed by sonication (15 μm oscillation, 5X 30 seconds on/ 30 seconds off) in lysis buffer (150 mM NaCl, 50 mM Hepes pH 8, 25% Glycerol, 0.5% igepal, 1 mM β-mercaptoethanol, 1X protease inhibitor tablet, 1X PMSF and 100 μg/ml RNase A) on ice. The lysate was cleared by ultracentrifugation (35,000 g, 45 minutes, 4ºC) and the supernatant was added to washed IgG sepharose 6 fast flow beads (GE Healthcare, 2 ml per litre of expression culture). The lysate was incubated with the sepharose, rotating at 4°C for 4 hours. The sepharose was washed 3X 10 minutes in wash buffer (150 mM NaCl, 1 mM Hepes pH 8, 10% Glycerol, 0.1% igepal, 1X PMSF) and 1X 10 minutes in cleavage buffer (wash buffer plus 1 mM DTT), pelleting the sepharose in-between each wash. The sepharose was incubated in 1.05 ml of cleavage buffer with 10.5 μl AcTEV protease overnight at 4°C. The supernatant was removed from the beads after centrifugation (4000g, 4 minutes, 4ºC) and either stored at 4ºC for protein analysis via SDS-PAGE gel and SimplyBlue staining or size-exclusion chromatography.

2.6.1.5 Analysis of NP via size-exclusion chromatography

500 µl of the NP sample was applied to an equilibrated Hi-Load Superdex 200 10/300 (GE healthcare) filtration column at room temperature in running buffer (150 mM NaCl, 50 mM Hepes pH 8, 25% Glycerol) connected to an ÄKTA protein purification system (GE Healthcare). Fractions from the column were collected every 0.5 ml and 10 µl of each fraction was analysed by adding 10 µl of 2X protein loading buffer (PLB) and running the samples on a SDS-PAGE followed by SimpleBlue staining (see section 2.7.2). A picture of the gel was taken and analysed using AIDA software (Raytest) for the band intensity in each fraction. The total band intensity across all fractions was summed and then the intensity of the band in each fraction was compared to the total intensity. This data was then plotted using Prism.

2.6.2 Ni-affinity purification of NP expressed in mammalian cells

Transfection was carried out in 10 cm dishes (approximately $9x10^6$ cells) with 9 µg each of the corresponding plasmids, 67.5 µl of Lipofectamine 2000 (Invitrogen) and 800 µl of opti-MEM (Invitrogen) in 8 ml of DMEM (10% FCS), to reconstitute RNP-like complexes with a PA-His6 tag. 30 hours post transfection the cells were harvested, lysed in 1.5ml of Tris-lysis buffer (50 mM Tris-HCl pH 8, 200 mM NaCl, 20 mM imidazole, 33% glycerol, 0.5% igepal CA-630, 1 mM DTT and 1X complete mini EDTA-free protease inhibitor cocktail tablet) at 4°C for 1 hour and centrifuged at 13000rpm for 5 minutes. The cell lysate was then diluted 1 in 5 in Ni-agarose binding buffer (20 mM Tris-HCl pH 8, 200 mM NaCl, 20 mM imidazole, 1 mM $MgCl_2$ and 1X complete mini EDTA-free protease inhibitor cocktail tablet) and incubated with 50 µl Ni-NTA agarose

(QIAGEN) for 4 hours at 4 ºC. After the Ni-NTA agarose was washed five times in Ni-agarose wash buffer (20 mM Tris-HCl pH 8, 200 mM NaCl, 20 mM imidazole, 1 mM $MgCl_2$, 10% glycerol, 0.1% igepal CA-630), 800 µl of cleavage buffer (Ni-agarose wash buffer, 250 mM imidazole) was added to the Ni-NTA agarose and incubated at 4 ºC for 2 hours before pelleting the Ni-NTA at 13000 rpm for 5 minutes and removing the supernatant for analysis by western-blot.

2.6.3 Myc immunoprecipitation

Transfection was carried out in 35 mm dishes (approximately $3x10^6$ cells) with 2 µg each of the corresponding plasmids, 4 µl of Lipofectamine 2000 (Invitrogen) and 500 µl of opti-MEM (Invitrogen) in 3 ml of DMEM (10% FCS) to reconstitute NP-NP polymers with a myc tag. 30 hours post transfection the cells were harvested and lysed in lysis buffer (50 mM Tris-HCl pH 8, 200 mM NaCl, 1 mM EDTA, 33% glycerol, 0.5% igepal, 1 mM DTT, 1X complete EDTA-free protease inhibitor mixture (Roche)) at 4°C. A small proportion of the lysate was kept and the remaining lysate was split into two fractions where one was treated with RNase A (100 µg/ml). The two fractions were then split again and to one of each of these two fractions 0.5 µl of myc-antibody (table 2.1) was added. These lysates were rotated for 4 hours at 4°C and then incubated with protein A sepharose beads (Sigma) overnight at 4°C. The protein A beads were washed in wash buffer (200 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% igepal, 1X PMSF) prior to the addition of cell lysates. The beads were then washed 4X 10 minutes in wash buffer. After the final wash the beads were pelleted by centrifugation (1000g, 4 minutes, 4 °C) and re-suspended in 100 µl 1X PLB.

2.6.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyse proteins. Each sample was mixed with an equal volume of 2X PLB (100 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 3 mM β-mercaptoethanol and bromophenol blue) and denatured by heating to 95°C for 5 minutes prior to loading onto the gel.

SDS-PAGE was performed using a 'mini-protean' protein gel apparatus (Bio-Rad). An 8% resolving gel (8% (v/v) acrylamide/bisacrylamide (29:1) in 375 mM Tris-HCl pH 8.8 and 0.1% SDS) and a 3.2% stacking gel (3.2% (v/v) acrylamide/bisacrylamide (29:1) in 375 mM Tris-HCl pH 8.8 and 0.1% SDS) were used in all cases. The gel was polymerised by the addition of a final concentration of 0.1% ammonium peroxodisulphate (APS) and 0.01% TEMED. The gels were run at 120 volts in SDS-PAGE running buffer (25 mM Tris base, 250 mM glycine and 0.1% SDS) for 1 hour 15 minutes. A 'precision plus protein all blue standards' (Bio-Rad) pre-stained protein marker was included in parallel with the samples. Proteins were visualised by staining with SimplyBlue SafeStain (Life Technologies), following the manufacturer's instructions.

2.6.5 Western blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose (Amersham Biosciences) using a mini trans-blot cell (Bio-Rad) at 100 volts for 1 hour. The nitrocellulose blot was then blocked in 2.5% non-fat milk (Marvel) in PBS containing 0.1% Tween-20 either for 1 hour at room temperature or overnight at 4°C on a rocker. The blot was then incubated with primary antibody (see table 2.1 for details of antibodies used). Blots were washed for 3X 10 minutes in PBS (0.1% Tween-20) and then incubated for 1 hour with the secondary antibody

conjugated to horseradish peroxidase. The blots were developed by chemiluminescence using Amersham ECL Western blotting system (Amersham Biosciences). Blots were exposed to X-ray film (Super RX Fuji Medical X-Ray film, Kodak) for varying lengths of time.

2.6.6 Immunofluorescence

Vero cells were seeded on 13 mm coverslips in 24-well plates. The Vero cells, when approximately 70% confluent, were transfected as a monolayer using 1 µg of the required plasmid and 1 µl Lipofectamine-2000 (Invitrogen). Cells were incubated at 37 °C, 5% CO_2 for 24 hours. After 24 hours the cells were washed with PBS and fixed (4% paraformaldehyde in 250 mM HEPES (pH 8)) for 15 minutes at room temperature. Following fixation the cells were permeabilised by adding 1 ml of 0.5% Triton X-100 for 15 minutes at room temperature and blocked for 1 hour at 4 °C in blocking solution (PBS containing 1% BSA and 0.2% gelatine). Immunostaining has been described previously (Fodor and Smith, 2004). Primary antibody (table 2.1) was added in blocking solution for 1 hour, followed by five PBS washes. Secondary antibody (Cy3-conjugated) was added in blocking solution for 1 hour, followed by five PBS washes. Coverslips were mounted in Mowiol containing 0.001% 4′,6′-diamidino-2-phenylindole (DAPI Sigma). Cells were viewed by using a Zeiss microscope with a Zeiss ×63/1.25 oil immersion objective, and the images were processed using ImageJ.

3 The role of NP in viral transcription and replication

3.1 Overview

NP is essential for viral transcription and replication to occur on a full-length vRNA template, and therefore plays a key role within the RNP complex. Yet there is a limited understanding of NP's contribution to RNP complex activity. Within the complex, NP binds to the viral RNA in a non-sequence specific manner and self-oligomerises along the length of the RNA that is not bound by the RNA polymerase. This chapter investigates the role of NP in RNA polymerase transcription and replication by studying polymerase activities on different length vRNA-like templates and their dependency on the presence of NP, the presence of NP-RNA binding and the presence of NP-oligomerisation. Taken together these data suggest that NP is not required to determine the initiation or termination mode of the viral RNA polymerase but that NP acts as an elongation factor in both viral transcription and replication. The results also suggest that different properties of NP, i.e. RNA binding and oligomerisation, may play differential roles in transcription versus replication.

3.2 Background

NP of influenza virus is a RNA binding protein and a major component of the RNP complex. The RNP complex, with a full-length genomic RNA template, forms a double helical structure which is stabilised though intra- and inter-strand interactions between NP monomers (Arranz et al., 2012; Moeller et al., 2012). Visualisation of a biologically active RNP-like structure, containing a 248-nucleotide template, showed that NP formed a loop structure rather than a double helix (Coloma et al., 2009; Martín-Benito et al., 2001). This suggests that the formation of the double helical structure is template length dependent and that the double helix is not required for replication or transcription of intermediate-length templates by the RNA polymerase. Unlike the formation of the NP double-helical structure, NP-RNA binding is essential for RNP complex activity and is required for the formation of the complex (Ng et al., 2008; Vreede et al., 2011). Oligomerisation of NP is also essential for RNP complex activity; however, due to the ability of oligomerisation mutants to stabilise cRNA (Vreede et al., 2011) it is thought that monomeric NP can bind to the RNA and form a RNP-like structure.

NP is essential for the formation of a vRNP complex, which acts as a template for both viral transcription and replication. It is unclear whether NP functions in the RNP complex as a structural factor stabilising the RNA in a conformation that is compatible with RNA polymerase activity and/or whether it functions as a cofactor along the length of the RNA, aiding the polymerase in processivity. NP has been implicated as an elongation factor in replication, both *in vitro* and *in vivo*; replication could occur in the absence of NP on short vRNA-like templates *in vivo* (Resa-Infante et al., 2010) and the addition of NP *in vitro* led to the synthesis of longer RNA products by the RNA polymerase (Honda et al., 1990).

NP may aid the RNA polymerase in the elongation phase of both viral transcription and replication by recruiting host factors required by the viral polymerase for the elongation phase. Several host factors have been implicated in viral transcription and replication such as the minichromosome maintenance replicative helicase complex which interacts with NP, stimulates cRNA synthesis *in vitro* and is required for cRNA synthesis *in vivo* (Kawaguchi et al., 2011; Kawaguchi and Nagata, 2007). NP has also been shown to interact with several other host-factors including importin α5, which binds to NP monomers while still retaining NP's RNA binding activity (Boulo et al., 2011; Mayer et al., 2007), and UAP56, acting as a chaperone to facilitate the binding of NP to RNA (Kawaguchi and Nagata, 2007; Momose et al., 2001).

The RNA polymerase carries out viral transcription and replication on the genomic vRNA template despite the two processes being mechanistically distinct (Resa-Infante et al., 2011). It remains unclear what determines the mode of the RNA polymerase in transcription versus replication. Several viral and cellular proteins have been proposed to play a role (see Chapter 1, section 1.4.3- Determining the mode of the viral polymerase). It was widely accepted in the literature that NP played a role in the regulation of transcription versus replication, as NP temperature sensitive mutants were shown to only be able to support transcription and not replication at non-permissive temperatures (Medcalf et al., 1999). The identification of NP-RNA polymerase binding led to a model whereby binding caused the polymerase to switch from a transcriptase to a replicase (Biswas et al., 1998; Newcomb et al., 2009). The binding of NP to the template RNA was also proposed to cause a switch in polymerase function (Klumpp et al., 1997). In contrast several other models suggest that NP is required for the

synthesis and stabilisation of replication products rather than directly altering the initiation mode of the viral polymerase. The encapsidation model proposed that NP not associated with the RNP complex is required for the synthesis of cRNA, through binding to the nascent RNA and preventing the addition of a poly(A) tail (Beaton and Krug, 1986; Shapiro and Krug, 1988), and the stabilisation model proposed that NP binding to cRNA, along with viral polymerase, is required to prevent the RNA from being degraded (Vreede et al., 2004). The findings that NP is not required for the viral polymerase to carry out initiation of transcription or replication *in vitro* (Honda et al., 1988) and the presence of replication products in the absence of NP when a short 46-nucleotide template is used (Resa-Infante et al., 2010) suggest that NP is not required to determine the mode of initiation of the viral polymerase.

The absolute requirement for NP bound along the length of the vRNA template for the formation and activity of RNP complexes makes it difficult to further study the different properties of NP and their role in transcription and replication. In order to gain a greater understanding of the role of NP in RNP complex activity, the ability of the RNA polymerase to carry out transcription and replication on different length vRNA-like templates in the presence of wildtype NP, absence of NP or the presence of different NP mutants was tested by RNP reconstitutions. RNP complexes are reconstituted in cells by the transfection of combinations of plasmids that express the minimal components of the RNP complex, allowing the ability of the RNA polymerase to carry out viral transcription and replication to be studied.

3.3 Aims

- Elucidate the role of NP in supporting RNA polymerase transcription and replication on different length vRNA-like templates
- Understand the requirement for NP oligomerisation and RNA binding in order for NP to support the RNA polymerase in transcription and replication

3.4 Results

3.4.1 The RNA polymerase is active on a short vRNA-like template in the absence of NP

It has been published that a short 46-nucleotide vRNA-like template can be replicated in the absence of NP *in vivo* (Resa-Infante et al., 2010). To investigate if NP plays a role in determining the initiation mode of the RNA polymerase several short vRNA-like templates were constructed. The internal region of segment 5 vRNA was deleted to different degrees, while always retaining the 5´ and 3´ termini containing the promoter regions, which is bound by the RNA polymerase, and the poly(U) stretch near to the 5´ end of the vRNA. These short vRNA-like templates were produced in 293T cells by transfection of plasmids that contained the positive sense sequence of the vRNA-like template under a polymerase I promoter. In addition to the plasmid encoding the vRNA-like template, combinations of plasmids encoding the three subunits of the RNA polymerase (PA, PB1 and PB2) and NP were also transfected to reconstitute RNP complexes. Following incubation to allow transcription and replication of the

reconstituted vRNP complexes to proceed, the RNA from these 293T cells was extracted and analysed by primer extension, allowing quantitative measurement of the level of mRNA and progeny vRNA produced from the vRNA-like template as a measure of transcription and replication, respectively. In agreement with the work published by Resa-Infante et al., a vRNA-like template of 47-nucleotides in length could be replicated in the absence of NP (figure 3.1). In addition the vRNA-like template could also be transcribed in the absence of NP, with mRNA accumulating to the same level as observed in the presence of NP.

Studying longer vRNA-like templates showed that the RNA polymerase was able to transcribe the 76-nucleotide long vRNA-like template at an equivalent level in the absence of NP compared to in the presence of NP. Replication of the 76-nucleotide template was significantly reduced, by approximately 20%, in the absence of NP compared to in the presence of NP. For vRNA-like templates of 101 nucleotides and longer, transcription is reduced by 80% and replication is reduced by 100% in the absence of NP compared to in the presence of NP, with the transcription and replication products barely detectable in the absence of NP for a 149-nucleotide template (figure 3.1). As the requirement for NP drastically increases between template lengths 76- and 101-nucleotides, another set of short vRNA-like templates 80, 85, 91 or 95-nucleotides in length were designed to test what the maximal template length was before the RNA polymerase becomes highly dependent on NP for activity. Replication was significantly reduced in the absence of NP on all length templates tested (80-95-nucleotides) whereas transcription was significantly reduced on the 85-95- nucleotide length templates, when compared to in the presence of NP (figure 3.2).

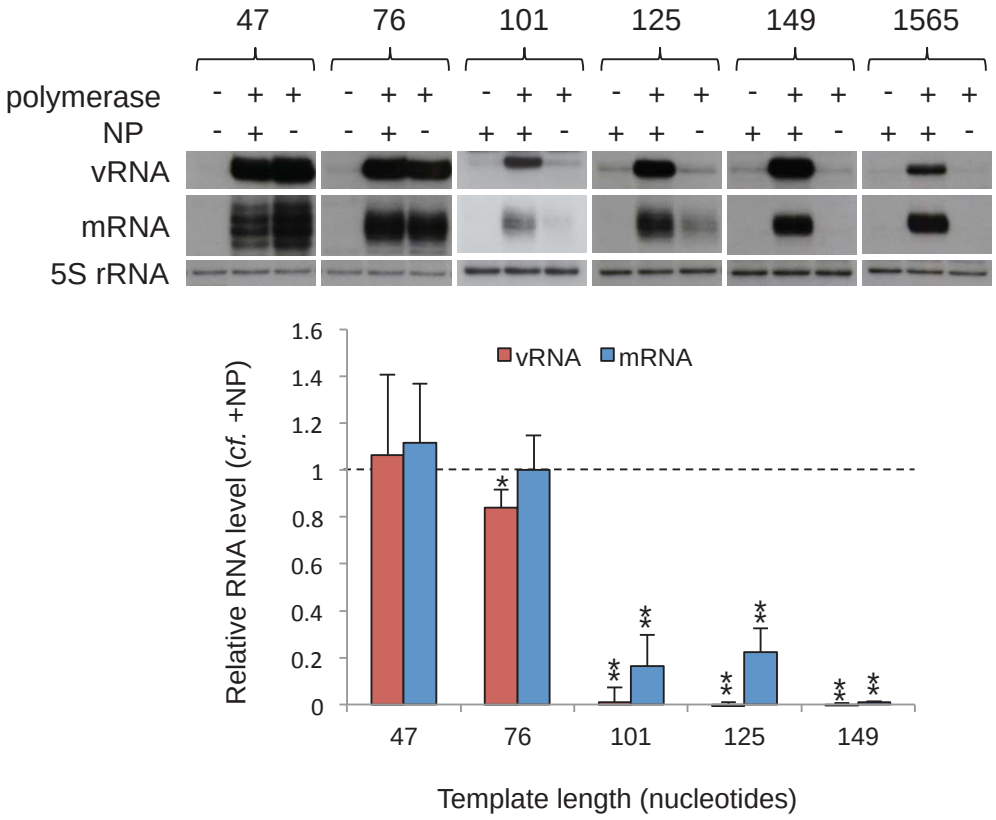


Figure 3.1. Influenza virus RNA polymerase replicates and transcribes short vRNA-like templates *in vivo* in the absence of NP. Combinations of plasmids, at a quantity of 1 µg, expressing the three polymerase subunits and nucleoprotein were transfected into 293T cells as indicated. The length of the template used in each transfection, generated by a pPOLI plasmid encoding vRNA-like templates of different lengths, is shown above each primer extension panel (nucleotides). Total RNA was harvested after 30-40 hours post transfection and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. The data is averaged from at least 3 independent experiments. During data analysis the signal in the negative control is subtracted from positive signals and the level of transcription and replication in the presence of wildtype NP is set to 1 for each length template. Error bars show standard deviation and asterisks are present when there is a significant difference from 1 (one sample *t*-test *p<0.05 **p<0.01). 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

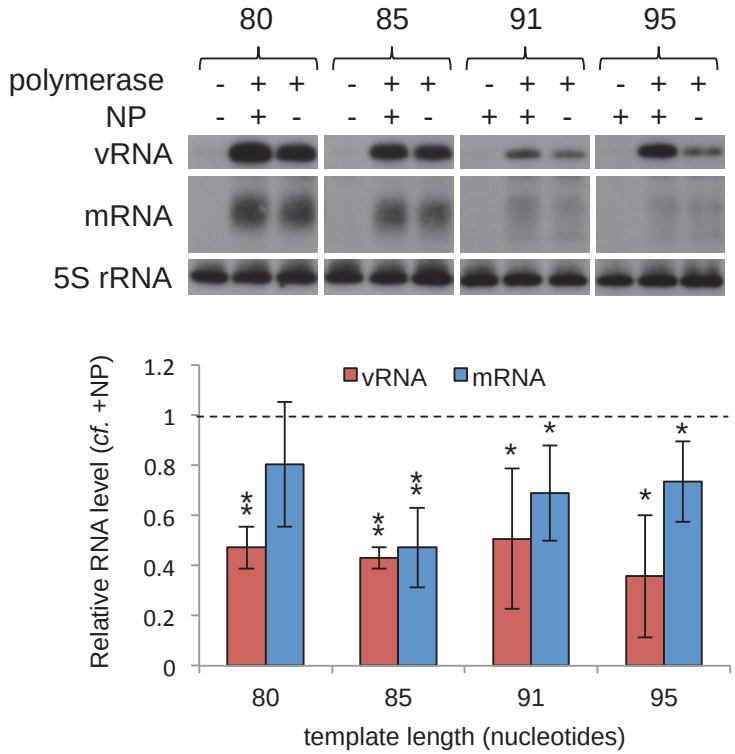


Figure 3.2. The RNA polymerase requires NP to carry out replication and transcription of vRNA-like templates longer than 85-nucleotides. Combinations of plasmids, at a quantity of 1 µg, expressing the three polymerase subunits and nucleoprotein were transfected into 293T cells as indicated. The length of the template used in each transfection, generated by a pPOLI plasmid encoding vRNA-like templates of different lengths, is shown above each primer extension panel (nucleotides). Total RNA was harvested after 30-40 hours post transfection and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. The data is averaged from 3 independent experiments. During data analysis the signal in the negative control is subtracted from positive signals and the level of transcription and replication in the presence of wildtype NP is set to 1 for each length template. Error bars show standard deviation and asterisks are present when there is a significant difference from 1 (one sample *t*-test *p<0.05 **p<0.01). 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

In order to characterise mRNA and vRNA products produced in the absence of NP and to compare them to products produced in the presence of NP, total RNA from each sample was separated by oligo(dT) chromatography and the fractions were analysed by primer extension (figure 3.3). There were no differences in the characteristics of RNA produced in the presence and absence of NP. vRNA and 5S rRNA were enriched in the poly(A)- fraction indicating that they did not contain a stretch of adenine residues. The majority of mRNA was present in the poly(A)+ fraction and this RNA contained a variable 5´ extension, as expected for viral mRNA, due to host cap-snatching.

3.4.2 The concentration of NP affects the activity of the RNA polymerase on a short vRNA-like template, but this is not dependent on NP-RNA binding

Short 47- and 76-nucleotide vRNA-like templates do not require NP for viral transcription and replication. To investigate if NP plays any role in the transcription and replication of these short vRNA-like templates NP titrations were carried out in the presence of the 76-nucleotide template with different concentrations of RNA polymerase (pcDNA-3P) (figure 3.4). As the concentration of NP present increased this led to an increase in transcription and replication of the 76-nucleotide vRNA-like template at all polymerase concentrations, but with the greatest effect at the lowest polymerase concentration. The increase in transcription and replication in the presence of increasing NP concentration was also observed on the shorter 47-nucleotide template (figure 3.5). The recently published polymerase structures show the RNA polymerase

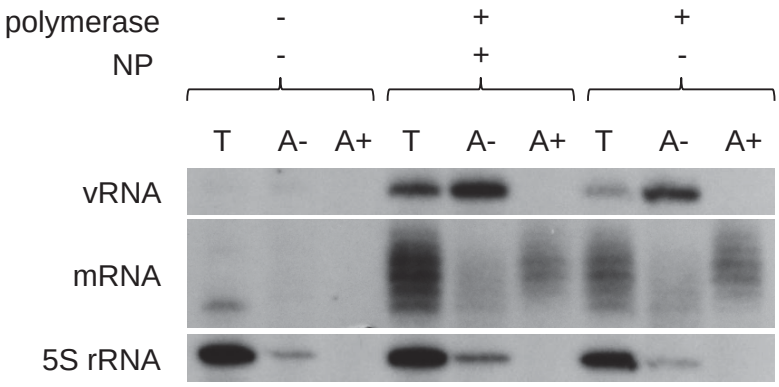


Figure 3.3. mRNA produced from short vRNA-like templates *in vivo* in the absence of NP is polyadenylated. RNP reconstitutions were carried out with 1 μg of each plasmid expressing the three polymerase subunits, and either including or omitting NP expressing plasmid in combination with the pPOLI plasmid that produces the 76-nucleotide template. 30-40 hours post-transfection the RNA from each sample was extracted. The RNA was analysed by primer extension prior to oligo dT chromatography (T Total) and after oligo dT chromatography (poly(A)+ fraction (A+) and poly(A)- fraction (A-)) with the detection of viral transcription (mRNA) and replication (vRNA) products and 5S rRNA.

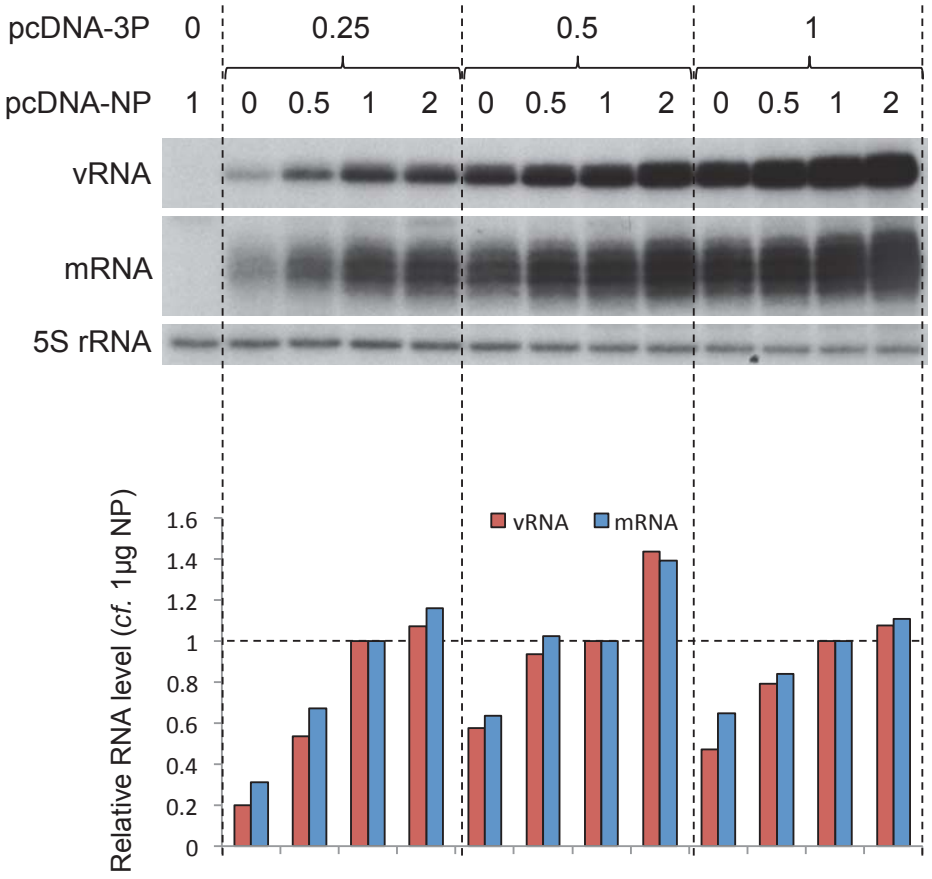


Figure 3.4. Replication and transcription of the short 76-nucleotide vRNA-like template *in vivo* is sensitive to the concentration of NP. Combinations of plasmids expressing the three viral polymerase subunits (pcDNA-3P) and nucleoprotein (pcDNA-NP), at the quantities indicated in µg, were transfected into 293T cells with pPOLI plasmid encoding the 76-nucleotide vRNA-like template. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. During data analysis the signal in the negative control is subtracted from positive signals and the level of transcription and replication in the presence of 1 µg of NP is set to 1 at each polymerase concentration. 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

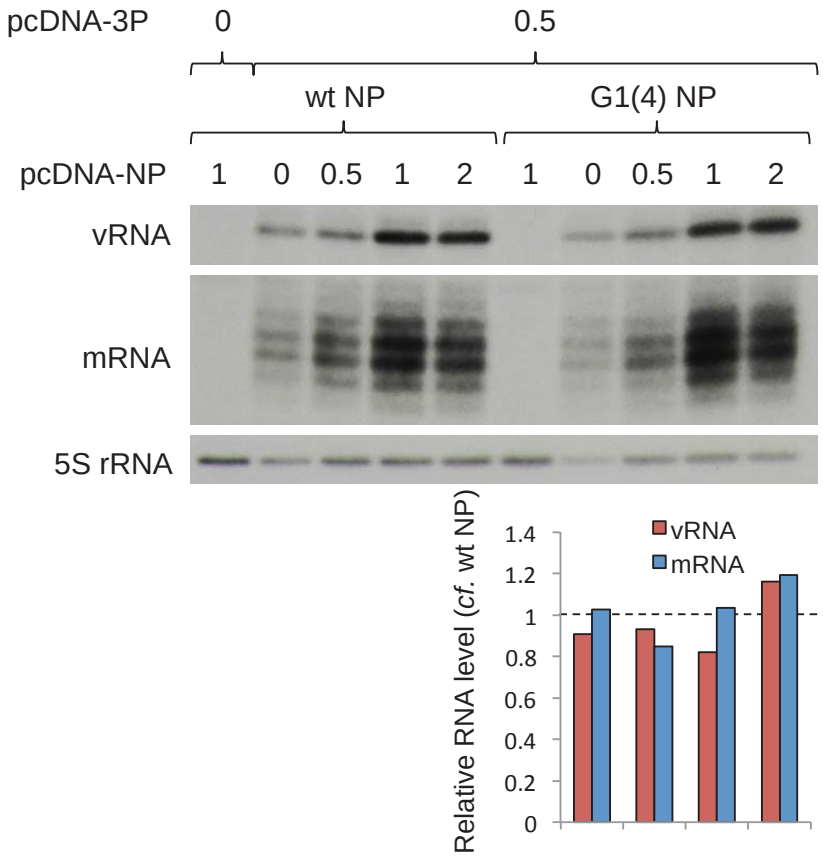


Figure 3.5. The sensitivity of replication and transcription to NP concentration on a short 47-nucleotide vRNA-like template is not dependent on NP binding RNA. Plasmids expressing the three viral polymerase subunits (pcDNA-3P) and nucleoprotein (pcDNA-NP (wildtype)wt/G1(4)), at the quantities indicated in μg, were transfected into 293T cells with pPOLI plasmid encoding the 47-nucleotide vRNA-like template. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. During data analysis the signal in the negative control is subtracted from positive signals. The level of transcription and replication in the presence of each concentration of wildtype NP is set to 1 and the level of transcription and replication at each concentration of mutant NP G1(4) is compared to this. 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

binding to 29 nucleotides of promoter RNA (Reich et al., 2014). It is, therefore, unlikely that the 47-nucleotide template can bind NP, as NP is thought to bind 24 nucleotides of RNA, and after polymerase binding there would be only approximately 18 nucleotides of RNA that could be bound by NP (Compans and Chopin, 1975; Ortega et al., 2000). To test if this NP effect on transcription and replication was dependent on NP-RNA binding the NP titration was carried out in the presence of the well characterised NP-RNA binding mutant G1(4) (Vreede et al., 2011). An increase in transcription and replication of the short 47-nucleotide template was observed in the presence of NP G1(4) (figure 3.5), suggesting that the increase in RNA polymerase activity, when increasing concentrations of NP are present, is not dependent on NP-RNA binding.

3.4.3 The RNA polymerase is active on an intermediate vRNA-like template in the presence of monomeric NP

Neighbouring NP monomers in the RNP complex interact via the tail-loop of one NP monomer binding to the insertion-groove of an adjacent NP monomer. This insertion leads to the formation of a salt-bridge between R416 in the tail-loop and E339 in the insertion-groove. When this salt-bridge is disrupted by mutation of either of these residues to alanine (R416A or E339A), NP can no longer oligomerise and these monomeric NP mutants cannot support RNP complex activity on a full-length viral gene segment (Chan et al., 2010). NP binds to approximately 24 nucleotides and the 76-nucleotide template and the 101-nucleotide template differ in length by 25 nucleotides. It was predicted that the addition of monomeric NP might be able to support RNA polymerase activity on

the 101-nucleotide template. Indeed, monomeric NP, R416A (figure 3.6a) and E339A (figure 3.6b), could support RNA polymerase activity on the 101-nucleotide template to higher levels compared to wildtype NP. In addition monomeric NP could support RNA polymerase replication of vRNA-like templates up to, and including, 221-nucleotides in length at similar levels to wildtype NP. Interestingly, templates of 149- to 221-nucleotides in length showed a significant decrease in transcription, by approximately 50%, in the presence of monomeric NP compared to the presence of wildtype NP. When the length of the RNA template was increased further to 246 nucleotides, the levels of transcription and replication were reduced by approximately 75% and 40%, respectively, when compared to wildtype NP. Only approximately 5% RNA polymerase activity was detected on a 287-nucleotide template in the presence of monomeric NP, when compared to wildtype NP. The NP monomeric double mutant, E339A/R416A, showed the same differential effects on transcription and replication and supported RNA polymerase replication on vRNA-like templates up to 221-nucleotides in length at similar levels to wildtype NP, whereas transcription was significantly reduced (figure 3.6c). The expression and cellular localisation of the oligomerisation NP mutants were similar to that of the wildtype NP (figure 3.7 and 3.8).

Monomeric NP is able to support replication at similar levels to wildtype NP on templates up to 221-nucleotides in length. This led to the question of whether the concentration requirement for monomeric NP was different from that of wildtype NP, on intermediate length templates. To address this, transfections with 0.5μg of each of the plasmids encoding the polymerase subunits and the pPOLI plasmid encoding the 221-nucleotide vRNA-like template in the presence of

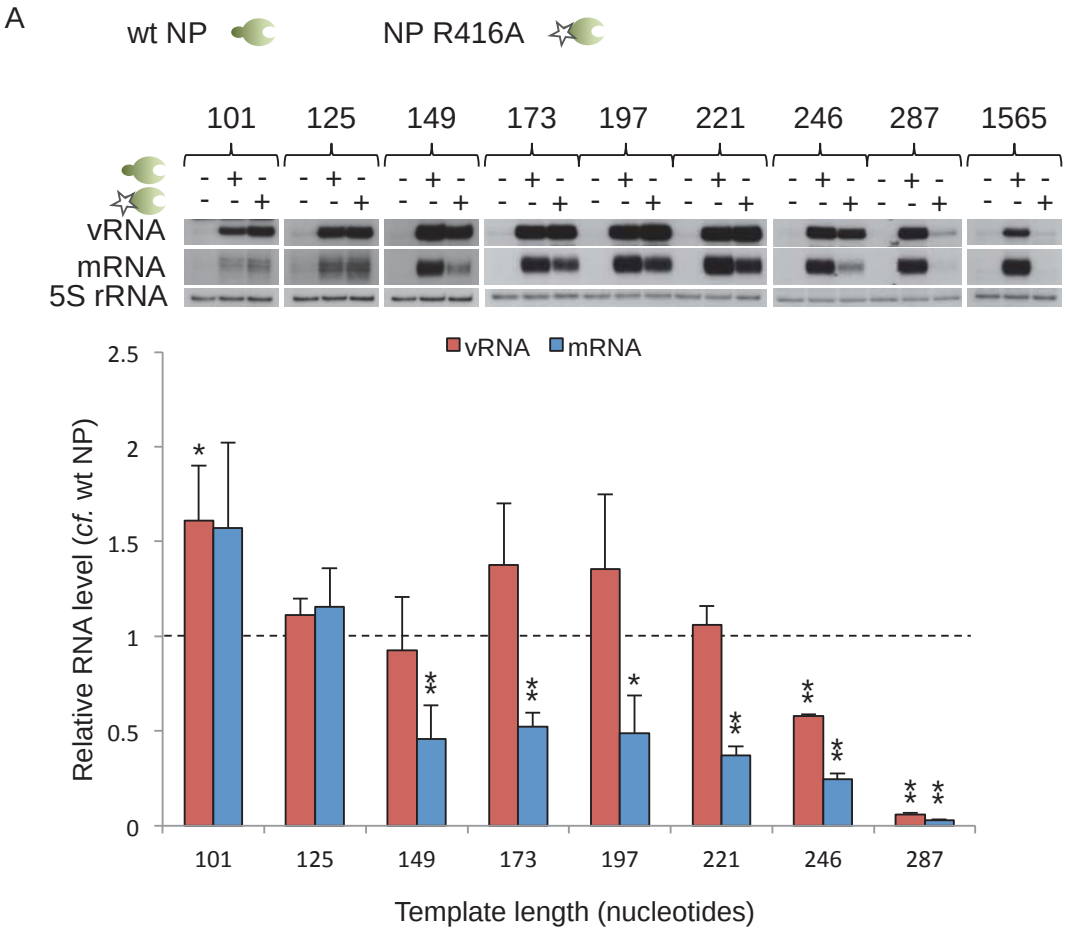


Figure 3.6a. Monomeric NP supports replication and transcription of intermediate length vRNA-like templates.
Plasmids expressing the three viral polymerase subunits in combination with either an empty plasmid or NP expressing plasmid, each at a quantitiy of 1 µg, were transfected into 293T cells. The length of the template used in each transfection, generated by a pPOLI plasmid encoding vRNA-like templates of different lengths, is shown above each primer extension panel (nucleotides). Either R416A (A) or E339A (B) or E339A/R416A (C) NP mutants were studied. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. The data is averaged from 3 independent experiments. During data analysis the signal in the negative control is subtracted from positive signals and the level of transcription and replication in the presence of wildtype (wt) NP is set to 1 for each length template. The level of transcription and replication observed in the presence of NP mutants is then compared to the presence of wildtype NP and this is shown on the graph. Error bars show standard deviation and asterisks are present when there is a significant difference from 1 (one sample *t*-test *p<0.05 **p<0.01). 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

B

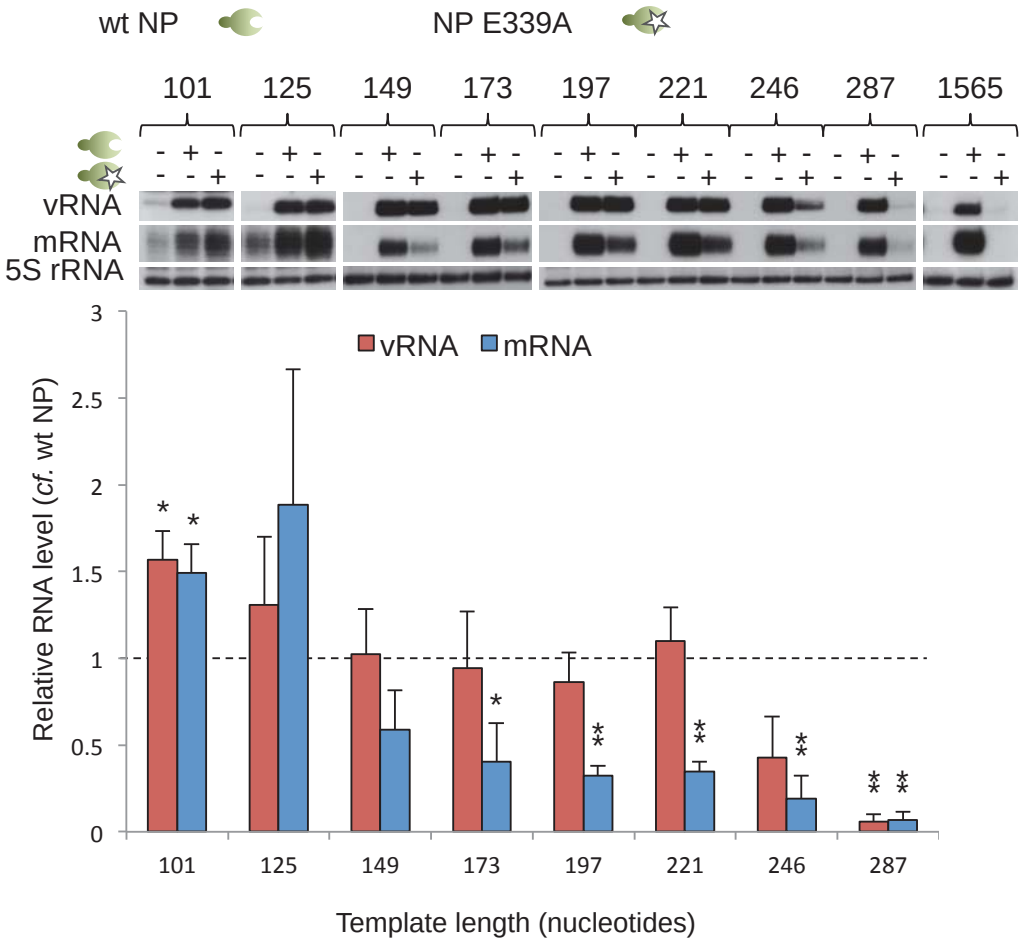


Figure 3.6b. Monomeric NP supports replication and transcription of intermediate length vRNA-like templates.

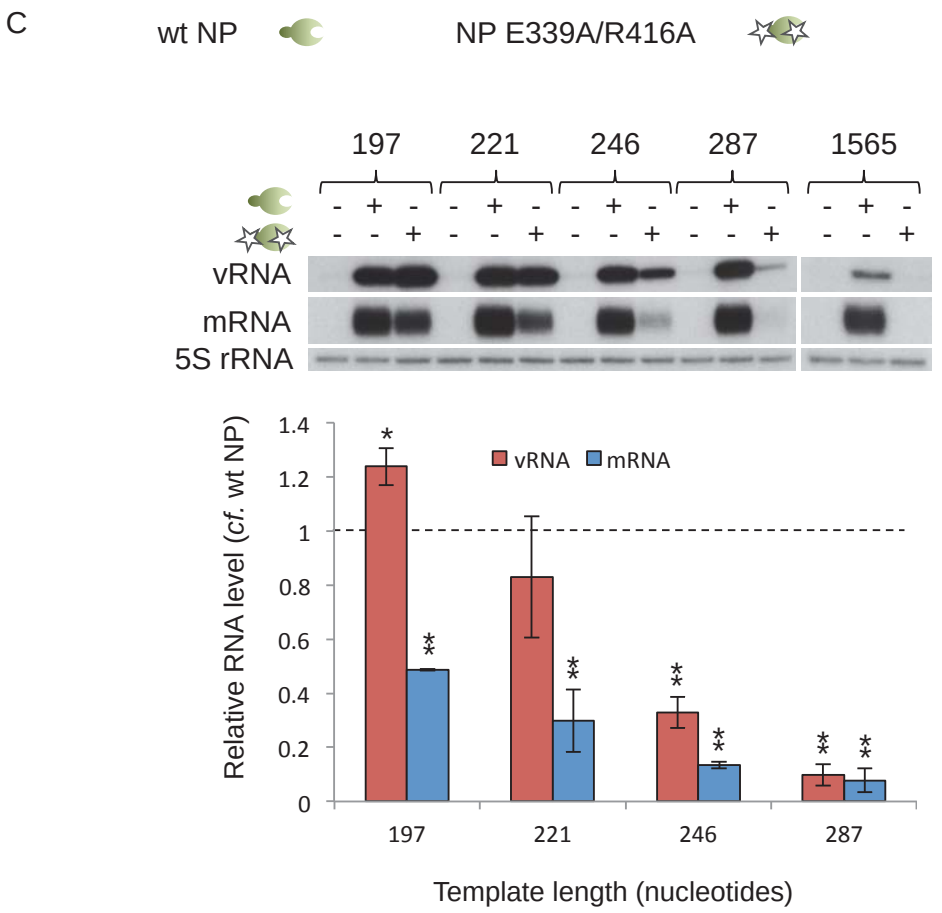


Figure 3.6c. Monomeric NP supports replication and transcription of intermediate length vRNA-like templates.

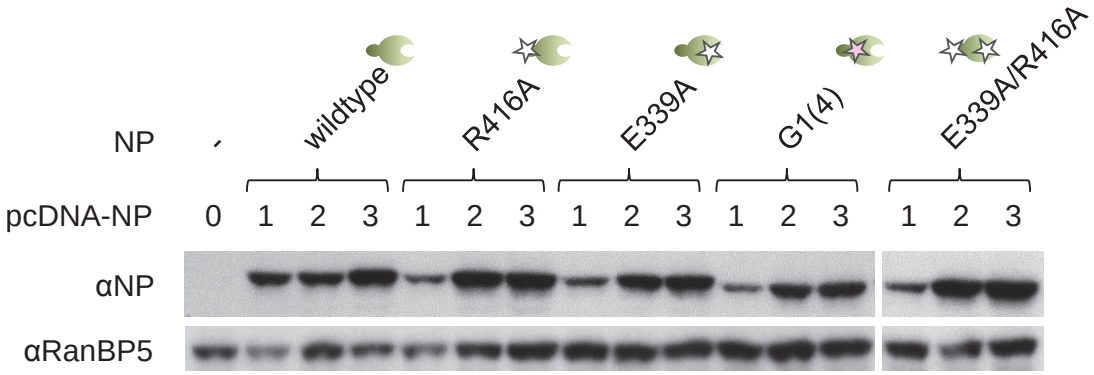


Figure 3.7. The expression levels of wildtype and mutant NPs. 293T cells were transfected with 1 μg of each of the plasmids encoding the polymerase subunits and nucleoprotein, at the quantities indicated in μg, with pPOLI plasmid encoding the 221-nucleotide vRNA-like template. Transfected cell lysates were analysed by western blot with an antibody against NP. An antibody against RanBP5 was used as a loading control.

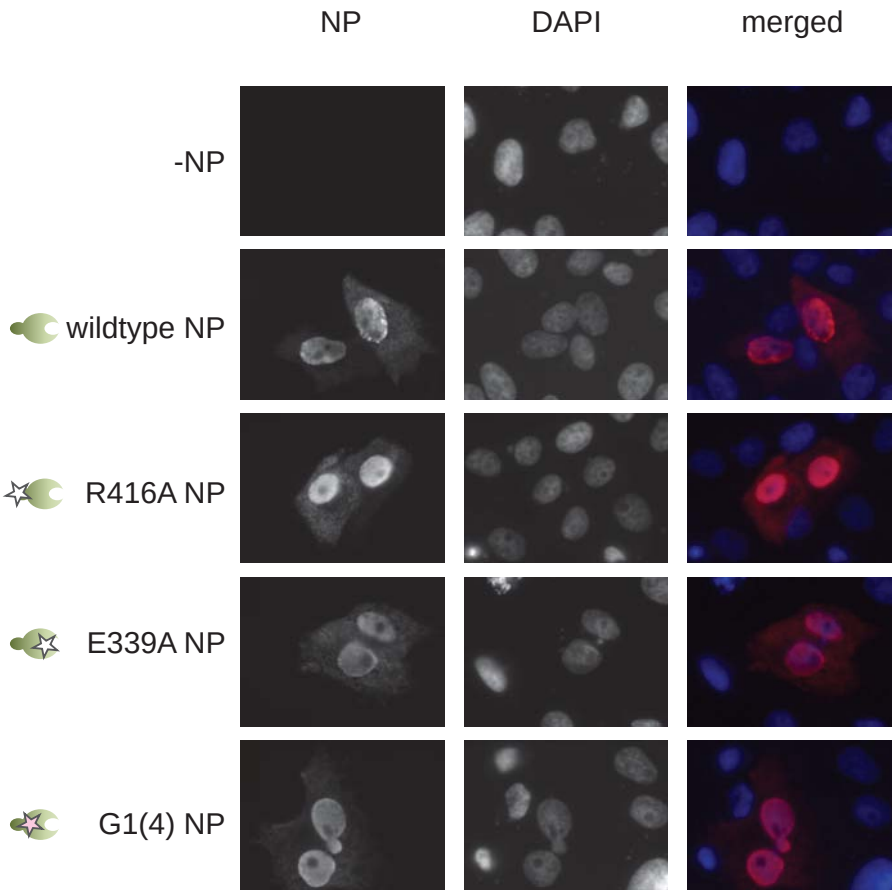


Figure 3.8. The cellular localisation of wildtype and mutant NPs. Vero cells were transfected with plasmids encoding wildtype and mutants NPs. The images above are representative of the localisation observed. DAPI staining (blue) and NP staining (red) were carried out.

increasing concentrations of either wildtype or NP R416A expressing plasmid, while keeping the overall concentration of DNA the same in each transfection, were carried out. The results show that a higher concentration of oligomerisation mutant NP R416A is required, compared to wildtype NP, in order for it to support transcription and replication (figure 3.9). The oligomerisation mutant is less able to support transcription and replication at lower concentrations compared to wildtype NP.

3.4.4 Monomeric NP binds to RNA and forms a functional RNP-like complex in the presence of an intermediate vRNA-like template

Monomeric NP is able to support viral replication of intermediate length templates (101-287-nucleotides) and it was hypothesised that non-oligomerising monomeric NP was binding to the RNA and forming a RNP-like complex. In order to address this, RNP reconstitutions were carried out with the 221-nucleotide template in the presence of a his-tagged PA RNA polymerase subunit. These RNP complexes were then purified by nickel affinity chromatography and analysed by western blot and primer extension. The results show that these RNP complexes contain both the vRNA template and either wildtype or NP R416A (figure 3.10a), providing evidence that monomeric NP can bind to the RNA and form a vRNP-like complex. As a control, the requirement for a complete and active (figure 3.10c) polymerase for the generation of the RNA products in the presence of wildtype and monomeric NP was confirmed.

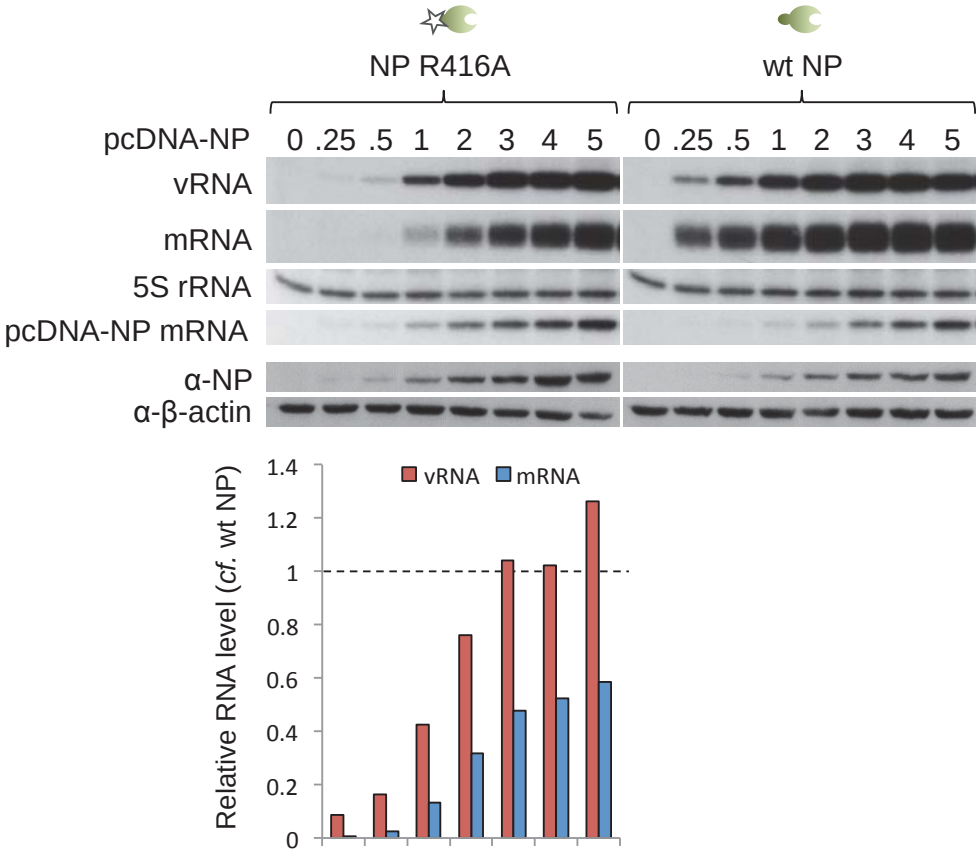


Figure 3.9. Oligomerisation mutant NP is less efficient than wild type NP at supporting replication and transcription of intermediate length vRNA-like templates *in vivo*. 0.5 µg of each plasmid expressing the three viral polymerase subunits and increasing quantities, as indicated in µg, of nucleoprotein (pcDNA-NP wt/R416A) were transfected into 293T cells with pPOLI plasmid encoding the 221-nucleotide vRNA-like template. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. During data analysis the signal in the negative control is subtracted from positive signals. The level of transcription and replication in the presence of each concentration of wildtype NP is set to 1 and the level of transcription and replication at each concentration of mutant NP R416A is compared to this. 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal. The level of both wildtype (wt) and mutant NP expression in each sample is shown in the panel below the primer extension panels and the protein samples were also analysed for the expression of β-actin as a loading control.

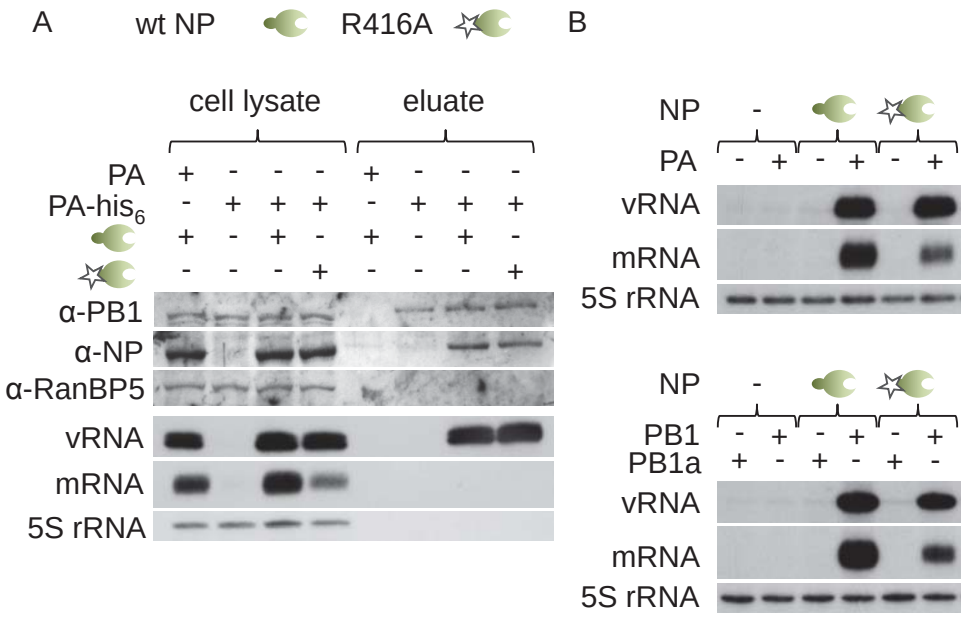


Figure 3.10. Replication and transcription of intermediate length templates requires assembly of polymerase and NP into functional RNP-like complexes. (A) Combinations of plasmids, each at a quantity of 1 μg were transfected into 293T cells as indicated with a pPOLI plasmid encoding the 221 nucleotide template. 48 hours post-transection the total cell-lysate was harvested. Ni-affinity chromatography was carried out on the cell lysate. Both the cell lysate and the eluate from the purification were analysed by western blot using antibodies against NP, PB1 and RandBP5. RanBP5 was used as a loading control for the cell lysates. RNA was isolated from the cell lysate and the eluate and the RNA was analysed by primer extension. (B) Combinations of plasmids, each at a quantity of 1 μg were transfected into 293T cells as indicated with a pPOLI plasmid encoding the 221 nucleotide template. PB1a contains a double mutation that disrupts the active site of PB1 and therefore inhibits RNA polymerase activity. 30 hours post-transection the total RNA was isolated and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. A primer detecting cellular 5S rRNA was used as a loading control.

3.4.5 Dimeric NP can support transcription and replication of intermediate vRNA-like template better than monomeric NP

It was hypothesised that when NP E339A and NP R416A were expressed together they would be able to form a dimer and that this dimer of NP may be able to support RNA polymerase activity of longer length RNA templates compared to monomeric NP. NP E339A and NP R416A theoretically should dimerise, as the wildtype tail-loop of NP E339A can interact with the wildtype insertion-groove of NP R416A (figure 3.11a). To test this transfections were carried out with NP E339A containing a C-terminal Myc tag and either wildtype NP, NP E339A or NP R416A, in the presence or absence of RNase A (figure 3.11b). In the absence of RNase A all un-tagged NPs are pulled down with NP-myc; their interaction is likely to be mediated by RNA. In the presence of RNase A, there is a reduction in the amount of NP E339A pulled down compared to wildtype NP, but the amount of NP R416A pulled down is similar to that of wildtype NP, suggesting that NP R416A and NP E339A can interact and form a dimer.

The ability of these two oligomerisation mutants when transfected together to support transcription and replication of longer length templates compared to transfecting them alone was then tested (figure 3.11c). The total amount of transfected NP expressing plasmid was kept constant and RNA polymerase activity was tested on the 246-, 287-, and 583-nucleotide templates. The presence of both NP mutants (R416A+E339A) resulted in a significant increase in transcription and replication, on both the 246- and 287-nucleotide templates, compared to the presence of either mutant alone. On the 583-nucleotide template, no activity could be detected in the presence of both of these mutants. These data

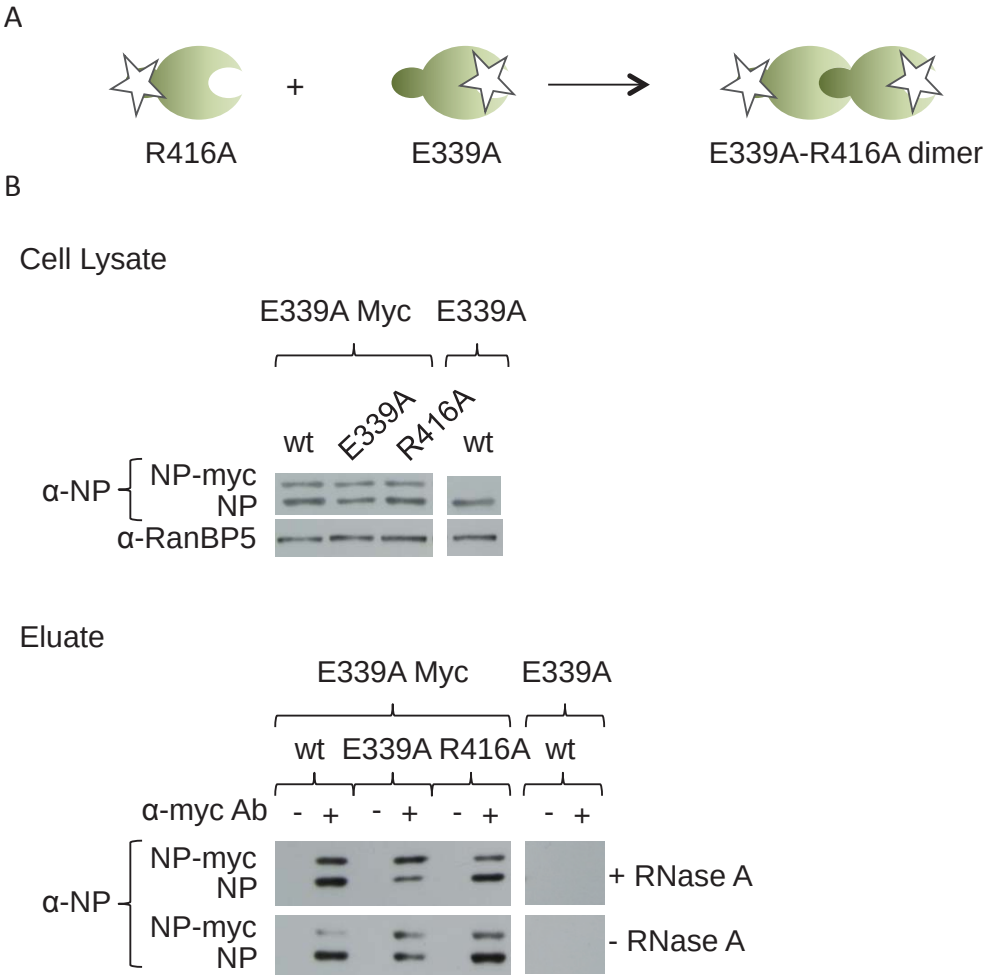


Figure 3.11. Dimeric NP can support transcription and replication of intermediate vRNA-like templates better than monomeric NP. (A) Schematic showing the possible dimer formation between NP E339A and NP R416A. (B) 293T cells were transfected with plasmids expressing C-terminally myc tagged NP E339A or untagged NP E339A NP with either wildtype NP, NP E339A or NP R416A. 48-hours post-transfection the cells were lysed. The total cell lysate was immunoprecipitated with a myc antibody (+) or no antibody (-) either in the presence or absence of RNase A. The cell lysate and immunoprecipitated sample (eluate) was analysed by western blotting using a NP antibody. A RanBP5 antibody was used as a loading control for the cell lysate.

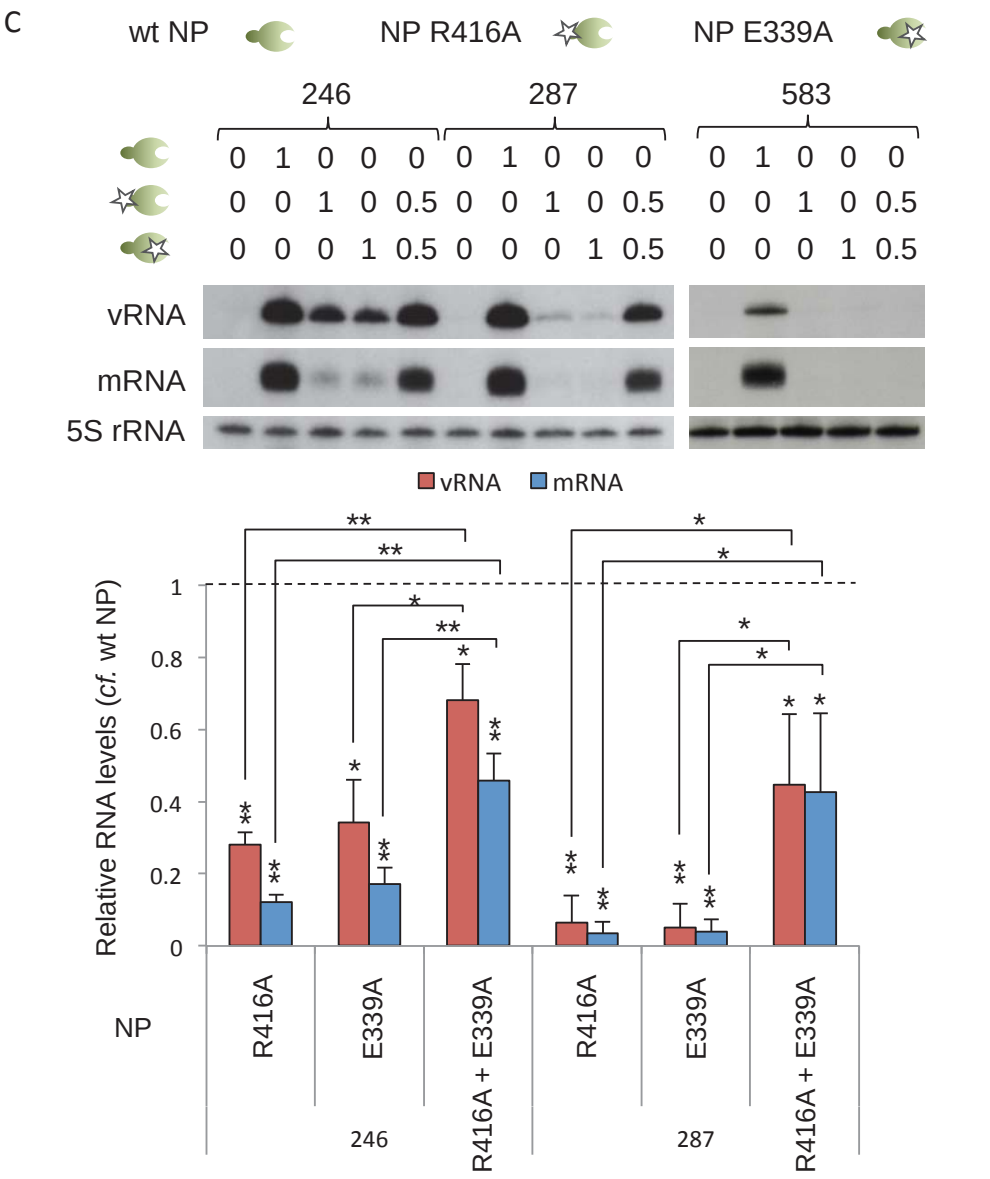


Figure 3.11. Dimeric NP can support transcription and replication of intermediate vRNA-like templates better than monomeric NP. (C) 293T cells were transfected with 1 μg of each of the plasmids encoding the polymerase subunits and different NP mutants at the quantities indicated in μg. The length of the template used in each transfection, generated by a pPOLI plasmid encoding vRNA-like templates of different lengths, is shown above each primer extension panel (nucleotides) and along the x-axis of the graph. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. The data is averaged from 3 independent experiments. During data analysis the signal in the negative control is subtracted from positive signals and the level of transcription and replication in the presence of wildtype (wt) NP is set to 1. Error bars show standard deviation and the asterisks are present when there is a significant difference from 1 (one sample *t*-test *p<0.05 **p<0.01). 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

suggest that a NP dimer is better at supporting RNA polymerase activity of intermediate length templates compared to monomeric NP.

3.4.6 RNA binding activity of NP is essential for transcription and replication of vRNA-like templates of various lengths that require NP

In order to test if NP is required to bind to the RNA to support RNA polymerase activity on intermediate length templates, a well characterised NP-RNA binding mutant G1(4) was used (Vreede et al., 2011). As shown in figure 3.12, replication in the presence of NP G1(4) was significantly reduced, compared to in the presence of wildtype NP, on all templates tested. Transcription was also significantly reduced in the presence of NP G1(4), compared to wildtype NP, on all templates apart from the 101-nucleotide template. With this template, surprisingly, transcription was equivalent in the presence of NP G1(4) compared to in the presence of wildtype NP. The expression and cellular localisation of NP mutant G1(4) was compared to wildtype NP and was found to be similar (figure 3.6 and figure 3.7).

3.4.7 The role of NP is conserved for different vRNA-like templates derived from different gene segments

The templates above are derived from gene segment 5 of the influenza A virus genome. In order to test if the results above were dependent on template sequence two additional templates were constructed, 60-nucleotides and 172-nucleotides in

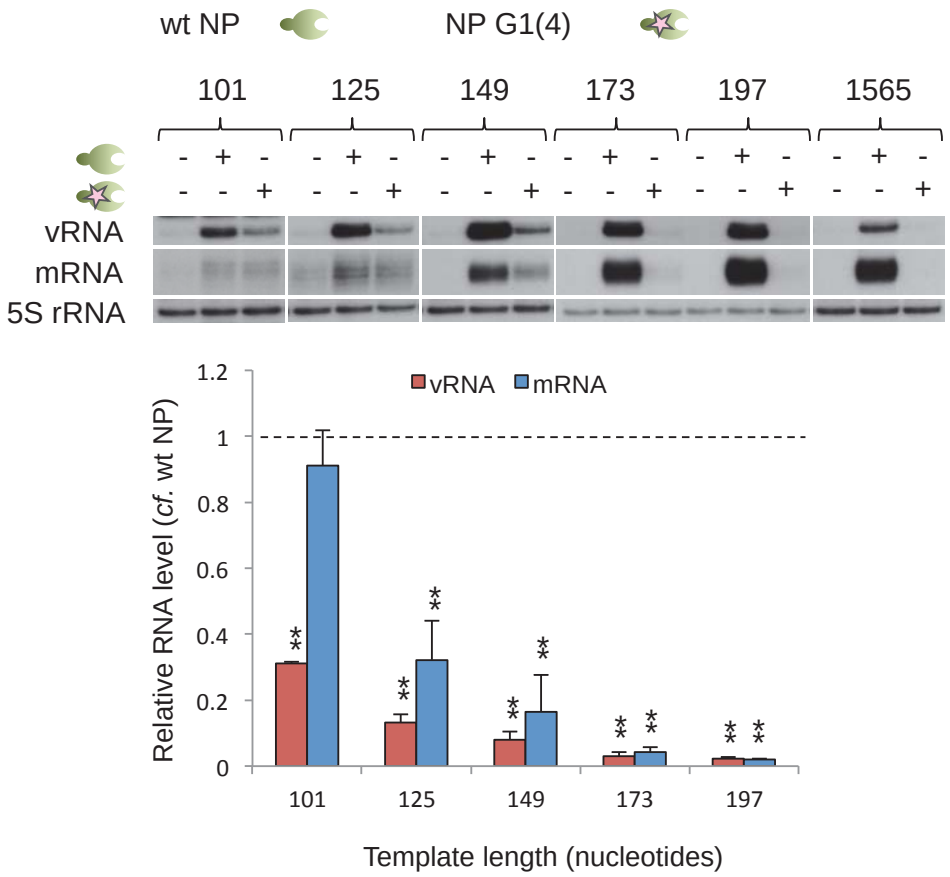


Figure 3.12. The RNA binding activity of NP is required for NP to support the RNA polymerase in replication. 293T cells were transfected with 1 µg of each of the plasmids encoding the polymerase subunits and 1 µg of the plasmid encoding either wildtype NP or RNA binding mutant NP G1(4) as indicated. The length of the template used in each transfection, generated by a pPOLI plasmid encoding vRNA-like templates of different lengths, is shown above each primer extension panel (nucleotides). Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. The data is averaged from 3 independent experiments. During data analysis the signal in the negative control is subtracted from positive signals and the level of transcription and replication in the presence of wildtype (wt) NP is set to 1 for each length template. Error bars show standard deviation and the asterisks are present when there is a significant difference from 1 (one sample *t*-test *p<0.05 **p<0.01). 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

length, derived from segment 8 of the influenza A virus genome. The RNA polymerase activity was then tested in the presence or absence of wildtype, monomeric (R416A) or RNA-binding mutant (G1(4)) NP on the 60-nucleotide, 172-nucleotide and full length gene segment 8 (890 nucleotides) templates (figure 3.13). The 60-nucleotide template can be both transcribed and replicated by the RNA polymerase in the absence of NP. The NP-RNA binding mutant G1(4) cannot support RNA polymerase activity on intermediate or full-length templates. In the presence of monomeric NP R416A transcription and to a lesser extent replication were significantly reduced compared to the presence of wildtype NP on the 172-nucleotide template. Neither NP R416A or NP G1(4) could support the viral polymerase in transcription or replication of the 890-nucleotide template.

3.5 Discussion

NP is an essential component of the RNP complex and without it the viral RNA polymerase cannot carry out transcription or replication of full length viral gene templates, *in vitro* or *in vivo* (Huang et al., 1990). The RNA polymerase alone, can carry out replicative activity *in vitro*, on short RNA templates (Honda et al., 1988), and it can also replicate a 46-nucleotide long template in the absence of NP, *in vivo* (Resa-Infante et al., 2010). In agreement, and in addition to these findings, this study has shown that both transcription and replication can occur in the absence of NP on short-RNA templates (47-76-nucleotides (figure 3.1 and 3.13)). The transcription and replication products are at the same ratio in the absence of NP compared to the presence of NP suggesting that NP does not play a role in determining whether the RNA polymerase carries out transcription versus replication (Krug et al., 1975; Medcalf et al., 1999), either through NP-polymerase

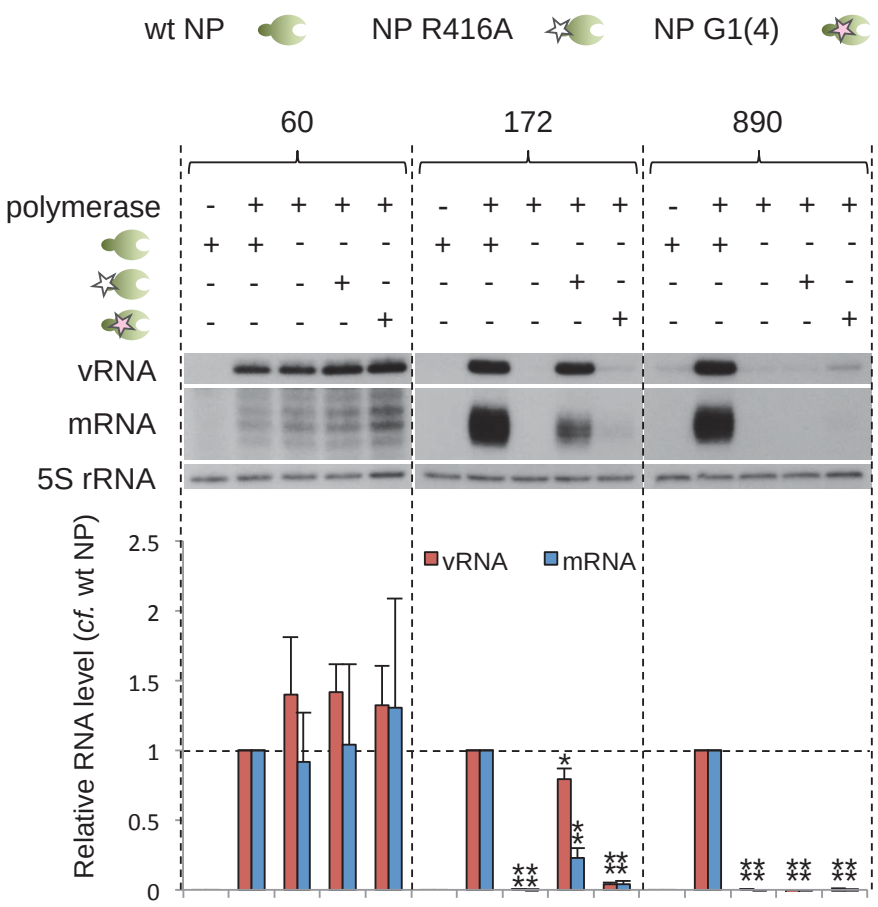


Figure 3.13.The role of NP in replication and transcription is conserved over gene segments. 293T cells were transfected with 1 µg of each of the plasmids encoding the polymerase subunits and 1 µg of the plasmid encoding either wildtype, R416A, G1(4) or no NP. The length of the template used in each transfection, generated by a pPOLI plasmid encoding vRNA-like templates of different lengths, is shown above each primer extension panel (nucleotides). Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. The data is averaged from 3 independent experiments. During data analysis the signal in the negative control is subtracted from positive signals and the level of transcription and replication in the presence of wildtype (wt) NP is set to 1 for each length template. Error bars show standard deviation and the asterisks are present when there is a significant difference from 1 (one sample *t*-test *p<0.05 **p<0.01). 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

binding (Biswas et al., 1998; Newcomb et al., 2009) or NP-RNA template binding (Klumpp et al., 1997). NP was also proposed to play a role in the synthesis of replication products by preventing the addition of the poly(A) tail (Beaton and Krug, 1986; Shapiro and Krug, 1988); however, this study shows that the absence of NP does not affect the polyadenylation status of the viral RNA (figure 3.3). It is likely that the binding of the polymerase to the short-RNA replication product is sufficient to protect it from degradation (Vreede et al., 2004). Short-RNA templates did not require NP for transcription and replication, however, the presence of NP promoted transcription and replication, especially at lower polymerase concentrations (figure 3.4). This dependency on NP concentration was independent of NP-RNA binding (figure 3.5). Although the mechanism for this effect is currently unknown, the RNA polymerase dependency on NP concentration independent of RNP complex formation may reflect a role of NP that is independent of viral RNA binding. For instance, NP may interact with the polymerase subunits leading to the stabilisation of the polymerase complex or allow more efficient trafficking of the RNA polymerase (Biswas et al., 1998; Marklund et al., 2012). Further work is required to address these hypotheses.

Rather than NP determining the initiation mode of the viral RNA polymerase, it was found that NP was functioning as an elongation factor in both transcription and replication. The polymerase required the presence of NP to efficiently transcribe and replicate templates longer than 101-nucleotides in length (figure 3.1-2). In agreement, NP has previously been proposed to function as an elongation factor by an *in vitro* study where the addition of NP facilitated the escape of the viral polymerase from the promoter (Honda et al., 1988). NP may function as an elongation factor by preventing RNA secondary structure from

forming, allowing full polymerase processivity. Alternatively or additionally, NP may recruit a host factor that allows the RNA polymerase to efficiently carry out its elongation phase, such as the helicase UAP56 that has been shown to interact with NP (Momose et al., 2001).

The roles of NP RNA binding and oligomerisation in transcription and replication were also studied in this chapter. Both RNA binding and oligomerisation of NP have been shown to be essential for NP to support RNA polymerase activity on full-length RNA templates (Chan et al., 2010; Ng et al., 2008; Vreede et al., 2011; Ye et al., 2006). The results above show that for templates of all lengths that require NP, NP-RNA binding is essential for the viral polymerase to carry out replication and transcription (figure 3.12 and 3.13). The interaction between NP and the RNA may stabilise the RNA in a conformation that is compatible with the processivity of the RNA polymerase. In contrast to the NP-RNA binding mutant, the NP oligomerisation mutant could stabilise cRNA; however, this cRNP-like complex was not compatible with polymerase activity (Vreede et al., 2011). In this chapter it was shown that monomeric NP was sufficient for polymerase processivity on intermediate length templates; however, as the length of the template increases the RNA polymerase required NP to have the ability to oligomerise for full-processivity (figure 3.6). Monomeric NP is recruited onto RNA to form a RNP-like complex despite its inability to oligomerise (figure 3.10); however, a higher concentration of monomeric NP R416A was required to support similar levels of replication when compared to wildtype NP, for the 221-nucleotide template (figure 3.9). The requirement for a higher concentration of monomeric NP compared to wildtype NP may be due to the ability of wildtype NP to be recruited to the RNP via oligomerisation, whereas monomeric NP

R416A can only be recruited via RNA/polymerase binding independent of oligomerisation. Therefore as the template length increases monomeric NP cannot be sufficiently recruited to the RNA to support viral transcription and replication. The ability of dimeric NP to support RNA polymerase transcription and replication significantly better than monomeric NP (figure 3.11) may be due to NP now being able to partly rely on oligomerisation for recruitment to the RNA. The differential effect on replication versus transcription on intermediate vRNA-like templates (101-287) in the presence of oligomerisation mutants (R416A, E339A and R416/E339A) suggests that transcription is more dependent on NP oligomerisation than replication (figure 3.6 and 3.13). This could be due to oligomerisation of NP being required for the stabilisation of the RNA template during the synthesis of the poly(A) tail, which is not formed in replication (reviewed in (Fodor, 2013)). This differential effect on transcription and replication in the presence of monomeric NP will be discussed further in Chapter 4.

This chapter has broadened the understanding of the role of NP in viral transcription and replication. NP is not required to determine the initiation or termination modes of the RNA polymerase in transcription versus replication. NP is, however, required during the elongation phase of viral transcription and replication, with RNA binding of NP being essential for its role as an elongation factor on any length vRNA-like template requiring NP, whereas, oligomerisation of NP is required in a length dependent manner. These results lead to the formulation of a model whereby as the length of the template increases so does the potential for it to form RNA secondary structures and NP-RNA binding is essential to prevent these secondary structures from forming either through direct

binding to the RNA or through the recruitment of a host-factor. In addition, as the template increases in length the number of NP monomers required to be recruited to the RNA in order for polymerase processivity will increase and for efficient recruitment to occur NP-oligomerisation may be required. Altogether NP-RNA binding and oligomerisation allow full processivity of the viral polymerase along the RNA.

3.6 Summary

- NP is not required for the viral RNA polymerase to carry out the two different mechanisms of transcription and replication
- NP is required for transcription and replication in a template length dependent manner suggesting that NP is functioning as an elongation factor in both processes
- NP plays an additional role, independent of RNP complex formation, which affects the activity of the RNA polymerase
- As the length of the vRNA-like template increases so does the requirement for oligomeric NP, possibly due to the requirement for efficient recruitment of NP onto the RNA as template length increases
- Transcription is more dependent on oligomeric NP than replication
- RNA binding activity of NP is required for transcription and replication of all vRNA-like templates that require NP

4. The recruitment of NP into the RNP complex

4.1 Overview

The RNP complex adopts a double-helical structure with NP bound along the length of the RNA that is not bound by the viral RNA polymerase. NP is required to encapsidate replication products for the production of progeny template RNP complexes. For these RNP complex structures to form, newly translated RNA polymerase subunits and NP must be recruited to the site where progeny RNA is produced and then assembled onto the RNA. How NP is recruited and assembled onto the newly synthesised cRNA and vRNA to form progeny RNP complexes is still not understood. In this chapter the role of NP oligomerisation and RNA binding in the encapsidation of RNA to form functional RNP complexes is investigated. The results point towards a model whereby NP is recruited via its tail-loop into the RNP complex. This recruitment of NP, via the tail-loop interacting with the insertion-groove of the resident NP, occurs prior to NP-RNA binding.

4.2 Background

During influenza virus infection the vRNP complex, which is present in the virion, is imported into the nucleus where the viral RNA polymerase carries out transcription and replication (Fodor, 2013). During replication the positive sense RNA intermediate, cRNA, is bound by the viral polymerase and NP to generate a cRNP complex, protecting cRNA from degradation (Vreede et al., 2004). Replication of the cRNA template leads to the production of progeny vRNA, packaged by the RNA polymerase and NP to form progeny vRNP complexes. Therefore, NP and the RNA polymerase have to be recruited and assembled onto RNA to form vRNP and cRNP complexes. vRNP and cRNP complexes have similar overall architectures, adopting a rod-like structure, with a central double-helical portion, maintained by NP intra- and inter-strand interactions, and the RNA polymerase and NP loop at opposite ends of the helix (Arranz et al., 2012; Moeller et al., 2012; York et al., 2013). The intra-strand contacts between NPs are thought to be maintained via the oligomerisation interaction between the tail-loop and insertion-groove, whereas it is currently unclear which residues of NP are responsible for the inter-strand interactions (Arranz et al., 2012; Moeller et al., 2012; Ye et al., 2006). The double helical structure has been shown to be dependent on NP-NP interactions as when NP is expressed in the absence of RNA, helical RNP-like complexes form *in vitro* (Ruigrok and Baudin, 1995; Yamanaka et al., 1990). The viral RNA polymerase is bound to the 5′ and 3′ ends of the RNA and this is essential for the double-helical structure to be maintained in the presence of RNA (Klumpp et al., 1997). The two structures of the full RNP complexes and the structure of the mini-RNP show that the terminal NPs, in the chain of NPs bound along the length of the RNA, bind to the RNA polymerase

(Arranz et al., 2012; Coloma et al., 2009; Moeller et al., 2012). NP has been shown to interact with PB1 and PB2 of the heterotrimeric RNA polymerase (Biswas et al., 1998; Poole et al., 2004) and NP residues responsible for the NP-polymerase interaction were shown to be essential for NP to support the activities of the viral RNA polymerase (Marklund et al., 2012).

The overall architecture of the RNP complex is well understood; however, how these complexes form in the host cell is unclear. The expression of the polymerase proteins, NP and viral RNA is sufficient for the assembly of RNP complexes *in vivo* (Huang et al., 1990; Moeller et al., 2012). When RNA was mixed with purified polymerase and NP *in vitro*, full-length RNA copies were produced, suggesting that a RNP complex could form (Honda et al., 1990; Parvin et al., 1989). Indeed *in vitro* packaged RNP complexes were incorporated into virions, when transfected in the presence of a helper virus (Enami et al., 1990; Luytjes et al., 1989). However, it has not been shown directly that RNP reconstituted complexes *in vitro* adopt the same structure as observed *in vivo* (Kingsbury et al., 1987; Yamanaka et al., 1990). The initial recruitment of NP onto the RNA template is hypothesised to occur via a NP-RNA polymerase interaction (Biswas et al., 1998; Poole et al., 2004), with NP oligomerisation and RNA binding playing further roles in the recruitment of NP. Monomeric, but not trimeric, NP could form a chain of NPs along the length of the RNA *in vitro*, this could be due to the use of the available oligomerisation domain on the resident NP monomer for recruitment (Tarus et al., 2012a). In agreement with this, it has been proposed that NP needs to be maintained as a monomer prior to RNP complex assembly for it to be encapsidation-competent (Chenavas et al., 2013). In addition to oligomerisation, NP-RNA binding is essential for RNP complex stabilisation; a

NP-RNA binding mutant could not stabilise replication intermediates and was unable to support RNA polymerase transcription and replication on a full-length RNA template (Ng et al., 2008; Vreede et al., 2011).

Little is known about how NP is assembled onto RNA to form RNP complexes and whether recruitment is dependent on oligomerisation and/or RNA binding. In order to study the recruitment of NP, RNP reconstitutions were used where plasmids expressing the components of the RNP complex were transfected and the ability of the viral polymerase to carry out transcription and replication was determined. The ability of the RNA polymerase to carry out transcription and replication of a full-length RNA template was used as a measure of RNP complex formation. The effect of NP oligomerisation and RNA binding mutants on the ability of the RNP complex to assemble and, therefore, replicate and transcribe was investigated.

4.3 Aims

- Determine the orientation in which NP is recruited into the RNP complex
- Investigate the role of NP oligomerisation in the recruitment of NP to the RNP complex
- Evaluate the role of NP-RNA binding in the recruitment of NP to the RNP complex

4.4 Results

4.4.1 Determining the optimal wildtype NP concentration for competition experiments

In order to determine a NP concentration in RNP reconstitutions where transcription and replication are in their exponential phase for further NP competition experiments, RNP reconstitutions were carried out with increasing amounts of plasmid expressing NP (pcDNA-NP) while maintaining a constant DNA concentration. As the level of NP expressed increases, viral replication increases proportionally (figure 4.1). In the case of transcription, mRNA levels increase dramatically between 0-1.5μg of pcDNA-NP transfected but as the level of NP is increased further the rate of mRNA accumulation declines. At 2μg of pcDNA-NP, the concentration of NP is limiting for transcription and, to a greater extent, replication. Therefore this condition was used for further experiments as it enabled detection of both an increase and a decrease in transcription and replication.

4.4.2 Recruitment of NP into the RNP complex is dependent on oligomerisation

In order for the oligomerisation of NP along the length of RNA to occur, the recruitment of NP has to be unidirectional so that all NPs are facing one way or the other, i.e. NP tail-loop facing the 5′ end of the RNA or NP insertion-groove facing the 5′ end of the RNA. In order to determine the orientation of NP in the RNP complex and the requirement for NP oligomerisation in the recruitment of

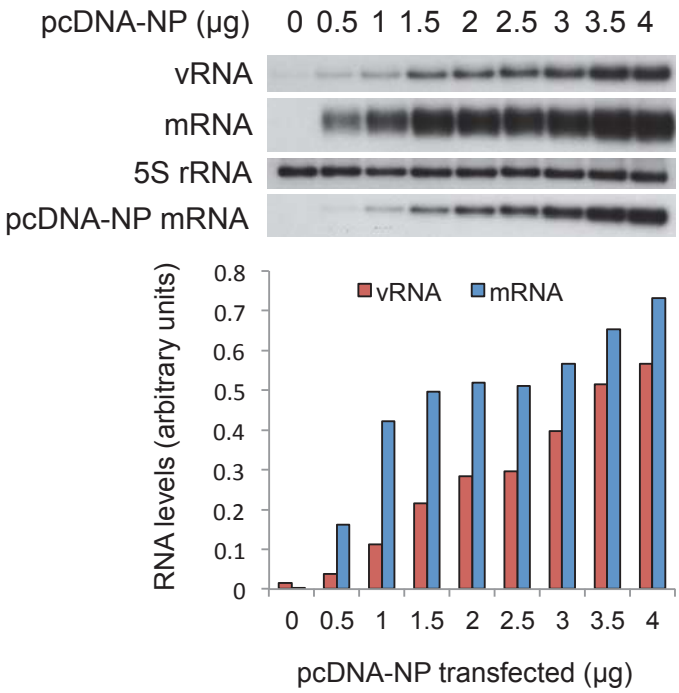


Figure 4.1. Determining the optimal NP concentration for competition RNP reconstitutions. Plasmids expressing the three viral polymerase subunits, each at a quantity of 0.5µg, and nucleoprotein (pcDNA-NP), at the quantities indicated in µg, were transfected into 293T cells with 0.5µg pPOLI plasmid encoding the 1565-nucleotide vRNA-like template. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. mRNA produced from the pcDNA-NP (wildtype and mutant) plasmid was detected. 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

NP competition experiments were set-up where well-characterised oligomerisation mutants of NP were competed with wildtype NP. The schematic in figure 4.2 shows the various scenarios and the effects that these scenarios would have on RNP complex formation and, therefore, activity. If recruitment occurs in a tail-loop first orientation then the insertion-groove mutant of NP would be recruited into the RNP complex via its wildtype tail-loop. This would prevent further wildtype NP from joining the NP chain and, therefore, a RNP complex could not form (figure 4.2a). However, if the recruitment occurs in the opposite way in an oligomerisation insertion-groove first orientation then the tail-loop mutant would be recruited to the RNP complex via its wildtype insertion-groove. This would prevent further wildtype NP from joining the NP chain and, therefore, a RNP complex could not form (figure 4.2b).

Competition RNP reconstitutions were set up with increasing amounts of plasmid expressing oligomerisation mutants NP R416A or NP E339A in the presence of 2μg of plasmid expressing wildtype NP, while keeping the concentration of transfected DNA constant. Analysis of the RNA from these transfections showed that in the presence of only 0.5μg of plasmid expressing the NP insertion-groove mutant E339A and 2μg of plasmid expressing wildtype NP, transcription and replication were inhibited by approximately 95% compared to in the presence of wildtype NP alone (figure 4.3a). In stark contrast, in the presence of even 2μg of plasmid expressing the NP tail-loop mutant R416A and 2μg of plasmid expressing wildtype NP, replication was approximately similar to in the presence of 2μg of plasmid expressing wildtype NP alone (figure 4.3b). These data suggest that the recruitment of NP occurs by the model presented in figure 4.2a, where NP joins the RNP complex in a tail-loop first orientation. The NP E339A mutant causes an

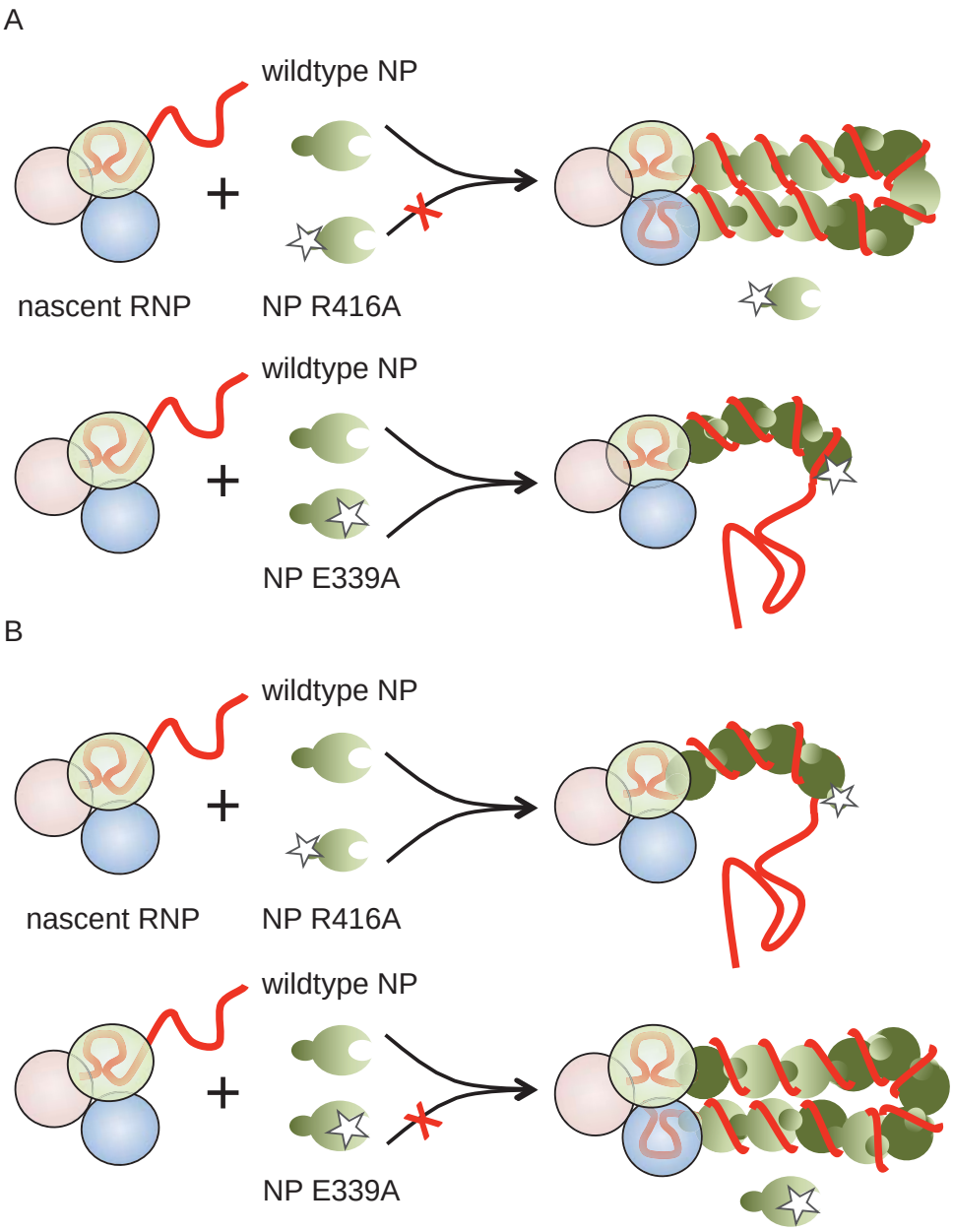


Figure 4.2. Schematic of the recruitment of NP into the RNP complex. (A) Schematic of NP being recruited via the tail-loop. The presence of NP E339A prevents a RNP complex from forming and therefore transcription and replication are inhibited. (B) Schematic of NP being recruited via the insertion-groove. The presence of NP R416A prevents a RNP complex from forming and therefore transcription and replication are inhibited.

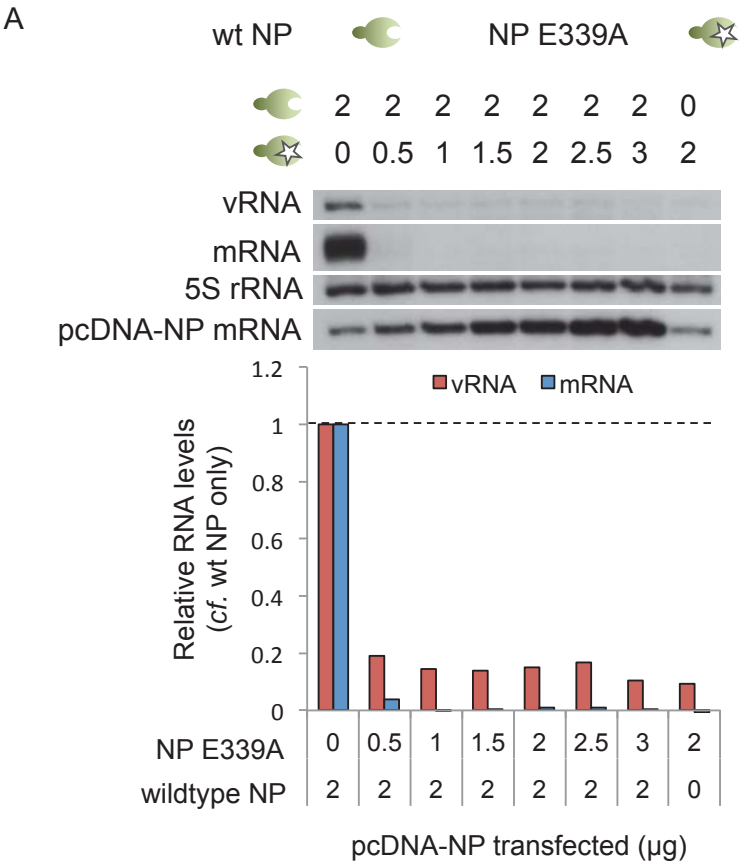


Figure 4.3a. Recruitment of NP into the RNP complex occurs in a 'tail-loop first' orientation via oligomerisation. Plasmids expressing the three viral polymerase subunits, each at a quantity of 0.5µg, and wildtype nucleoprotein at 2µg throughout were transfected. In addition a plasmid expressing either NP E339A (A) or R416A (B), at the quantities indicated in µg, were transfected into 293T cells with 0.5µg pPOLI plasmid encoding the 1565-nucleotide vRNA-like template. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. mRNA produced from the pcDNA-NP (wildtype (wt) and mutant) plasmids was detected. 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

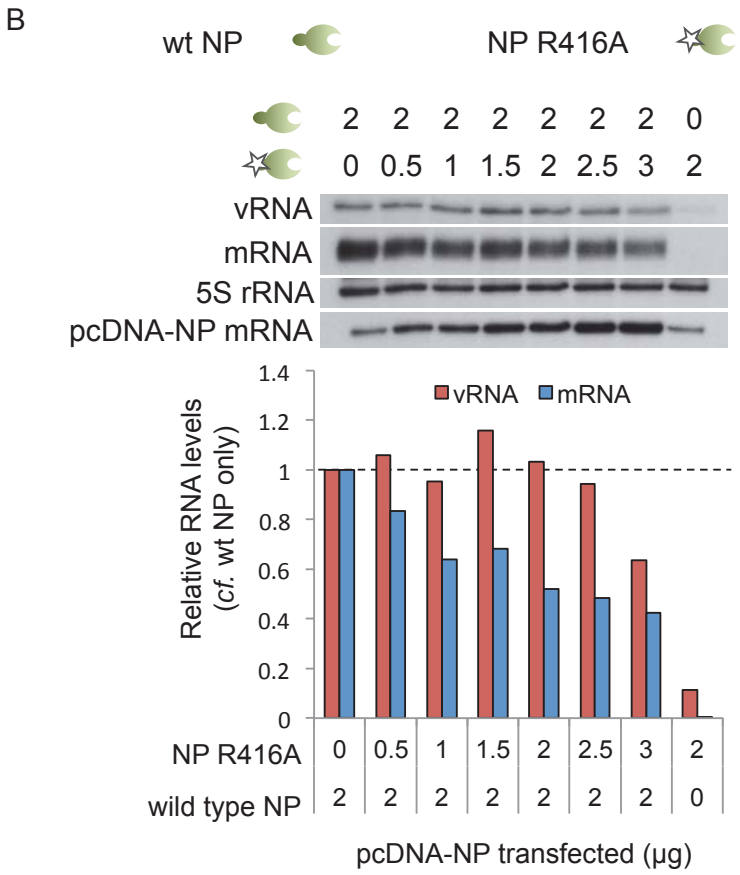


Figure 4.3b. Recruitment of NP into the RNP complex occurs in a 'tail-loop first' orientation via oligomerisation.

inhibition in transcription and replication by joining the NP oligomer, through its wildtype tail-loop, preventing further recruitment of NP due to its mutation in the insertion-groove, thereby prematurely terminating the formation of the RNP complex. This prematurely terminated complex cannot carry out transcription or replication.

Interestingly, in the competition experiment where the tail-loop mutant NP R416A is competed with wildtype NP, there is a differential effect on transcription versus replication at higher concentrations of plasmid expressing NP R416A (figure 4.3b). In the presence of 2μg of plasmid expressing the oligomerisation tail-loop mutant NP R416A and 2μg of plasmid expressing wildtype NP, replication is similar to the presence of wildtype NP alone, whereas transcription is reduced by 50%. This effect was also observed in further experiments later in this chapter (figure 4.8) and will be discussed later. For all competition experiments the level of mRNA from the pcDNA NP plasmids (both wildtype and mutant), presented in each panel, demonstrates that the pcDNA-NP mRNA signal is proportional to the amount of NP plasmid transfected. The expression levels and cellular localisation of these two mutants, NP R416A and NP E339A, are similar to wildtype NP (figure 4.4 and 4.5).

4.4.3 Recruitment of NP into the RNP complex via oligomerisation occurs independently to RNA binding

The results above suggest that NP is recruited into the RNP complex in a 'tail-loop first' orientation via oligomerisation. It was questioned whether this recruitment via oligomerisation was dependent on NP binding to the viral RNA.

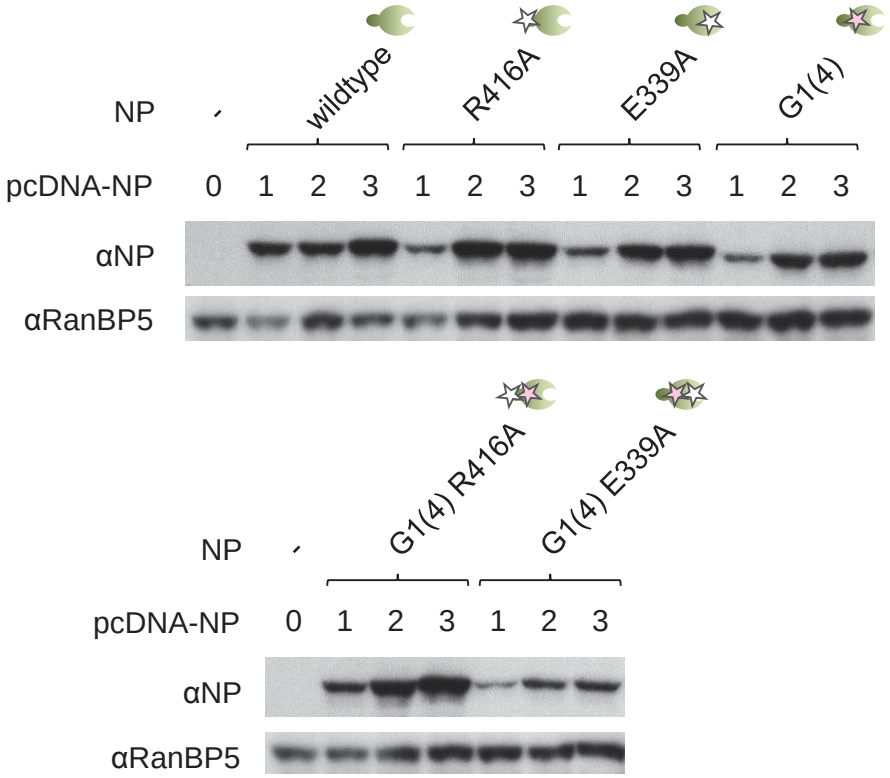


Figure 4.4. The expression levels of wildtype and mutant NPs. 293T cells were transfected with 1μg of each of the plasmids encoding the polymerase subunits and nucleoprotein, at the quantities indicated in μg, with pPOLI plasmid encoding the 221-nucleotide vRNA-like construct. Cell lysates were analysed by western blot with an antibody against NP. An antibody against RanBP5 was used as a loading control.

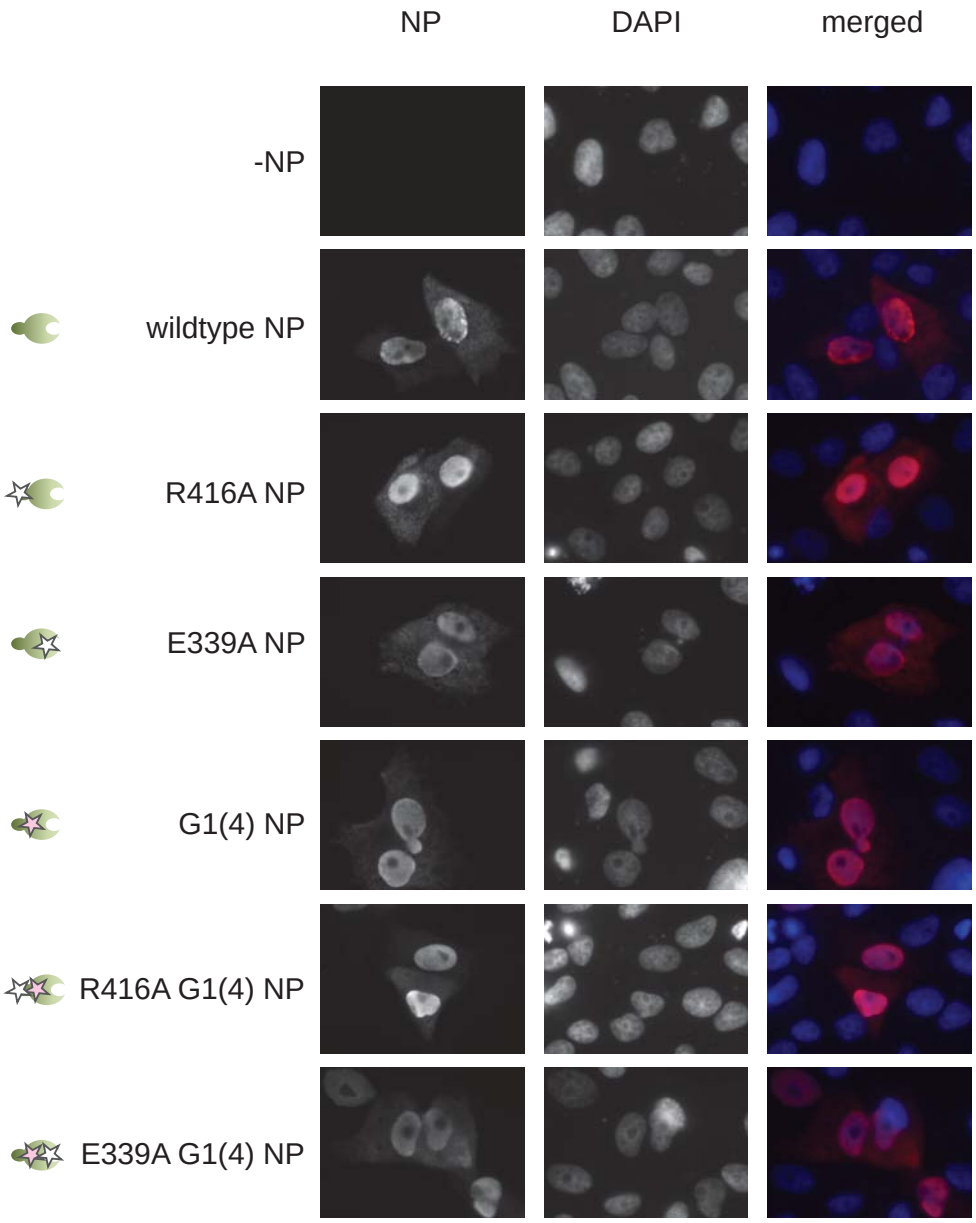


Figure 4.5. The cellular localisation of wildtype and mutant NPs. Vero cells were transfected with plasmids encoding wildtype and mutants NPs. The images above are representative of the localisation observed. DAPI staining (blue) and NP staining (red) were carried out.

To determine if this was the case four mutations that are known to disrupt the ability of NP to bind RNA (G1(4)- R74A, R74A, R174A and R175A) were introduced into both of the oligomerisation mutants to generate NP G1(4)-R416A and NP G1(4)-E339A. These RNA-binding/oligomerisation mutants were then tested in competition assays as before. In the presence of wildtype NP and increasing amounts of NP G1(4)-E339A an inhibition in transcription and replication was observed compared to in the presence of wildtype NP alone (figure 4.6a). The insertion-groove mutant can still be recruited into the RNP complex independently of RNA binding and inhibit RNP complex formation. The inhibition brought about by 0.5μg of plasmid expressing NP G1(4)-E339A is not as dramatic as the inhibition caused by 0.5μg of plasmid expressing NP E339A. This may be due to the expression level of NP G1(4)-E339A being reduced compared to NP E339A (figure 4.4); however, its cellular localisation was similar (figure 4.5). In the presence of increasing amounts of NP G1(4)-R416A there was no significant effect on the level of transcription and replication compared to in the presence of wildtype NP alone (figure 4.6b). The differential effect on transcription versus replication in the presence of wildtype NP and increasing amounts of NP R416A (figure 4.3a) was no longer present when the RNA binding mutations were introduced, suggesting that this effect is dependent on the oligomerisation mutant binding RNA. The expression level and cellular localisation of NP G1(4)-R416A and NP R416A were similar (figure 4.4 and 4.5). Next the RNA binding mutant NP G1(4) was competed with wildtype NP. NP G1(4) can oligomerise through its wildtype tail-loop and insertion-groove (Ng et al., 2008; Vreede et al., 2011). In the presence of increasing concentrations of NP

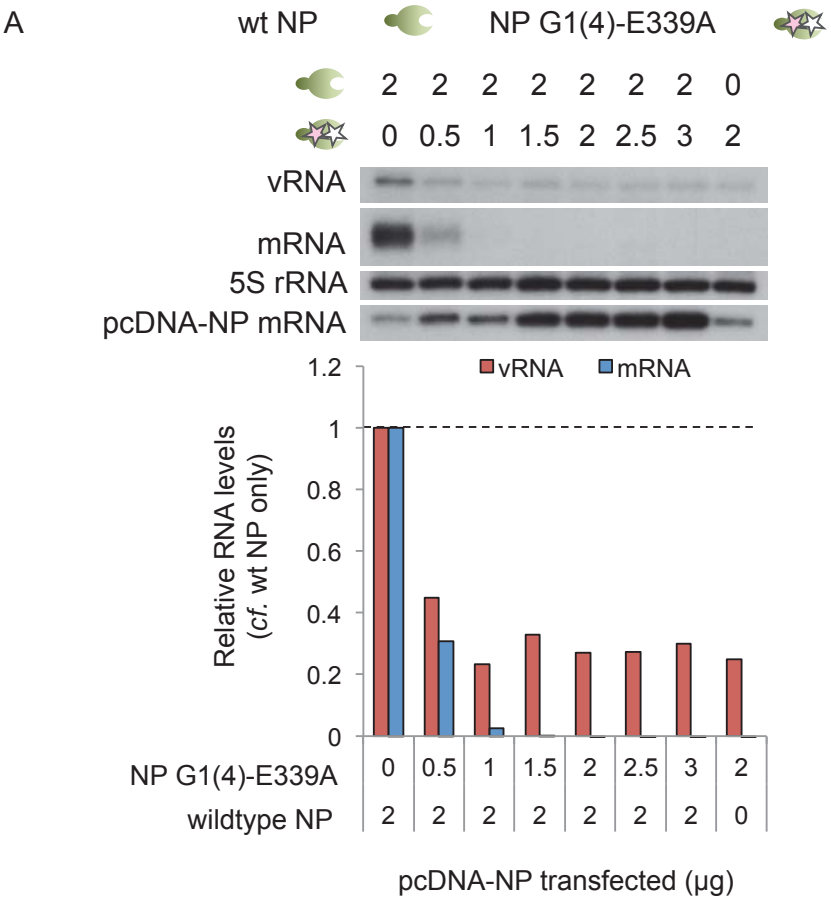


Figure 4.6a. Recruitment of NP to the nascent RNP is independent of RNA binding. Plasmids expressing the three viral polymerase subunits, each at a quantity of 0.5µg, and wildtype nucleoprotein at 2µg throughout were transfected. In addition a plasmid expressing (A) NP G1(4)-E339A or (B) NP G1(4)-R416A, at the quantities indicated in µg, were transfected into 293T cells with 0.5µg pPOLI plasmid encoding the 1565 nucleotide vRNA-like template. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. mRNA produced from the pcDNA-NP (wildtype (wt) and mutant) plasmids was detected. 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

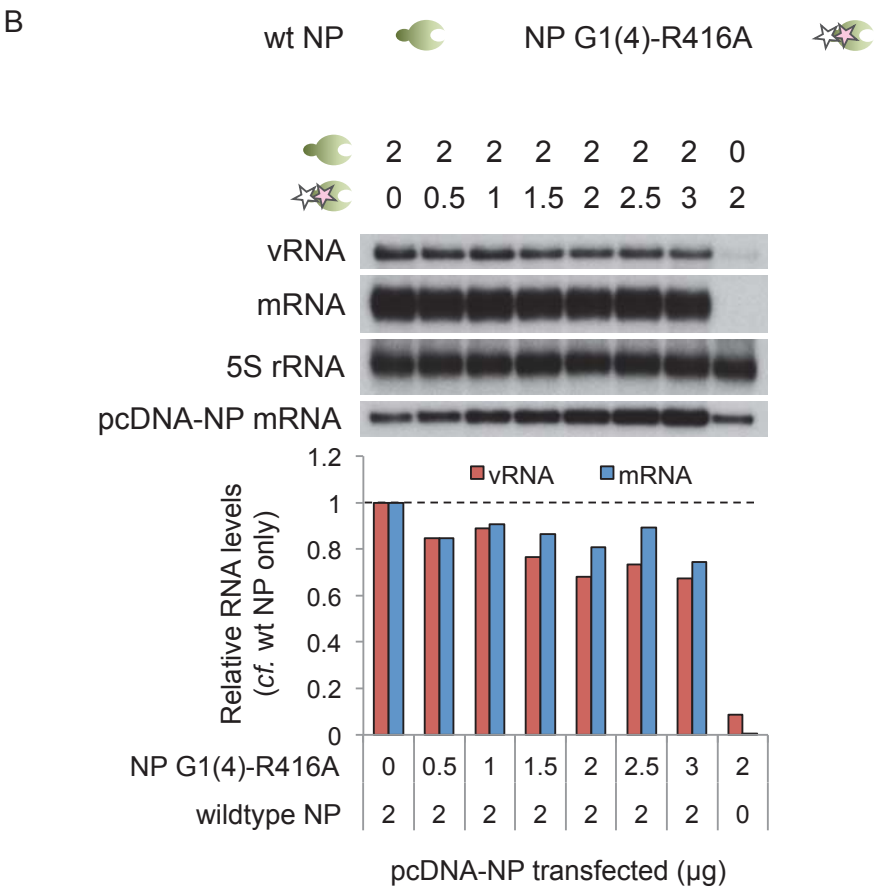


Figure 4.6b. Recruitment of NP to the nascent RNP is independent of RNA binding.

G1(4), in competition with wildtype NP, replication was similar to in the presence of wildtype NP alone, whereas transcription was increased approximately 2-fold at higher NP G1(4) concentrations (figure 4.7). This effect was also observed in further experiments later in this chapter (figure 4.8) and will be discussed later (figure 4.8). The expression and cellular localisation of NP G1(4) was found to be similar to that of wildtype NP (figure 4.4 and 4.5).

4.4.4 The recruitment of different NP mutants into the RNP complex causes differential effects on transcription versus replication

As shown above, differential effects on transcription and replication were observed in the presence of NP oligomerisation or RNA binding mutants in competition with wildtype NP. In order to do a direct comparison transfections were carried out in the presence of 2μg of plasmid expressing wildtype NP and 2μg of plasmid expressing the different NP mutants, in addition to the plasmids expressing the trimeric RNA polymerase and the full-length RNA template. Little or no RNA polymerase activity was observed in the absence of NP and in the presence of NP E339A or NP G1(4)-E339A in competition with wildtype NP (figure 4.8), as expected. There was a significant decrease in the mRNA/vRNA ratio in the presence 2μg of plasmid expressing NP R416A compared to 2μg of plasmid expressing wildtype NP alone, caused by a decrease in mRNA levels while vRNA levels remained constant. The mRNA/vRNA ratio was restored to 1 by the introduction of the RNA binding mutations into NP R416A (NP G1(4)-R416A), suggesting that the effect on mRNA/vRNA ratio in the presence of NP

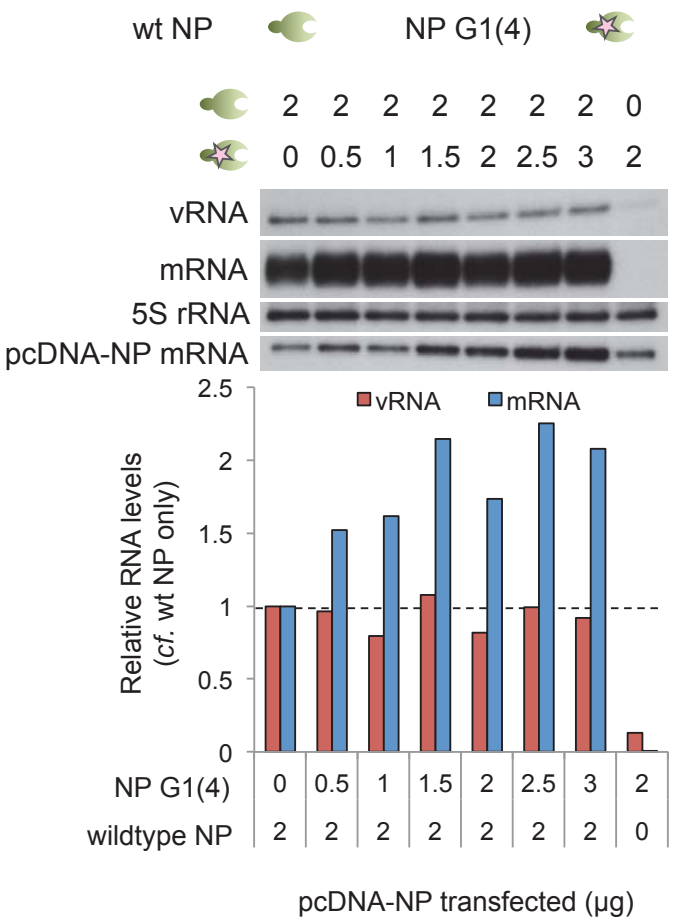


Figure 4.7. RNA binding mutant of NP does not competitively inhibit viral transcription and replication. Plasmids expressing the three viral polymerase subunits, each at a quantity of 0.5µg, and wildtype nucleoprotein at 2µg throughout were transfected. In addition a plasmid expressing NP G1(4) at the quantities indicated in µg, were transfected into 293T cells with 0.5µg pPOLI plasmid encoding the 1565-nucleotide vRNA-like template. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. mRNA produced from the pcDNA-NP (wildtype (wt) and mutant) plasmids was detected. 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

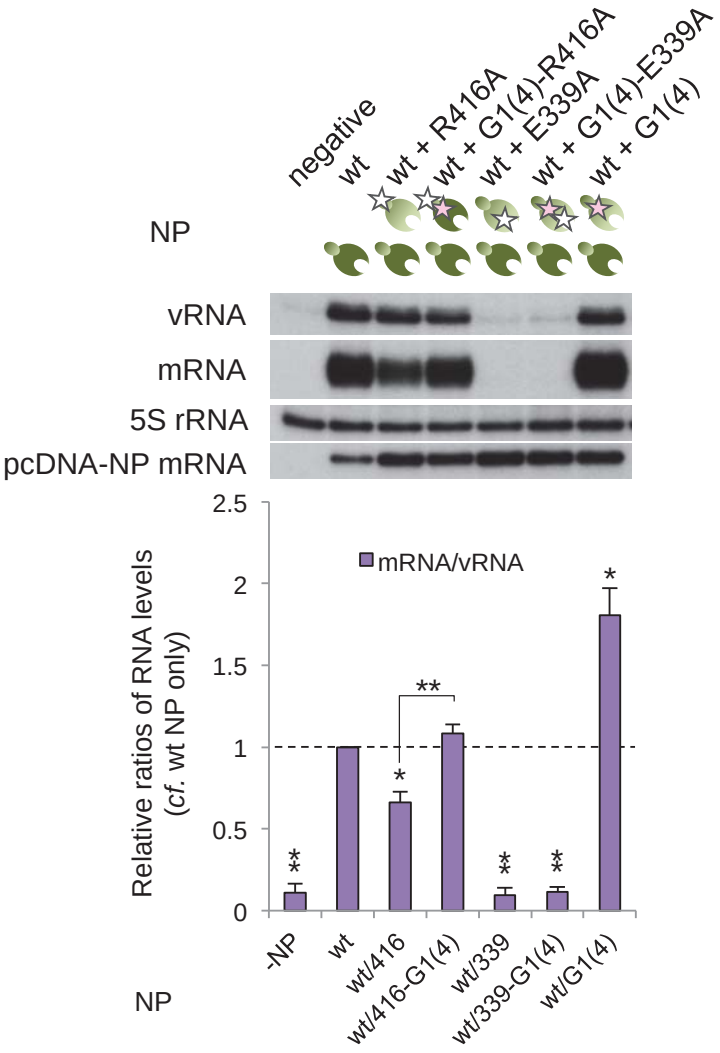


Figure 4.8. The effect of different NP mutants in competition with wildtype NP on the ratio of transcription to replication. Plasmids expressing the three viral polymerase subunits, each at a quantity of 0.5µg, and wildtype nucleoprotein at 2µg throughout were transfected. In addition plasmids expressing NP mutants, as indicated, at a quantity of 2µg were transfected into 293T cells with 0.5µg pPOLI plasmid encoding the 1565-nucleotide vRNA-like template. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. mRNA produced from the pcDNA-NP (wildtype (wt) and mutant) plasmid was detected. 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal. The data is averaged from 3 independent experiments. The ratio of mRNA/vRNA in the presence of wildtype NP is set to 1. Error bars show standard deviation and asterisks are present when there is a significant difference from 1 (one sample *t*-test *p<0.05 **p<0.01).

R416A is dependent on RNA binding. In contrast, in the presence of 2μg of plasmid expressing NP G1(4) in competition with wildtype NP there was a significant increase in the ratio of mRNA/vRNA compared to in the presence of wildtype NP alone, caused by a significant increase in mRNA levels while vRNA levels remained constant.

4.5 Discussion

During viral infection an early transcription dominant phase and a later replication dominant phase occur (Scholtissek and Rott, 1970; Taylor et al., 1977). The virion derived vRNP can carry out transcription; no additional viral proteins are required for the generation and stabilisation of viral mRNA (Vreede et al., 2004). Contrary to this, progeny RNA generated in replication requires newly translated RNA polymerase subunits and NP, from viral transcripts, to form RNP complexes and stabilise the RNA (Vreede et al., 2004). While determining the optimal NP concentration for competition experiments it was observed that in RNP reconstitutions as the concentration of NP increased the ratio of vRNA to mRNA increased (figure 4.1). This suggests that replication is more dependent on NP concentration than transcription, presumably due to the requirement for NP to bind cRNA and vRNA during replication. The findings are in contrast to a study that found no detectable increase in replication as the concentration of expressed NP increased, both in RNP reconstitution and infection (Mullin et al., 2004). This may be due to the ratios of polymerase to NP being different in these studies.

The structures of both vRNP and cRNP complexes have been analysed by EM (Arranz et al., 2012; Moeller et al., 2012; York et al., 2013). The intra-strand interaction of NP monomers along the length of the RNA is thought to be

maintained by the insertion of the NP tail-loop into the insertion-groove of the adjacent monomer (Chan et al., 2010; Ng et al., 2008; Ye et al., 2006). For the recruitment of NP to be compatible with the oligomerisation of NP along the length of the RNA it must bind the RNA in one orientation (figure 4.2). The data presented above where the inhibition of oligomerisation through mutation of the insertion-groove prevents RNP complex formation (figure 4.3b) suggests that NP is orientated with the tail-loop pointing towards the 5′ end of the RNA (figure 4.3). It is unclear how the orientation of the first NP recruited to the nascent RNA is determined such that the insertion groove is directed towards the 3′ end and available for subsequent NP oligomerisation; it may be through direct NP polymerase binding or through NP binding to a host-factor associated with the viral polymerase. Further work is required to address these hypotheses.

Once the orientation of the initial NP is determined, further recruitment of NP onto the RNA could occur via polymerase binding, RNA binding, oligomerisation, or a combination of all three. The data suggest that recruitment of NP onto the RNA occurs via oligomerisation independently of NP-RNA binding (figure 4.6b). In agreement, when NP lacking the tail-loop region was competed with wildtype NP there was no reduction in the transcription of a luciferase reporter gene (Shen et al., 2011). However, these authors also found that both NP R416A and NP E339A competitively inhibited transcription and replication in the presence of wildtype NP. This discrepancy may be due to the use of tagged wildtype and tagged mutant NPs in the study published by Shen et al., whereas untagged NPs were used in the assays above. The very different results observed in the competition experiments with oligomerisation mutants NP R416A and NP E339A (figure 4.3) are unlikely to be due to differences in their ability to bind

RNA as they have very similar RNA binding affinities (K_D of 975 and 853nM for NP R416A and NP E339A, respectively, with wildtype NP having a K_D of 15.8nM) (Ng et al., 2008). This is supported by cRNA stabilisation experiments showing that oligomerisation mutants NP E339A and NP R416A could both stabilise cRNA (Vreede et al., 2011).

Overall a model can be proposed whereby the initial NP is recruited onto the RNA in a tail-loop first orientation, the next NP to be recruited would then insert its tail-loop into the insertion-groove of the resident NP. The newly recruited NP then binds to the RNA allowing further recruitment of NP, until it is bound along the length of the progeny RNA. The data suggest that recruitment of NP into the RNP complex occurs via NP oligomerisation. However, oligomerisation mutants can form RNP-like structures as NP R416A and NP E339A can stabilise cRNA (Vreede et al., 2011), NP R416A and NP E339A could support transcription and replication of RNA templates up to 221-nucleotides in length (Chapter 3- figure 3.6) and NP R416A in the presence of a 221-nucletide template and RNA polymerase could form a RNP-like complex (Chapter 3-figure 3.10). Taken together, these data suggest that both NP oligomerisation mutants can be recruited onto the RNA independently of oligomerisation. Therefore, although NP oligomerisation may play a role in NP recruitment, in the absence of oligomerisation NP can rely on RNA binding/polymerase binding for recruitment.

The competition assay and the use of intermediate-length RNA templates allowed us to test if different properties of NP were important for transcription versus replication. The differential effect on transcription and replication observed in the presence of both NP R416A and NP G1(4) on the intermediate RNA templates (chapter 3) is maintained in the competition experiments. The presence of NP

oligomerisation mutants (in this case R416A) causes a decrease in the ratio of mRNA/vRNA, and the NP-RNA binding mutant (NP G1(4)) causes an increase in the ratio of mRNA/vRNA (figure 4.8). These mutants could be incorporated into the RNP complex disrupting its architecture in a way that affected transcription but not replication; however the reasons for the differential effects on transcription and replication are still unclear and further work is required to investigate these observations.

Overall the data presented in this study suggest that NP encapsidation of cRNA and vRNA is dependent on NP recruitment via its 'tail loop', in an oligomerisation dependent manner, to form the RNP complex. This initial recruitment of NP into the RNP complex via the 'tail-loop' can occur independently of RNA binding; however, it seems that in the absence of NP oligomerisation recruitment can still occur via a secondary mechanism, for example NP-RNA binding.

4.6 Summary

- NP is orientated in the RNP complex with its tail-loop directed towards the 5′ end of the RNA
- NP is recruited into the RNP complex via its tail-loop
- Recruitment of NP via the tail-loop can occur independently of NP-RNA binding
- The presence of an RNA binding mutant causes an increase in mRNA/vRNA ratios whereas the presence of an oligomerisation mutant causes a decrease in mRNA/vRNA ratios by mechanisms unknown

5 The regulation of NP oligomerisation

5.1 Overview

NP in the RNP complex forms oligomers through an interaction between the tail-loop of one NP monomer and the insertion-groove of an adjacent NP monomer and binds to the viral RNA in a non-sequence specific manner. A phosphorylation site within the oligomerisation insertion-groove of NP has been identified in both influenza A (serine (S)165) and influenza B (S223) viruses. It was hypothesised that phosphorylation of these sites may regulate NP oligomerisation. This chapter investigates the role of phosphorylation within the NP insertion-groove in NP oligomerisation, RNA polymerase activity and the viral life-cycle. The results show that constitutive phosphorylation inhibits NP oligomerisation leading to an inhibition in viral transcription and replication and viral growth. When this site cannot be phosphorylated, NP oligomerisation is unregulated resulting in reduced viral transcription and replication and viral growth. Therefore, these results have led to a proposal whereby NP oligomerisation is regulated by reversible phosphorylation within the oligomerisation insertion-groove to ensure that when NP reaches the site of RNP complex formation it is encapsidation-competent.

5.2 Background

The RNP complex of influenza virus forms a rod like-structure with the viral RNA polymerase at one end, a NP-loop at the opposite end and a double-helical NP structure in the central portion (Arranz et al., 2012; Moeller et al., 2012; York et al., 2013). Within the RNP complex neighbouring NPs form an intra-strand interaction via the tail-loop of one NP monomer interacting with the adjacent NP oligomerisation insertion-groove (Arranz et al., 2012; Moeller et al., 2012).

In order for the virus to complete its life-cycle and generate progeny RNP complexes, newly translated viral RNA polymerase and NP must be transported into the nucleus and assembled onto the replicative intermediate cRNA, for the generation of progeny vRNA. Progeny vRNA is also encapsidated by the RNA polymerase and NP forming progeny vRNP complexes. It is thought that the initial recruitment of NP onto the RNA occurs via its interaction with the viral RNA polymerase (Biswas et al., 1998; Poole et al., 2004). Subsequent NP recruitment occurs via oligomerisation, in a 'tail-loop first' orientation, prior to RNA binding (chapter 4) (Turrell et al., 2013). Recent work investigating the oligomerisation pathways of NP showed that monomeric NP could assemble onto the RNA and form a ring of NPs on a RNA template; however, when trimers of NP were present, the trimer readily assembled onto the RNA but no chain of NPs could form along the length of the RNA (Chenavas et al., 2013; Tarus et al., 2012a). In addition, small molecule drugs that either inhibit or induce NP oligomerisation have been shown to inhibit vRNP complex assembly and viral replication (Gerritz et al., 2011; Kao et al., 2010; Shen et al., 2011; Su et al., 2010). Altogether, these data suggest that NP is maintained in a monomeric form

prior to assembly onto the RNA, possibly in order for NP recruitment to occur via oligomerisation.

It has been previously proposed that NP is maintained as a monomer prior to RNP complex assembly by binding to importin α5 (Boulo et al., 2011). It has also been hypothesised that phosphorylation at S165 within the insertion-groove of NP may prevent oligomerisation by inhibiting the tail-loop insertion-groove interaction (Hutchinson et al., 2012). Mutation of S165 to glutamic acid, a phosphomimetic, resulted in a significant decrease in RNA polymerase activity (figure 5.1a). In addition, a phosphorylation site was also identified in the oligomerisation insertion-groove of influenza B NP, at position S223, suggesting that phosphorylation at this site is conserved between influenza types (Hutchinson et al., 2012). Phosphorylation of S165 in ANP was also identified in an independent study and mutation of S165 to aspartic acid, another residue used as a phosphomimetic, caused NP to behave in a similar fashion to monomeric wildtype NP (Chenavas et al., 2013). In this chapter the hypothesis that NP oligomerisation is regulated by phosphorylation within the oligomerisation groove is addressed and the importance of this regulation in the viral life-cycle is investigated.

5.3 Aims

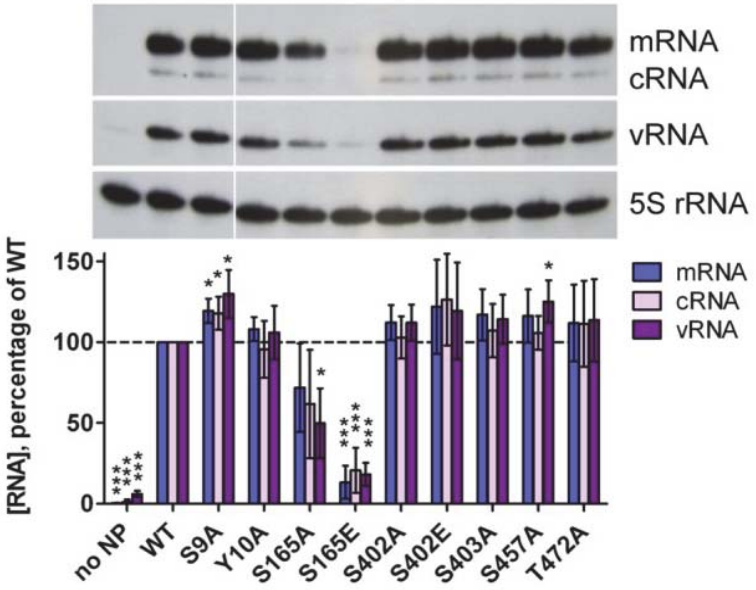
- Determine if mutating the phosphorylation site within the NP insertion-groove affects influenza viral growth
- Investigate the oligomerisation status of different recombinant NP mutants expressed from baculovirus in insect cells
- Evaluate the ability of the phosphorylation mutants to support RNA polymerase activity

5.4 Results

5.4.1 Mutating S165 within NP results in a decrease in viral titre compared to wildtype influenza virus

A study carried out in our laboratory identified many phosphorylated residues in the proteins of influenza A and B viruses by mass spectrometry (Hutchinson et al., 2012). Seven phosphorylation sites were identified within NP of influenza A virus including S165 within the insertion-groove of NP (figure 5.1b). This phosphorylation site was also identified in an independent study, where NP from influenza A/PR/8/34 virus grown in embryonated chicken eggs was analysed by mass spectrometry (Chenavas et al., 2013). In order to test if this phosphorylation site was important in the virus life-cycle, recombinant influenza viruses were generated where phosphorylation was either prevented or mimicked. To prevent phosphorylation S165 of NP was mutated to alanine (A) and to mimic phosphorylation S165 was mutated to either glutamic acid (E) or aspartic acid (D) (figure 5.1c). Reverse genetics was carried out using the pHW2000 plasmids, where the cDNA of each of the eight viral gene segments is inserted in-between a human polI and polII promoter, leading to the generation of both negative sense vRNA and positive sense mRNA. Plasmids encoding each segment were transfected into 293T cells with the exception of pHW2000-NP. In place of this plasmid a wildtype pcDNA-NP plasmid was transfected which leads to the expression of wildtype NP protein to enable the assembly of active RNP complexes. In addition a pPOLI-NP plasmid was transfected which produced the NP vRNA template. The plasmid that encoded the NP vRNA template was mutated to include mutations S165A, S165E or S165D. Three attempts to rescue

A



B

Sample	S165 (S223)
Purified virus (without enrichment; A+B)	N/A
Purified virus (with enrichment; A+B)	WSN, (B/Bris)
TAP-NP (with enrichment; WSN only)	N/A
RNP (with enrichment; WSN only)	WSN

C

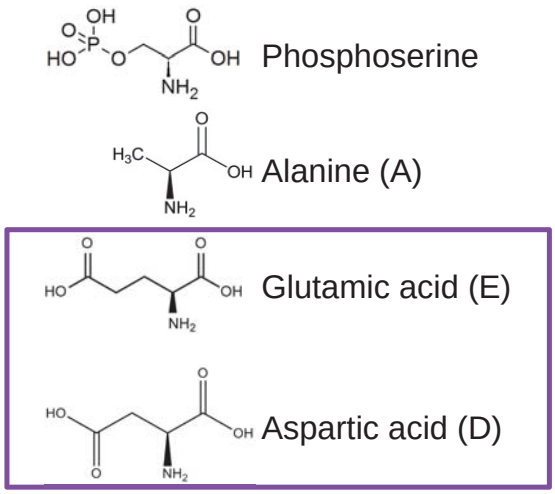


Figure 5.1. Identification and characterisation of phosphorylation sites within NP. (A) RNP reconstitution data of the effects of mutating phosphorylated serine, tyrosine and threonine residues within NP to alanine or glutamic acid. Figure adapted from Hutchinson et al., 2012. (B) Description of samples used for the identification of phosphorylation at S165 and S223 in influenza A WSN and influenza B B/Bris, respectively (Hutchinson et al., 2012). (C) Structure of phosphoserine and amino acids. Purple box highlights the two amino acids which are used as phosphomimetic residues.

virus were carried out; in all cases wildtype, S165A and S165D viruses could be rescued. A virus with serine mutated to glutamic acid at position 165 of NP could not be rescued. Recombinant wildtype and mutant viruses were sequenced confirming the amino acid present at position 165. These viruses were then analysed for their ability to grow in MDBK cells. Cells were infected at an MOI of 0.001, with either wildtype or mutant viruses, and a sample of the supernatant was harvested every 12 hours, up to 72 hours, to determine viral growth. Each sample was analysed by plaque assay and the titre of the virus in the sample was determined (figure 5.2). Wildtype virus reached a maximal titre of $10^{7.5}$ PFU/ml 60 hours post infection, whereas viruses with NP mutations S165A or S165D were inhibited in their viral growth compared to wildtype virus, both yielding maximal viral titres of $10^{5.5}$ PFU/ml 60 hours post infection. This significant decrease in viral titre, when S165 is mutated to alanine or aspartic acid and the inability to rescue virus when the site is mutated to glutamic acid suggests that this site plays a significant role in the viral life-cycle. Neither constitutive phosphorylation nor the absence of phosphorylation, at position S165, is compatible with optimal viral growth.

5.4.2 The presence of a phosphomimetic amino acid residue within the insertion-groove inhibits NP oligomerisation

In order to characterise how mutations at S165 affected the oligomerisation status of NP large-scale recombinant NP expression was carried out in Sf9 cells through rescuing recombinant baculoviruses that encoded wildtype or mutant NPs with a protein A tag (Materials and methods- section 2.6.1). NP was purified using IgG

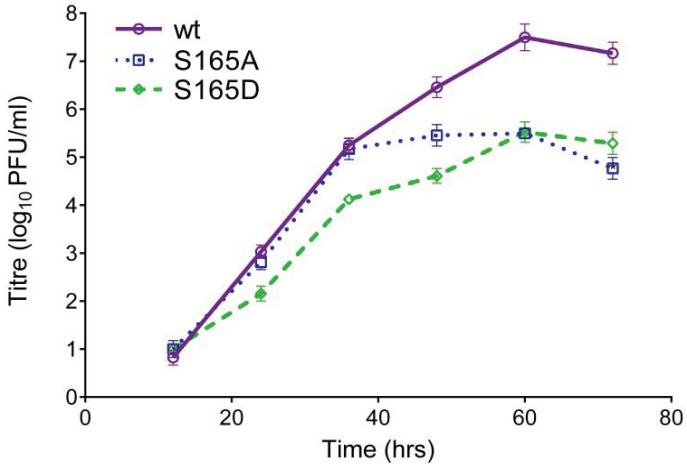


Figure 5.2. Growth kinetics of recombinant influenza viruses with mutations at position S165 of NP. MDBK cells were infected with wildtype (wt) or mutant viruses at a multiplicity of infection (MOI) of 0.001. The amount of released virus at the indicated times points post-infection was determined by plaque assay on MDBK cells.

sepharose chromatography and analysed by size exclusion chromatography (figure 5.3). The mutants NP E339A and NP G1(4) were not used further. Wildtype NP and oligomerisation mutant NP R416A were used as controls. The cell lysates were treated with RNase A, so that only oligomerisation dependent on a direct protein-protein interaction could occur. After purification, the different NP mutants were loaded onto a superdex S200 10/300 column and the fractions were analysed for the presence of NP. It was found that 80% of wildtype NP elutes from the column in the early fractions, due to it forming higher order oligomers, approximately 341 kDa in size. The remaining 20% of wildtype NP is present in the monomeric fraction, corresponding to a size of approximately 50 kDa, within the range of monomeric NP (figure 5.4). In contrast, NP R416A is separated into three fractions but with the highest proportion of NP being present in the monomeric fraction. One peak corresponds to the approximate size of 50kDa, the second peak corresponds to an approximate size of 113kDa, within the range of dimeric NP, and the third peak corresponds to an approximate size of 341kDa, within the range of higher order oligomers. When S165A is expressed and run on a superdex column the protein is in the higher order form, with a peak at 341kDa; no S165A NP can be detected in the monomeric fraction, unlike wildtype NP. NP mutants S165E and S165D are mostly present within the monomeric NP fraction, with an approximate size of 50kDa, and this peak aligns with the monomeric peak of the mutant NP R416A.

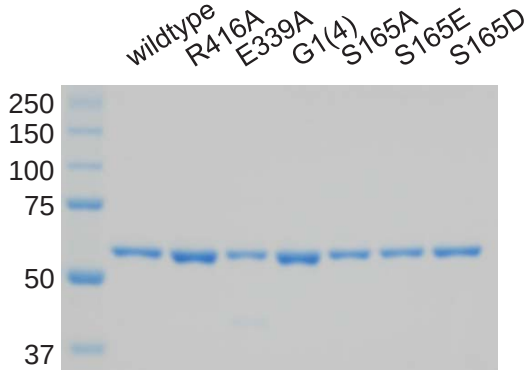


Figure 5.3. Purification of soluble wildtype and mutant NP from insect cells infected with recombinant baculovirus. Coomassie staining of SDS-PAGE gel, each lane is a separate purification of different NP constructs.

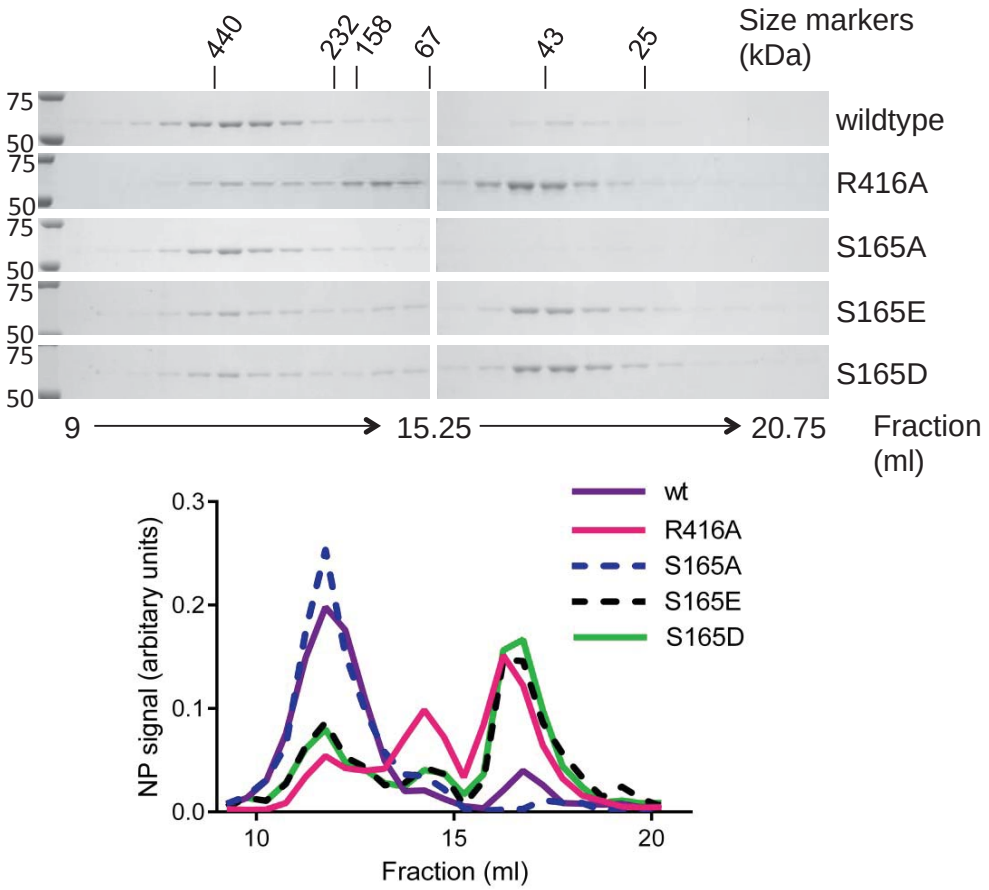


Figure 5.4. Analysis of wildtype and mutant NPs by size-exclusion chromatography. Approximately the same concentration of NP, wildtype or mutants, was loaded onto a Superdex S200 column 10/300 (GE Healthcare). The fractions collected were analysed using SDS-PAGE and staining with Coomassie Brilliant Blue. The position at which different molecular weight proteins were eluted from the column is indicated above the panel (proteins used from LMW and HMW gel filtration calibration kits (Amersham Biosciences)). Quantitation of NP present in the fractions was performed by densitometry and the data was analysed using Aida. The experiment shown is a representative of two independent experiments.

5.4.3 Mutating residue S165 of NP affects its ability to support viral transcription and replication

To assess the ability of the NP mutants to support viral transcription and replication RNP reconstitutions were carried out using different length RNA templates. A significant reduction in transcription and replication with all three mutants, S165A/E/D, was observed on a 2340-nucleotide long template when compared to the presence of wildtype NP (figure 5.5a). Mutating serine to glutamic acid has the most dramatic effect on the ability of NP to support transcription and replication, with the mRNA and vRNA levels reduced to 20% when compared to levels in the presence of wildtype NP on templates longer than 1409 nucleotides, explaining the inability of a virus to be rescued with the NP mutation S165E. NP with mutations S165D and S165A have similar effects on viral transcription and replication, both supporting 50% of RNA polymerase activity when compared to wildtype NP on the 2340-nucleotide long template. This may explain why recombinant viruses with NP S165D or S165A mutations have similar growth kinetics, as they are able to support similar levels of transcription and replication. As NP oligomerisation mutants R416A, E339A and R416A/E339A, are able to support transcription and replication of intermediate length RNA templates (Chapter 3- section 3.4.3), it was hypothesised that oligomerisation S165 mutants would also support transcription and replication of intermediate length templates. Indeed this was the case, in the presence of NP mutants S165A, E or D transcription and replication were similar to levels observed in the presence of wildtype NP on templates up to 221-nucleotides in

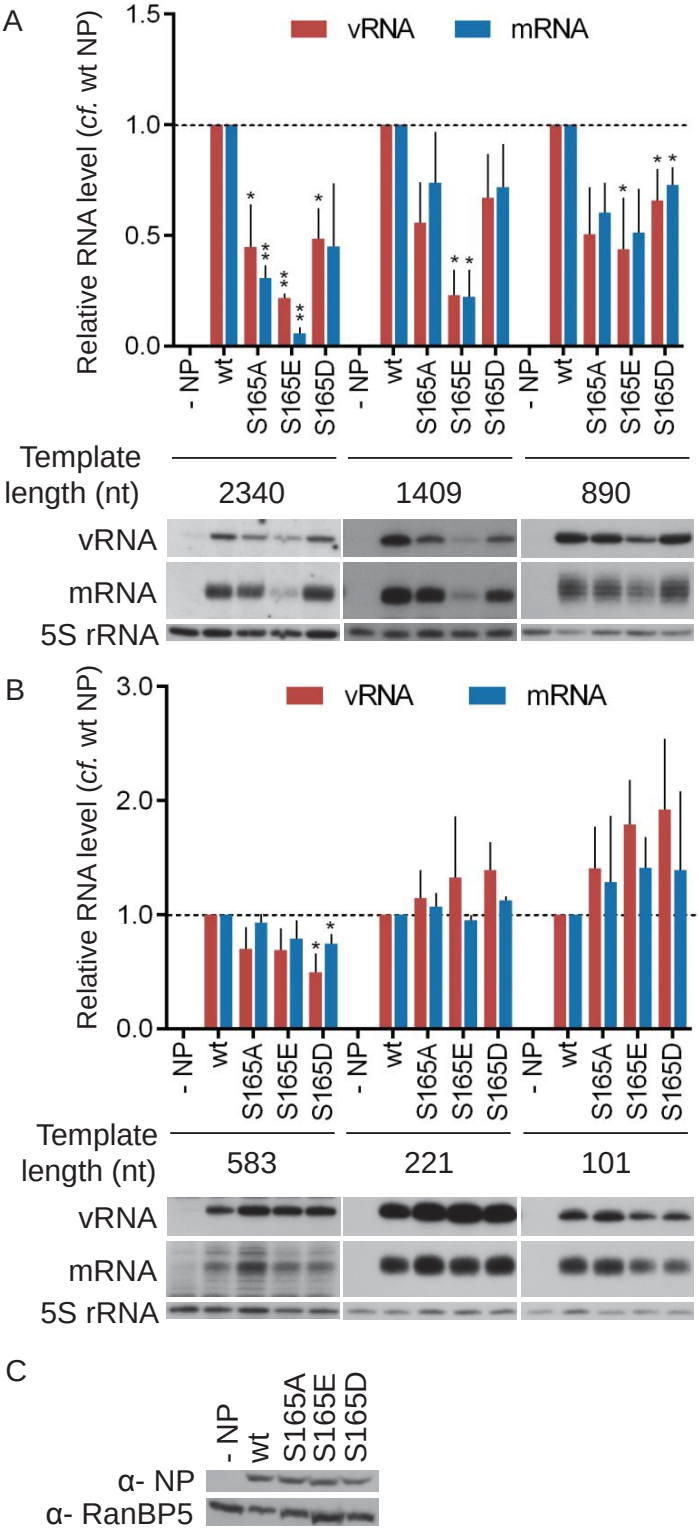

A
vRNA
mRNA
Relative RNA level (cf. wt NP)
1.5
1.0
0.5
0.0
- NP
wt
S165A
S165E
S165D
Template length (nt)
2340
1409
890
vRNA
mRNA
5S rRNA
B
vRNA
mRNA
Relative RNA level (cf. wt NP)
3.0
2.0
1.0
0.0
- NP
wt
S165A
S165E
S165D
Template length (nt)
583
221
101
vRNA
mRNA
5S rRNA
C
- NP
wt
S165A
S165E
S165D
α- NP
α- RanBP5

Figure 5.5. Effect of mutations at S165 of influenza A NP on transcription and replication by the viral RNA polymerase. RNP reconstitutions were carried out in 293T cells by transfecting plasmids to express the viral polymerase, wildtype (wt) or mutant NP, and a plasmid to express full-length genomic vRNA (PB2, 2340 nucleotides long; NA, 1409 nucleotides long; NS, 890 nucleotides long) (A) or NP vRNA deletion mutants of the indicated length (B). A negative control without NP was included for each template. RNA harvested 30 hours post transfection was analysed by primer extension. The amount of mRNA or vRNA was quantified using phosphorimage analysis and the level of 5S rRNA was used as a loading control. The signal of mRNA and vRNA in the negative control was set to 0 and the signals in the presence of wildtype NP were set to 1. Error bars represent the variation observed in the 3 repeats and statistical analysis was carried out using one-tailed student's t-test (* $P<0.05$ and ** $P<0.01$). (C) The expression of NP mutants in the RNP reconstitution was analysed by western blot using a rabbit polyclonal NP antibody. A RanBP5 antibody was used as a loading control.

length (figure 5.5b). The expression levels of the different NP mutants were shown to be similar to wildtype NP (figure 5.5c).

5.4.4 S165E does not compete with wildtype NP and inhibit viral transcription and replication

The insertion-groove mutant, NP E339A, inhibited RNP complex assembly in the presence of wildtype NP, hypothetically due to blocking further recruitment of NP into the RNP complex (Chapter 4- section 4.4.2). Therefore, it was hypothesised that a NP with a phosphomimetic residue within the insertion-groove could also cause an inhibition in RNP complex formation when competed with wildtype NP as the NP phosphomimetic would be recruited via its wildtype tail-loop and this would block further recruitment of wildtype NP. RNP reconstitutions, where a constant amount of wildtype NP expressing plasmid was transfected with increasing amounts of plasmid expressing NP S165E were carried out and the RNA was analysed by primer extension. No inhibition of transcription or replication was observed as the concentration of plasmid expressing NP S165E was increased from 0.5μg to 3μg (figure 5.6). The inability of S165E to inhibit transcription and replication in the presence of wildtype NP suggests that it cannot form part of the RNP complex and inhibit further recruitment of wildtype NP.

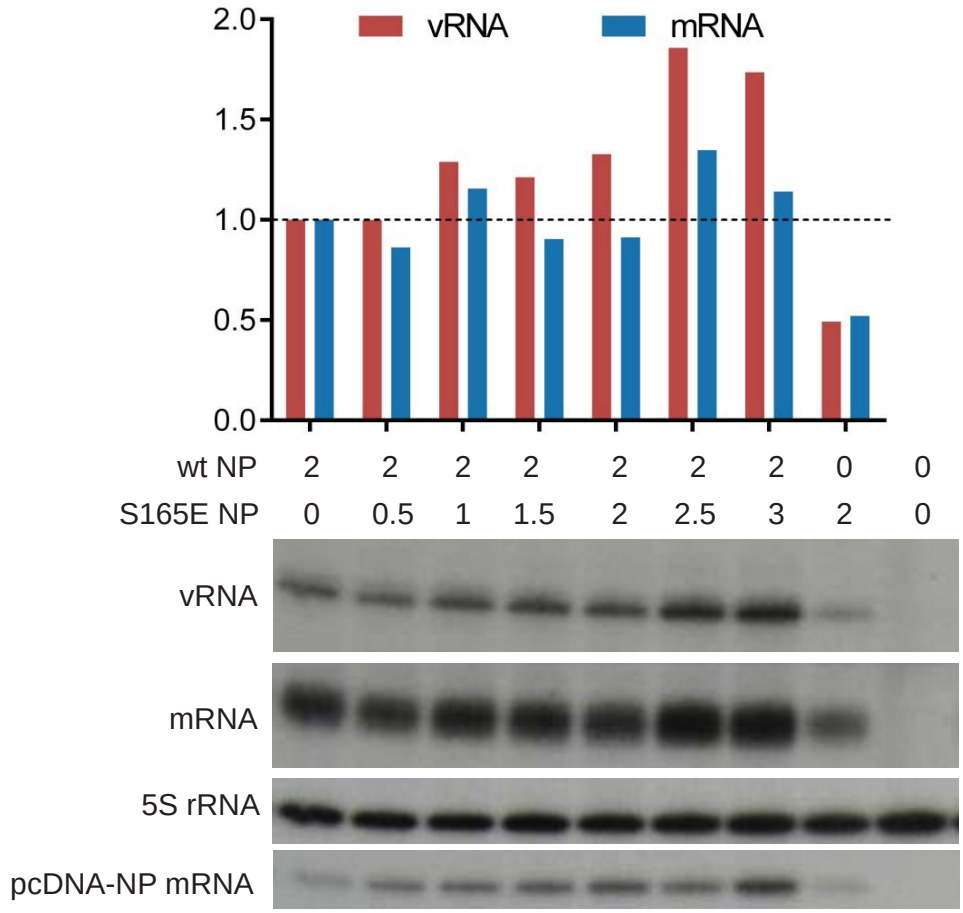


Figure 5.6. Competition of S165E with wildtype NP. Plasmids expressing the three viral polymerase subunits, each at a quantity of 0.5µg, and wildtype (wt) NP at 2µg throughout were transfected. In addition a plasmid expressing S165E, at the quantities indicated in µg, were transfected into 293T cells with 0.5µg pPOLI plasmid encoding the 1565-nucleotide vRNA-like template. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. mRNA produced from the pcDNA-NP (wildtype and mutant) plasmid was detected. 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal.

5.4.5 Mutating residue S233 of influenza B NP affects its ability to support viral transcription and replication

A phosphorylation site within the insertion-groove of influenza B virus NP (BNP) was also identified at position S223 (figure 5.1) (Hutchinson et al., 2012). To test if this phosphorylation site plays a role in RNP complex formation and, therefore, RNA polymerase transcription and replication, S223 was mutated to alanine, glutamic acid or aspartic acid. These BNP mutants were then tested in influenza B virus RNP reconstitutions for their ability to support viral transcription and replication of an influenza B virus NA gene segment. The intermediate length influenza A virus templates were also tested as influenza B virus polymerase is compatible with an influenza A virus gene segment promoter (Crescenzo-Chaigne et al., 1999; Iwatsuki-Horimoto et al., 2008). The RNA from the RNP reconstitutions, on different length templates, was analysed by primer extension (figure 5.7). In the case where S223 was mutated to alanine, so that this site could no longer be phosphorylated, viral transcription and replication were reduced by approximately 40% compared to wildtype NP on a full-length influenza B virus NA gene segment and the two intermediate-length templates, 221- and 583-nucleotides in length. In the case of the phosphomimetic mutations, NP S223E was unable to support transcription or replication of any length template tested and NP S223D was able to support a degree of transcription and replication but only to approximately 40% compared to wildtype NP on all length templates tested. The expression of the three mutants was confirmed to be similar to wildtype NP (figure 5.7).

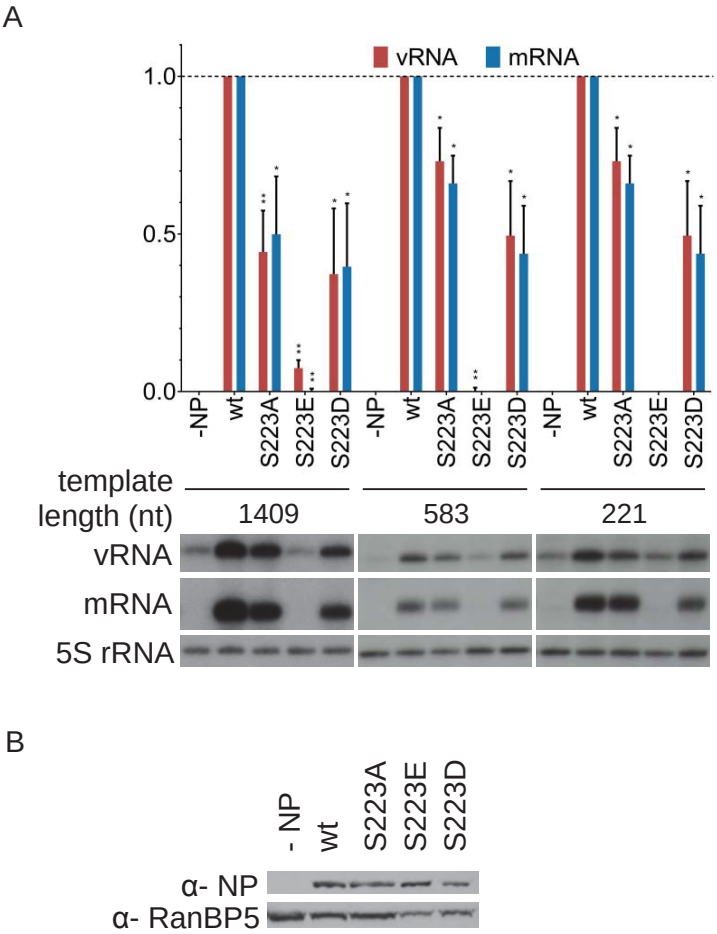


Figure 5.7. Effect of mutations at S223 of influenza B NP on transcription and replication by the viral RNA polymerase. (A) RNP reconstitutions were carried out in 293T cells by transfecting plasmids to express the viral polymerase, wildtype (wt) or mutant NP, and a plasmid to express full-length genomic vRNA (NA, 1554 nucleotides long or NP vRNA deletion mutants of the indicated length). A negative control without NP was included for each template. RNA harvested 30 hours post transfection was analysed by primer extension. The amount of mRNA or vRNA was quantified using phosphorimage analysis and the level of 5S rRNA was used as a loading control. The signal of mRNA and vRNA in the negative control was set to 0 and the signals in the presence of wildtype NP were set to 1. Error bars represent the variation observed in the 3 repeats and statistical analysis was carried out using one-tailed student's t-test (* $P<0.05$ and ** $P<0.01$). (B) The expression of NP mutants in the RNP reconstitution was analysed by western blot using a rabbit polyclonal NP antibody. A RanBP5 antibody was used as a loading control.

5.4 Discussion

Formation of RNP complexes during the viral life-cycle is essential for viral replication, leading to the generation of progeny viruses. For the formation of RNP complexes, newly translated NP and the viral polymerase subunits have to be imported in the nucleus and assemble onto the newly synthesised RNA in viral replication. It has been proposed that for NP to be RNA encapsidation-competent, it must be maintained as a monomer (Chenavas et al., 2013; Tarus et al., 2012a). In addition, NP recruitment into the RNP complex can occur via the tail-loop of incoming NPs interacting with the insertion-groove of the resident NP (Turrell et al., 2013). For this to occur the oligomerisation domains of NP would have to be available for recruitment; therefore, NP that had already oligomerised would not form a chain of NPs along the length of the RNA (Tarus et al., 2012a). The identification of a phosphorylation site within the insertion-groove of influenza A NP, conserved in influenza B NP (Hutchinson et al., 2012) (figure 5.1), led to the hypothesis that NP oligomerisation, within the host-cell, could be regulated by reversible phosphorylation of NP within its oligomerisation domain. Phosphorylation at S165 in influenza A virus was also identified by an independent group and mutation of S165 to aspartic acid resulted in recombinant NP S165D to behave in a similar fashion to wildtype monomeric NP (Chenavas et al., 2013).

The results presented in this study highlight the importance of the phosphorylation site S165 in viral growth as mutation of this site significantly reduces viral growth compared to wildtype virus (figure 5.2). In agreement with Chenavas et al., mutation of S165 to aspartic acid results in an inhibition in recombinant NP oligomerisation when expressed in insect cells (figure 5.4). In contrast, mutation

of S165 to alanine within the insertion-groove does not affect the ability of recombinant NP to oligomerise (figure 5.4). Despite the oligomerisation status of NP S165A and NP S165D being very different they have very similar phenotypes in their ability to support transcription and replication (figure 5.5) and viral growth (figure 5.2). In the case of NP S165D, NP is monomeric and monomeric NP has been shown to be unable to support transcription and replication of full-length RNA templates (Chan et al., 2010). However, when S165 is mutated to alanine NP oligomerisation can no longer be regulated and this deregulation inhibits the ability of NP to support transcription and replication. This reduction in transcription and replication in the presence of S165A may be due to a reduction in the monomeric NP pool that is required for NP assembly onto the RNA (Tarus et al., 2012a).

Even though both glutamic acid and aspartic acid are often used as phosphomimetics in the literature there was a stark difference between the two residues in their effect on the ability of NP to support viral growth despite them having the same oligomerisation profiles (figure 5.4). In the case when NP S165 is mutated to glutamic acid no virus could be rescued, whereas when NP S165 is mutated to aspartic acid the recombinant virus was able to support a degree of viral growth, in agreement with the differences in their ability to support transcription and replication on full-length RNA templates. This difference in phenotypes between S165E NP and S165D NP could be due to the larger size of the glutamic acid side chain compared to aspartic acid, with an extra carbon-carbon bond (figure 5.1). Due to the polypeptide of NP running back and forth between the head and body domains, it can be presumed that changes in the structure within the insertion-groove could affect the overall structure of NP (Ng

et al., 2008; Ye et al., 2006). It may, therefore, be that the larger size of glutamic acid within the groove compared to aspartic acid may affect the overall structure of NP. The inability of S165E to inhibit RNP complex activity in competition with wild-type NP (figure 5.6), suggests that the tail-loop of this mutant is not in a conformation that can support its recruitment into the RNP complex. Further work is required to characterise the two phosphomimetic NP mutants in order to understand their different phenotypes in recombinant virus rescue and RNP reconstitution.

Mutation of the phosphorylation site within the oligomerisation insertion-groove of influenza B NP, S223, had a dramatic effect on transcription and replication (figure 5.7). Again the two phosphomimetics gave different phenotypes, with influenza B virus NP S223E unable to support transcription and replication of any length RNA template, whereas NP with the aspartic acid mutation could support transcription and replication to approximately 50% compared to wildtype NP on all length RNA templates tested. The complete absence of these activities observed in the presence of S223E mutant on the 221-nucleotide template suggests that this NP cannot carry out an essential NP function required to support transcription and replication of an intermediate-length template (Turrell et al., 2013). The reason for the differences in phenotypes observed on the intermediate-length RNA templates when phosphorylation sites within the insertion-groove of influenza A and B virus NP are mutated is unclear.

The results have led to a model whereby influenza NP mRNA, produced by transcription of the incoming vRNP, is translated by the host-cell machinery to produce NP. Oligomerisation of soluble newly expressed NP in the host cell is prevented by phosphorylation of NP within the insertion-groove, resulting in a

pool of monomeric NP. The phosphorylated monomeric NP could then be dephosphorylated when in the vicinity of a replicating RNP allowing NP recruitment and oligomerisation along the length of the RNA. Identification and antiviral targeting of the host-cell kinase responsible for phosphorylation of NP within the insertion-groove would allow a way in which to inhibit viral growth; however, there are clear obstacles in inhibiting host-cell proteins that would have to be overcome. Likewise, for dephosphorylation of S165 within the insertion-groove of NP to occur a host-cell phosphatase must play a role, as influenza virus does not encode a phosphatase. The viral polymerase interacts with several host-factors including protein phosphatase 6, whose activity was shown to play a role in RNA polymerase activity (York et al., 2014). Protein phosphatase 6 may be a possible candidate for the dephosphorylation of NP in the vicinity of the viral polymerase; however, this requires further investigation. Once dephosphorylated, NP could join the growing chain of NPs via its tail-loop, with its insertion-groove would then be available for recruitment of further NPs. Alternatively, phosphorylated NP could join the growing chain of NP and then get dephosphorylated allowing further recruitment of NP. The presence of phosphorylated S165 NP within preparations of purified viruses and RNP complexes (Hutchinson et al., 2012) does not fit with this model; however, only the presence of phosphorylation was identified and not the frequency. The presence of this phosphorylation at S165 in NP within these preparations could be due to several reasons, including carry-over in the purification or monomeric NP associated with the RNP complex.

Recently, further evidence in support of NP phosphorylation being the mechanism by which oligomerisation is regulated was provided (Mondal et al., 2015). In

addition to phosphorylation at S165, this study identified a phosphorylation site within the tail-loop of NP, at position S407. Mutation of this site to aspartic acid inhibited oligomerisation and mutation of this site to alanine allowed NP oligomerisation in mammalian cells, in a similar way to the data for S165 in figure 5.4. Therefore, it may be that both of these phosphorylation sites, within the oligomerisation domains of NP, play roles in regulating NP oligomerisation during viral infection. In addition, this recent study further highlights the importance of regulating NP oligomerisation and provides additional evidence, in agreement with the data above, that S165 within the insertion groove of NP plays a role in this regulation.

4.5 Summary

- The regulation of NP oligomerisation is required to support viral replication
- A phosphomimetic amino acid within the oligomerisation insertion-groove of NP inhibits NP oligomerisation
- An alanine mutation within the insertion-groove results in unregulated oligomerisation and in NP existing predominantly as a multimer
- Monomeric phosphomimetic NP is less able to support transcription and replication of full-length RNA templates, compared to wildtype NP
- Multimeric NP, where oligomerisation is not regulated, is less able to support transcription and replication of full-length RNA templates, compared to wildtype NP
- The phosphorylation site within the insertion-groove of BNP is important for NPs function in supporting viral transcription and replication carried out by the RNA polymerase

6 Compatibility between the RNA polymerase and NP

6.1 Overview

It is known that NP from influenza A (ANP) and influenza B (BNP) viruses are unable to support RNA synthesis in the presence of a heterotypic viral RNA polymerase. The inability of the viral polymerase to carry out transcription and replication in the presence of a heterotypic NP suggests that there has to be compatibility between the viral RNA polymerase and NP. This chapter focuses on three NP sequence elements that may play a role in this compatibility: two unstructured loops, one between α-helices 2 and 3 (L1) and the other between α-helices 8 and 9 (L2), and the N-terminus. The results suggest that L1 and L2 from ANP may be required for compatibility with the influenza A polymerase. In addition, it was determined that the N-terminus does not play a role in compatibility as it can be interchanged between ANP and BNP without affecting RNA polymerase activity. Overall, comparing the compatibility of NP with the two different influenza polymerase types provides us with further information about NP's mode of action in supporting RNA polymerase activity.

6.2 Background

Influenza A and influenza B virus infections cause annual epidemics and both types of virus are included in the seasonal influenza vaccines. Even though these two types of influenza virus co-exist in nature, there has been no report of reassortants nor have reassortants been generated *in vitro* (Kaverin et al., 1983; Mikheeva and Ghendon, 1982). Influenza A and influenza B viruses can form homotypic but not heterotypic reassortants suggesting that there must be incompatibility between the two influenza virus types either at the RNA level, protein level or both (Kaverin et al., 1983; Mikheeva and Ghendon, 1982). The incompatibility was shown not to be due to RNA recognition as influenza A virus polymerase subunits can bind to the promoter of an influenza B virus RNA gene segment and vice versa (Crescenzo-Chaigne et al., 1999; Iwatsuki-Horimoto et al., 2008). Instead the incompatibility occurs at the protein level between the two influenza virus types, as demonstrated by studies where the protein components of the RNP complex of influenza A virus were interchanged with those of influenza B virus causing an inhibition in polymerase activity (Crescenzo-Chaigne et al., 1999; Iwatsuki-Horimoto et al., 2008). When all possible combinations of heterotypic polymerase protein subunits were reconstituted in the presence of a chloramphenicol acetyltransferase (CAT) reporter gene no CAT expression was detected suggesting that the three-polymerase protein subunits have to be homotypic for them to form a functional RNA polymerase (Crescenzo-Chaigne et al., 1999). In addition to this, the study showed that CAT expression was not detected when NP was expressed in the presence of a heterotypic polymerase complex suggesting that a homotypic NP is essential to support the RNA polymerase in carrying out RNA synthesis. Iwatsuki-Horimoto et al., reported that

when the RNA polymerase from influenza A virus was combined with NP from two different strains of influenza B virus (B/Panama/45/90 or B/Lee/40), through transfection of plasmids encoding the heterotypic RNP components, the polymerase was unable to carry out transcription of a luciferase reporter gene. Therefore, the results point towards the existence of a type-specific interaction between NP and the viral polymerase that is essential for the viral polymerase to replicate and transcribe the viral genome (Crescenzo-Chaigne et al., 1999).

The crystal structures of both ANP and BNP have been solved (Ng et al., 2012; Ng et al., 2008; Ye et al., 2006). They have very similar overall architectures with head and body domains and a positively charged groove, which has been shown through mutagenesis to be responsible for RNA binding in both cases. Both structures contain a RNA binding loop between alpha helices 2 and 3 (L1) and deletion of this loop in either structure results in significantly reduced NP-RNA binding and RNA polymerase activity (Ng et al., 2012; Ng et al., 2008). In addition it has been suggested that the RNA binding loop in BNP plays a more central role in RNA binding than the loop in ANP, as deletion of this loop in BNP results in a greater fold reduction in RNA binding when compared to wildtype BNP than deletion of the loop in ANP when compared to wildtype ANP (Ng et al., 2012). ANP was crystallised as a trimer (Ng et al., 2008; Ye et al., 2006) whereas BNP was crystallised as a tetramer (Ng et al., 2012); however, in both conformations, neighboring NPs interact via a salt-bridge between a residue in the tail-loop of one NP and a residue in the insertion-groove of the neighboring NP. These data, therefore, suggest that influenza A and influenza B virus NPs oligomerise via the same mechanism and that their RNA binding properties are very similar. In influenza A virus, NP has been shown to bind to the viral

polymerase via three residues, R204, W207 and R208, within the unstructured loop between alpha helices 8 and 9 (L2) (Marklund et al., 2012) and via D88 present within L1 (Gui et al., 2014). The interaction between NP and the viral polymerase is essential for NP to support RNA polymerase activity but at which stage the interaction is required during the viral replication cycle is unclear.

NP contains nuclear localisation signals (NLSs) that are required for the import of RNP complexes into the nucleus and the import of soluble NP into the nucleus to support transcription and replication. Three NLSs have been proposed in ANP: an unconventional NLS within the N-terminus (Wang et al., 1997), a bipartite NLS between residues 198-216 (L2) (Neumann et al., 1997) and a NLS between residues 320-400 (Bullido et al., 2000). Studies into BNP NLSs have identified a NLS within the N-terminus (Sherry et al., 2014; Wanitchang et al., 2013). This is contrary to a paper published proposing that the influenza B N-terminus does not function as a NLS as fusion of the N-terminus from BNP to GFP did not result in its localisation to the nucleus (Stevens and Barclay, 1998). A major difference between ANP and BNP is the length of the N-terminus; the ANP N-terminus is 23 amino acids long whereas the BNP N-terminus is 70 amino acids long. The N-terminus, in both types, is thought to be largely unstructured as it is not present in either crystal structure (Ng et al., 2012; Ng et al., 2008; Ye et al., 2006). The reason for the much longer N-terminus in BNP compared to ANP is unclear.

It is known that NP cannot support RNA synthesis in the presence of a heterotypic RNA polymerase, suggesting that there is a requirement for compatibility between NP and the RNA polymerase. When monotypic NP is expressed in the absence of the RNA polymerase it can bind RNA and oligomerise (Chenavas et al., 2013; Tarus et al., 2012a). However, whether NP can oligomerise and bind RNA when

in the context of a RNP reconstitution in the presence of a heterotypic viral polymerase is unclear. The stage at which compatibility between NP and the viral polymerase is required in viral RNA synthesis is also unclear. Several residues within ANP have been identified which are thought to play a role in ANP polymerase binding; however, whether these regions are involved in NP-polymerase compatibility has not been studied. This chapter identifies and investigates regions within both ANP and BNP that may be required for homotypic compatibility between NP and the viral polymerase.

6.3 Aims

- Evaluate the ability of heterotypic NP to support transcription and replication in a RNP reconstitution
- Determine sequence elements within ANP and BNP, through analysis of their structures, that may be required for compatibility between NP and the viral polymerase
- Investigate the ability of heterotypic NP chimeras, where these sequence elements have been swapped between the two influenza virus types, to support viral transcription and replication in both heterotypic and homotypic polymerase systems

6.4 Results

6.4.1 NP incompatibility with the heterotypic RNA polymerase

To test if both viral transcription and replication were inhibited when the viral polymerase was expressed in the presence of a heterotypic NP, RNP reconstitutions were carried out with polymerase subunits and NP derived from either influenza A or influenza B virus. When influenza A virus RNA polymerase subunits were expressed in the presence of influenza B virus NP and an influenza A virus NA gene segment no viral transcription or replication could be detected (figure 6.1a). In addition, when influenza B virus RNA polymerase subunits were expressed in the presence of influenza A virus NP and an influenza B virus NA gene segment no viral transcription or replication could be detected (figure 6.1b) This is in agreement with previous findings that NP is not interchangeable between the two influenza virus types (Crescenzo-Chaigne et al., 1999; Iwatsuki-Horimoto et al., 2008) and shows that both viral transcription and replication are inhibited in the presence of a heterotypic NP.

6.4.2 Structural analysis of ANP and BNP

It has been hypothesised that intertypic incompatibility between NP and the viral polymerase is caused by their inability to interact (Crescenzo-Chaigne et al., 1999). To identify regions in NP that are required for compatibility between NP and the viral polymerase the structures of ANP from influenza A/WSN/33 virus (Ye et al., 2006) and the structure of BNP from influenza B/Managua/4577.01/2008 (Ng et al., 2012) were analysed. For the reasons described below three key regions were identified which were proposed to play a

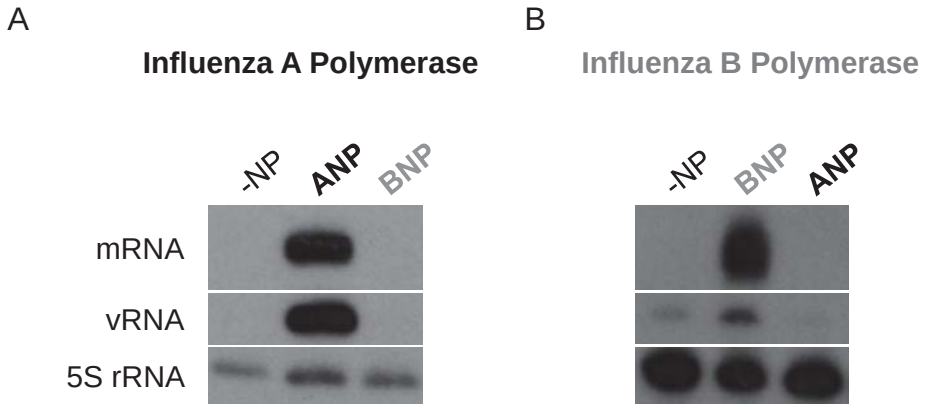


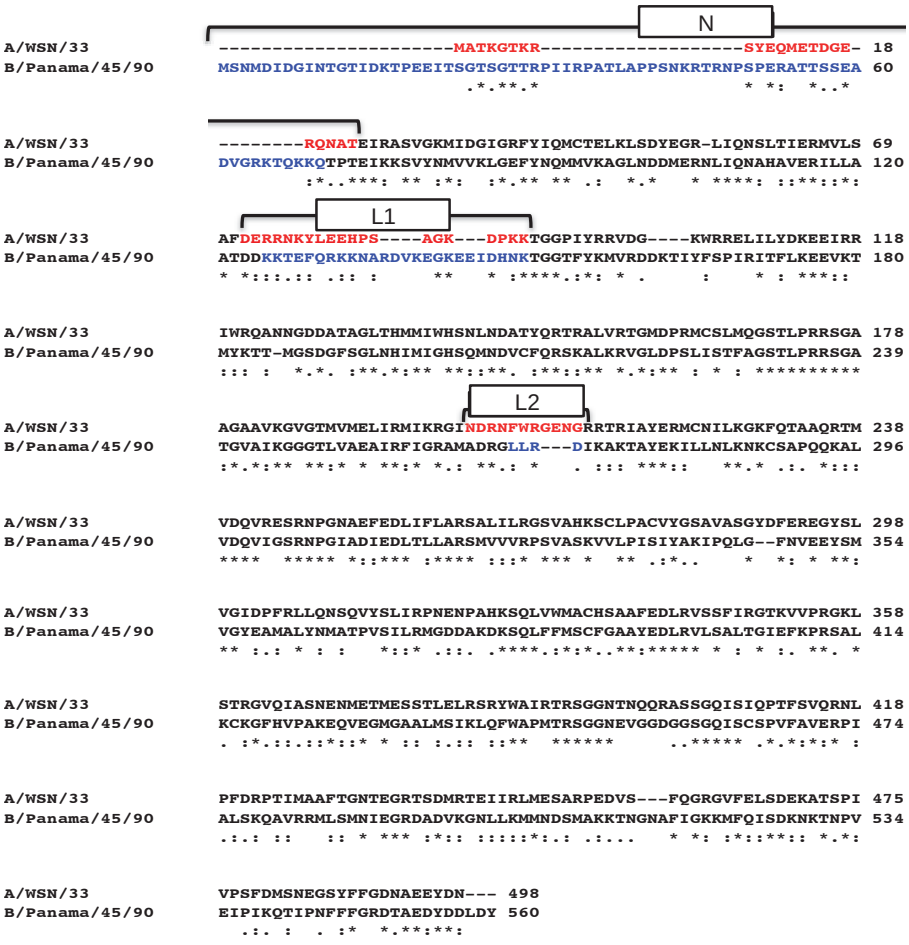
Figure 6.1. Incompatibility of influenza A and influenza B RNA polymerase with heterotypic NP. Plasmids expressing the three viral polymerase subunits of influenza A (A) and influenza B (B) virus, each at a quantity of 0.5 μg, and homotypic or heterotypic nucleoprotein, at 1 μg, were transfected with 0.5 μg pPOLI or pPR- plasmid encoding the corresponding NA vRNA. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. 5S rRNA was used as a loading control.

role the homotypic compatibility between NP and the viral polymerase: the loop between alpha helices 2 and 3 (L1), the loop between alpha helices 8 and 9 (L2) and the N-terminus. In ANP residues R204, W207 and R208 have been shown to play a role in viral RNA polymerase binding (Marklund et al., 2012) and are within L2 (figure 6.2a). L2 is not visible in the crystal structures of ANP (11 amino acids) presumably due to its flexible nature, whereas in BNP L2 (4 amino acids) is visible in the structure forming a tight loop between the two helices. L1 is in close proximity to L2 and D88 within L1 has been proposed to play a role in polymerase binding (Gui et al., 2014). The N-terminus was also studied due to the large difference in amino acid length between the N-terminus of the two influenza types. Both L1 and the N-terminus are not visible in the crystal structures of ANP or BNP suggesting that they are flexible. To assay if these three regions are required for NP polymerase compatibility NP chimeras were constructed where these regions were interchanged between the two influenza types (figure 6.2b). N-termini deletion mutants of both ANP and BNP were also constructed. The ability of these NP chimeras and deletion mutants to support transcription and replication by the viral RNA polymerase in their heterotypic and homotypic systems was tested.

6.4.3 The ability of NP chimeras to support RNA synthesis in the presence of a heterotypic RNA polymerase

It was hypothesised that if any of the sequence elements identified were required for compatibility between NP and its homotypic RNA polymerase then a heterotypic NP chimera that contained the homotypic determining sequence

A



B

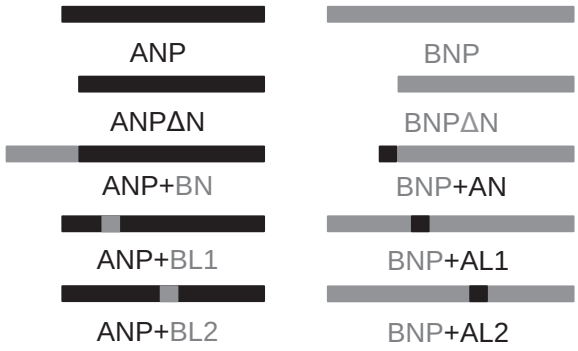


Figure 6.2. Identification of sequence elements that may determine the incompatibility between NP and a heterotypic RNA polymerase. (A) Alignment of ANP (A/WSN/33- GenBank: M30746.1) and BNP (B/Panama/45/90- GenBank: CY115338.1) using Clustal W2. The three sequence elements identified through analysis of the structures of ANP are highlighted; N-terminus (N), loop between alpha helices 2 and 3 (L1) and loop between alpha helices 8 and 9 (L2). (B) Schematic of the NP chimeras. ANP chimeras with the ANP amino acid sequence as the backbone that are modified by either deletion or replacement of sequence elements with those from BNP. BNP chimeras with the BNP amino acid sequence as the backbone that are modified by either deletion or replacement of sequence elements with those from ANP.

element may now be compatible with a heterotypic polymerase. To test if any of the NP chimeras were now compatible with a heterotypic polymerase due to the insertion of the homotypic sequence element RNP reconstitutions were set up. The ability of the RNA polymerase, from one influenza type, to support transcription and replication of a NA gene segment from the same type was determined in the absence of NP, in the presence of homotypic NP or in the presence of heterotypic wildtype and chimeric NP. Figure 6.3 shows that when the amino acid sequence of L1 or L2 or the N-terminus is replaced in BNP with the cognate amino acid sequences from ANP, the BNP chimera is still incompatible with its heterotypic influenza A polymerase. Similarly, when the amino acid sequences of L1 or L2 or the N-terminus are replaced in ANP with the cognate amino acid sequences from BNP, the ANP chimera is still incompatible with heterotypic influenza B polymerase. This suggests that none of these regions alone are responsible for the compatibility between the RNA polymerase and homotypic NP. BNP chimeras were then tested in the heterotypic influenza A system with an intermediate-length RNA template, as the RNA polymerase requirement for a fully functional NP is less stringent on an intermediate template compared to a full-length template (Turrell et al., 2013). Figure 6.3 shows that there is a low level of transcription and replication of the 149-nucleotide template by influenza A virus RNA polymerase in the presence of heterotypic BNP; however, compared to the presence of homotypic ANP, RNA polymerase activity is approximately 100-fold less. This low level of transcription and replication in the presence of heterotypic BNP is equivalent to the level observed in the presence of BNP chimera where the N-terminus has been replaced with the ANP N-terminus (BNP+AN). There is a significant increase in the level of transcription products when BNP L1 or L2 is

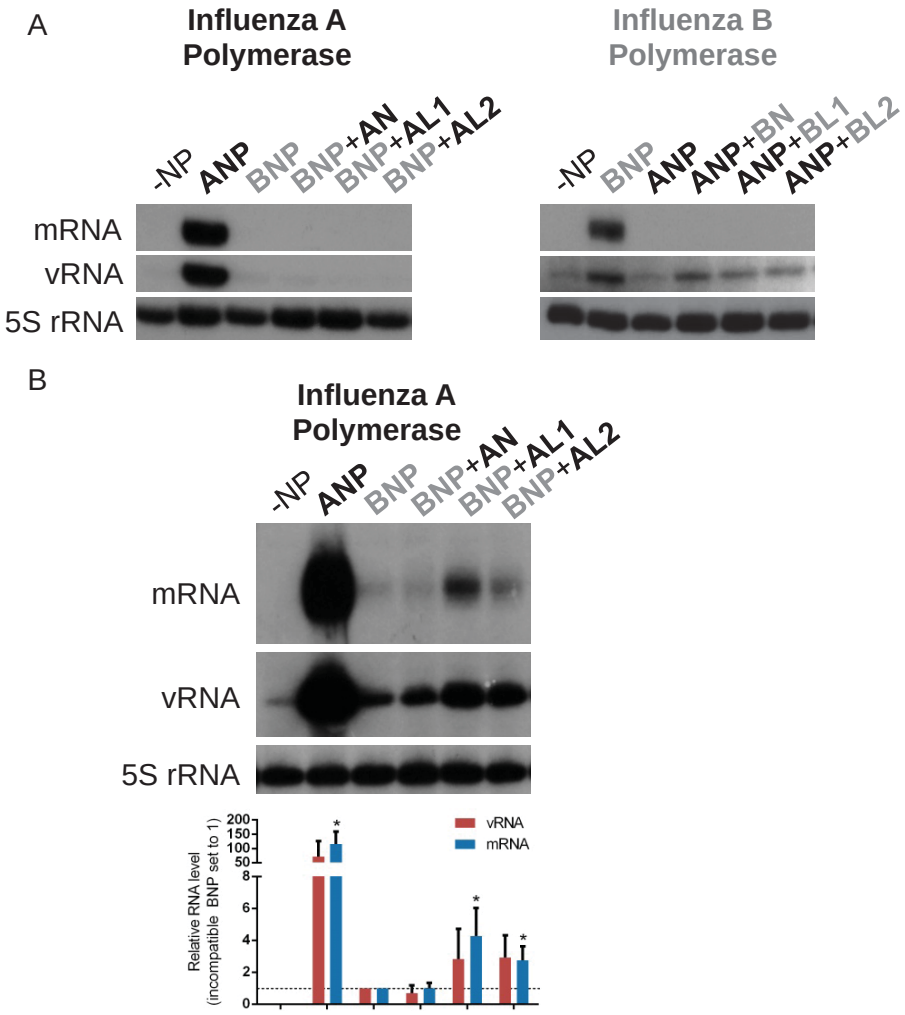


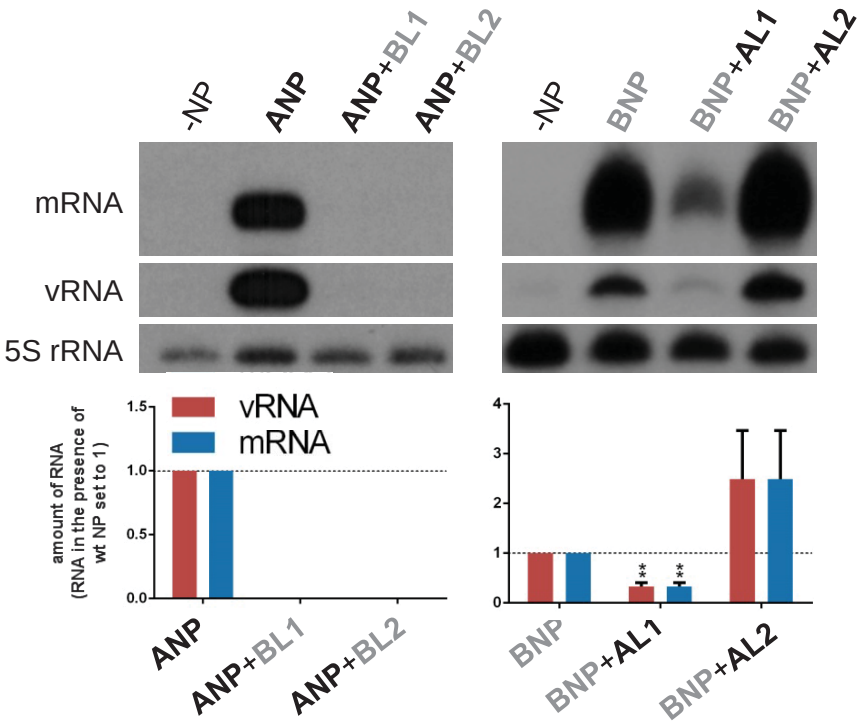
Figure 6.3. The ability of NP chimeras to support RNA synthesis in the presence of a heterotypic RNA polymerase (A) RNP reconstitutions were carried out in 293T cells where plasmids encoding the viral RNA polymerase, nucleoprotein and NA template were transfected. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. The level of 5S rRNA in each sample was used as an internal control. The left panel shows the influenza A system in the presence of the BNP chimeras with ANP as the positive control. The right panel shows the influenza B system in the presence of the ANP chimeras with BNP as the positive control. (B) RNP reconstitutions in the A system with a pPOLI plasmid encoding the 149 nucleotide vRNA-like template. The wildtype BNP and BNP chimeras were tested with ANP as a positive control. The amount of mRNA or vRNA was quantified using phosphorimage analysis and the level of 5S rRNA was used as an internal control. The signal of mRNA and vRNA in the negative control was set to 0 and the signals in the presence of wildtype heterotypic BNP was set to 1. Error bars represent the variation observed in the 3 repeats and statistical analysis was carried out using one-tailed student's t-test (* $P<0.05$).

replaced with the cognate amino acid sequence from ANP L1 or L2 (BNP+AL1 and BNP+AL2) by approximately 3-4 fold compared to wildtype heterotypic BNP. However, transcription and replication are still reduced by 96% when compared to the presence of homotypic ANP. Therefore, loops L1 and L2 may play a partial role in the compatibility between NP and the polymerase; however, these data suggest that a combination of sequence elements may be required for compatibility.

6.4.4 The ability of NP L1 and L2 chimeras to support RNA synthesis in the presence of a homotypic RNA polymerase

As none of the sequence elements alone were solely responsible for compatibility between NP and the RNA polymerase, the ability of the NP chimeras to support RNA polymerase activity in their homotypic system was tested to investigate if these sequence elements were required for polymerase function. Firstly the loops were investigated where L1 and L2 in ANP had been replaced with the cognate amino acid sequences of L1 and L2 from BNP and these ANP chimeras were tested for their ability to support influenza A homotypic polymerase activity. The replacement of L1 in ANP with L1 from BNP (ANP+BL1) resulted in an ANP chimera that could not support RNA polymerase activity in its homotypic influenza A system on a full-length RNA gene segment (figure 6.4a). Similarly, the replacement of L2 in ANP with L2 from BNP (ANP+BL2) resulted in another ANP chimera that could not support RNA polymerase activity in its homotypic influenza A system on a full-length RNA gene segment (figure 6.4a). Analysis of the cellular localisation of ANP where L2 has been replaced showed that

A


-NP
ANP
ANP+BL1
ANP+BL2
-NP
BNP
BNP+AL1
BNP+AL2
mRNA
vRNA
5S rRNA
amount of RNA (RNA in the presence of wt NP set to 1)
vRNA
mRNA
1.5
1.0
0.5
0.0
4
3
2
1
0
**
**
ANP
ANP+BL1
ANP+BL2
BNP
BNP+AL1
BNP+AL2

B

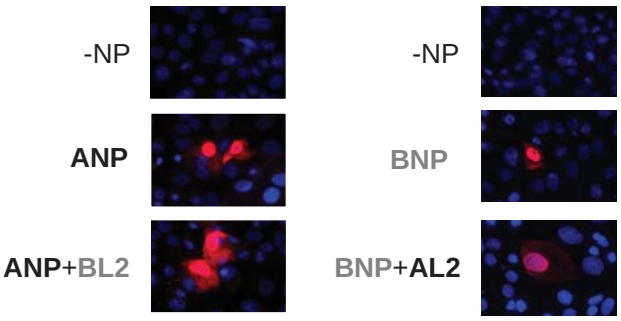

-NP
ANP
ANP+BL2
-NP
BNP
BNP+AL2

Figure 6.4. The ability of NP L1 and L2 chimeras to support RNA synthesis of a full-length template in the presence of a homotypic RNA polymerase (A) RNP reconstitutions were carried out in 293T cells where plasmids encoding the viral RNA polymerase, nucleoprotein and NA template were transfected. The left panel shows the influenza A system (black) in the absence of NP, presence of compatible NP (ANP), presence of ANP where the loop between alpha helices 2 and 3 has been replaced with the loop from BNP (ANP+BL1) or presence of ANP where the loop between alpha helices 8 and 9 has been replaced with the loop from BNP (ANP +BL2). The right hand panel shows the influenza B system (grey) in the absence of NP, presence of compatible NP (BNP), presence of BNP where the loop between alpha helices 2 and 3 has been replaced with the loop from ANP (BNP+AL1) or presence of ANP where the loop between alpha helices 8 and 9 has been replaced with the loop from BNP (BNP+AL2). The amount of mRNA or vRNA was quantified using phosphorimage analysis and the level of 5S rRNA was used as an internal loading control. The signal of mRNA and vRNA in the negative control was set to 0 and the signals in the presence of wildtype NP was set to 1. Error bars represent the variation observed in the 3 repeats and statistical analysis was carried out using one-tailed student's t-test (** $P<0.01$). The expression of the different NP deletion mutants and chimeras in the RNP reconstitution was tested by western blot analysis with RanBP5 used as an loading control.

swapping the loop does not result in a change of NP localisation (figure 6.4b), despite L2 containing the bi-partite NLS sequence in ANP. As no mRNA or vRNA was detected in the presence of the ANP with BNP L1 or L2 their ability to support RNA polymerase activity on an intermediate 149-nucleotide template was tested to determine if the ANP chimeras were folded correctly to form a functional NP. The requirement for NP properties, such as oligomerisation, in supporting transcription and replication is less stringent on an intermediate length template compared to a full-length template (Turrell et al., 2013). Both ANP chimeras with either L1 or L2 sequence elements replaced with the cognate amino acid sequence of L1 or L2 from BNP (ANP+BL1 and ANP+BL2) were able to support transcription and replication of the intermediate 149-nuleotide template when the plasmid expressing the NP chimeras was transfected at high concentrations (figure 6.5). This therefore suggests that both ANP loop chimeras do fold to form functional NPs but they are unable to support transcription and replication on a full-length template.

BNP chimeras were also tested in their homotypic influenza B system on a full-length RNA template. The loops were investigated where L1 and L2 in BNP had been replaced with the cognate amino acid sequence of L1 and L2 from ANP and these BNP chimeras were tested for their ability to support influenza B homotypic polymerase activity. The replacement of L1 in BNP with L1 from ANP (BNP+AL1) resulted in a BNP chimera that could support transcription and replication to approximately 40% compared to wildtype homotypic BNP (figure 6.4a). In contrast, the replacement of L2 in BNP with L2 from ANP (BNP+AL2) resulted in a BNP chimera that was able to support transcription and replication of a full-length RNA template at a similar level to homotypic wildtype BNP

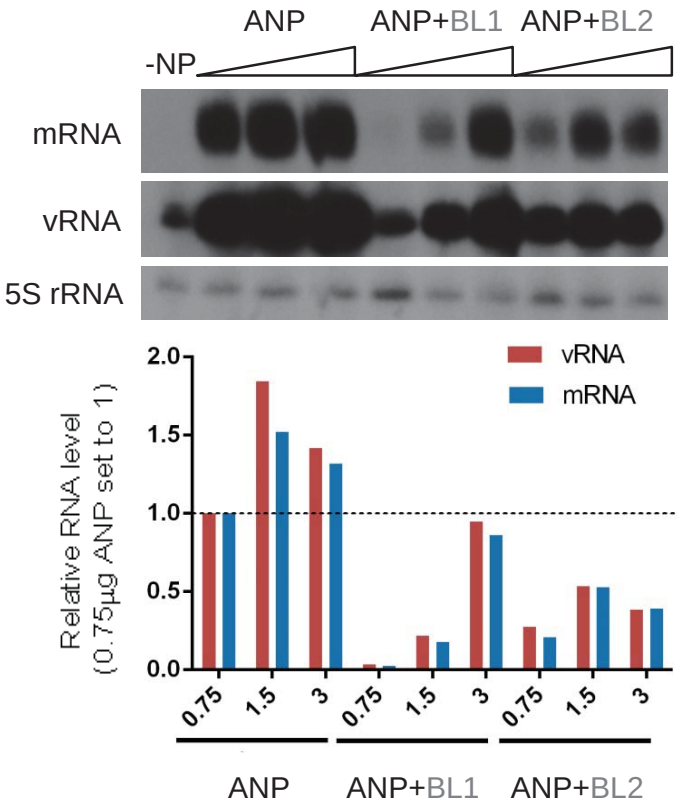


Figure 6.5. The ability of NP L1 and L2 chimeras to support RNA synthesis of an intermediate-length template in the presence of a homotypic RNA polymerase. Plasmids expressing the three influenza A viral polymerase subunits, each at a quantity of 0.5 µg, and increasing quantities of NP expressing plasmids were transfected into 293T cells with 0.5 µg pPOLI plasmid encoding the 149 nucleotide vRNA-like template. Total RNA was harvested after 30-40 hours and analysed by primer extension with the detection of viral transcription (mRNA) and replication (vRNA) products. 5S rRNA was used as a loading control and the mRNA and vRNA levels were normalised to the 5S rRNA signal. The signal of mRNA and vRNA in the negative control was set to 0 and the signals in the presence of wildtype NP was set to 1. Error bars represent the variation observed in the 3 repeats and statistical analysis was carried out using one-tailed student's t-test (* $P<0.05$ and ** $P<0.01$).

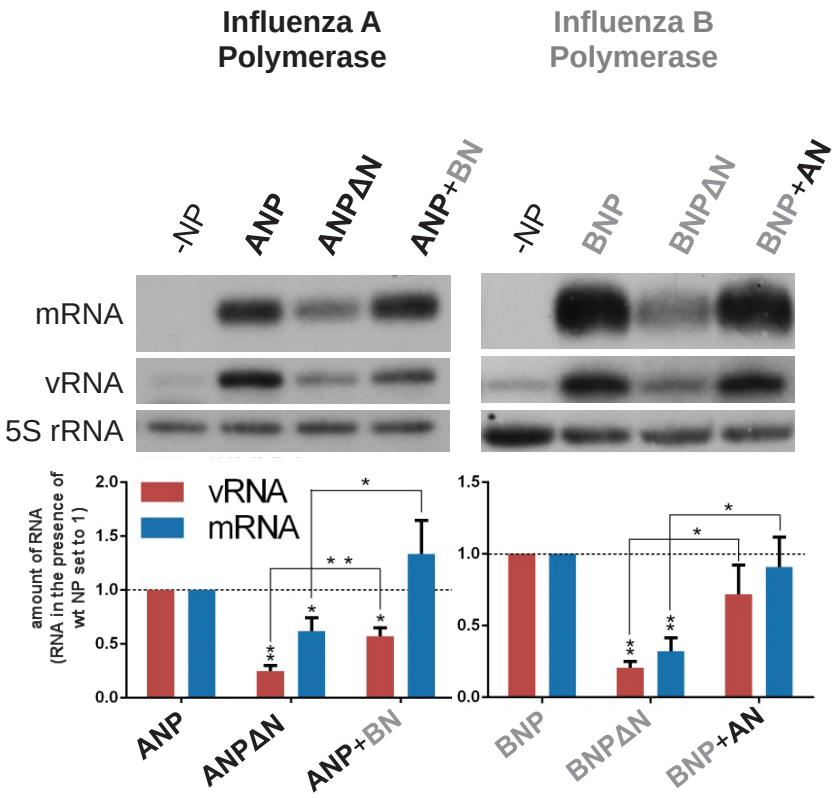
(figure 6.4a). Analysis of the cellular localisation of BNP where L2 had been replaced showed that swapping the loop does not result in a change of NP localisation (figure 6.4b).

Altogether the requirement for the L1 and L2 sequence elements is different between the two influenza types. In ANP L1 and L2 from ANP are essential to support homotypic influenza A polymerase activity on a full-length gene segment as they cannot be replaced with BNP L1 and L2. The requirement for these sequence elements is different in BNP. L1 from BNP is required for BNP to support transcription and replication at levels similar to homotypic wildtype BNP although BNP+AL1 still shows some activity indicating that in BNP L1 from BNP is not essential for activity. In contrast, L2 can be replaced with L2 from ANP and the BNP chimera is able to still support transcription and replication at similar levels to homotypic wildtype BNP.

6.4.5 The ability of NP N-terminal chimeras to support RNA synthesis in the presence of a homotypic RNA polymerase

Next the ability of NP chimeras where the N-terminus has been replaced with the N-terminus from the opposite type were tested in their homotypic system. Deletion mutants were also tested. When the N-terminus was deleted from ANP (ANPΔN), this resulted in a significant decrease in transcription and replication when compared to wildtype ANP in the homotypic influenza A system on a full-length template (figure 6.6a). Deletion of the N-terminus results in a shift in cellular localisation; ANPΔN has a predominantly cytoplasmic localisation, whereas wildtype ANP has a nuclear/cytoplasmic localisation (figure 6.6b).

A


Influenza A Polymerase
Influenza B Polymerase
-NP
ANP
ANPΔN
ANP+BN
-NP
BNP
BNPΔN
BNP+AN
mRNA
vRNA
5S rRNA
amount of RNA (RNA in the presence of wt NP set to 1)
vRNA
mRNA
ANP
ANPΔN
ANP+BN
BNP
BNPΔN
BNP+AN

B

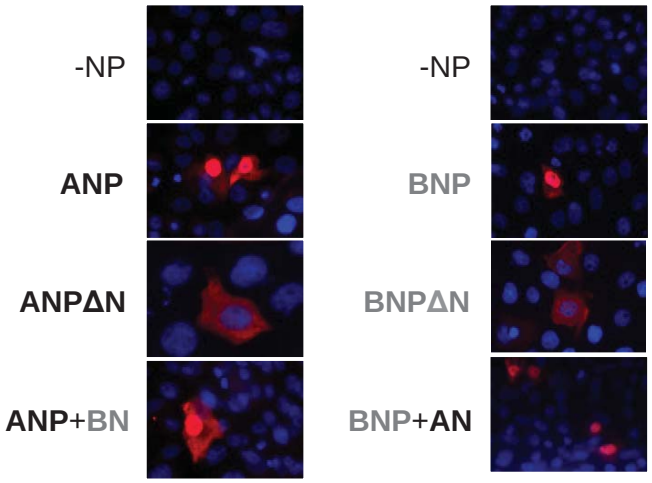

-NP
ANP
ANPΔN
ANP+BN
-NP
BNP
BNPΔN
BNP+AN

Figure 6.6. The ability of NP N-terminal chimeras to support RNA synthesis in the presence of a homotypic RNA polymerase. (A) RNP reconstitutions were carried out in 293T cells where plasmids encoding the viral RdRp, nucleoprotein and NA template were transfected. The left panel shows the influenza A system (black) in the absence of NP, presence of compatible NP (ANP), presence of ANP with a deleted N-terminus (ANPΔN) or presence of ANP with its N-terminus replaced with that of the BNP N-terminus (ANP+BN). The right hand panel shows the influenza B system (grey) in the absence of NP, presence of compatible NP (BNP), presence of BNP with a deleted N-terminus (BNPΔN) or presence of BNP with its N-terminus replaced with that of the ANP N-terminus (BNP+AN). The amount of mRNA or vRNA was quantified using phosphorimage analysis and the level of 5S rRNA was used as an internal loading control. The signal of mRNA and vRNA in the negative control was set to 0 and the signals in the presence of wildtype NP was set to 1. Error bars represent the variation observed in the 3 repeats and statistical analysis was carried out using one-tailed student's t-test (* $P<0.05$ and ** $P<0.01$). (B) Immunofluorescence was used to determine the location of the NP deletion mutants and chimeras. A representative of the observed localisation is shown here. DAPI staining (blue) and NP staining (red) were carried out.

Replacement of the N-terminus of ANP with the BNP N-terminus (ANP+BN) resulted in a significant increase in both transcription and replication compared to ANP N-terminal deletion mutant (ANPΔN) (figure 6.6a) and a significant decrease in replication when compared to wildtype ANP in the homotypic influenza A system on a full-length template. ANP with an N-terminus derived from BNP (ANP+BN) has a nuclear/cytoplasmic localisation, similar to wildtype ANP (figure 6.6b).

These experiments were also carried out in the influenza B system with the BNP N-terminus deletion mutant (deletion of the first 70 amino acids) and the BNP N-terminus chimera. Like in the influenza A system, the deletion of the N-terminus of BNP (BNPΔN) resulted in a significant decrease in both transcription and replication (figure 6.6a) in the homotypic influenza B system on a full-length template and the NP deletion mutant was localised to the cytoplasm (figure 6.6b). The replacement of the N-terminus of BNP with the ANP N-terminus (BNP+AN) results in a significant increase in transcription and replication when compared to the BNP N-terminal deletion mutant (BNPΔN) and similar levels of transcription and replication when compared to wildtype BNP in the homotypic influenza B system on a full-length template (figure 6.6a). BNP with an N-terminus derived from ANP (BNP+AN) has nuclear/cytoplasmic localisation similar to wildtype BNP (figure 6.6b).

Therefore, the N-terminus of ANP can carry out the same function as the N-terminus of BNP in supporting RNA polymerase activity, and this suggests that the N-terminus is not the sequence element that is required for compatibility between NP and the viral RNA polymerase.

6.5 Discussion

Influenza A and influenza B viruses coexist in nature but only homotypic reassortment occurs; heterotypic reassortment has never been found in nature or been generated *in vitro* (Kaverin et al., 1983; Mikheeva and Ghendon, 1982). This is thought to be due to incompatibility of the two influenza types at the protein level, with the requirement for a homotypic polymerase, where all of the polymerase subunits are derived from the same influenza type, and compatibility between the polymerase and NP (Crescenzo-Chaigne et al., 1999; Iwatsuki-Horimoto et al., 2008). In agreement with previous findings, it was found in this study that ANP is not compatible with the heterotypic influenza B polymerase and BNP is not compatible with the heterotypic influenza A polymerase (figure 6.1). It was proposed that inhibition of polymerase function in the presence of a heterotypic NP is due to the absence of an interaction between NP and the polymerase (Crescenzo-Chaigne et al., 1999). The essential interaction between NP and the viral polymerase that confers compatibility could be required at several stages in the influenza virus life-cycle such as in the recruitment of the first NP onto the progeny RNA and/or for the processivity of the polymerase and/or for the stabilisation of the RNA polymerase independent of NP-RNA binding. It was hypothesised in this study that a sequence element of NP may be responsible for the interaction between NP and the viral polymerase which confers compatibility. Therefore, the introduction of this sequence element in a heterotypic NP may cause this NP chimera to then become compatible with a heterotypic polymerase.

NP was shown to interact with the polymerase (Biswas et al., 1998) and more recently residue D88 within L1 (Gui et al., 2014) and residues R204, W207 and

R208 within L2 have been shown to play a role in the NP polymerase interaction (Marklund et al., 2012). Mutation of R204, W207 and R208 residues to alanine inhibited the interaction between NP and the viral polymerase and this mutant NP was no longer able to support RNA polymerase activity (Marklund et al., 2012). At what stage the replication cycle is inhibited when the interaction between the polymerase and NP is blocked is unclear. In this chapter NP chimeras were constructed where L1 or L2 was swapped with L1 or L2 from the opposite influenza type in order to see if the presence of either of these loops in a heterotypic NP conferred compatibility between the RNA polymerase and a heterotypic NP with a homotypic sequence element (figure 6.2). The undetectable polymerase activity in the presence of heterotypic NP with a homotypic loop for either influenza A or influenza B virus polymerase on a full-length template suggests that neither L1 nor L2 are solely responsible for compatibility between NP and the polymerase that is required for RNA synthesis (figure 6.3). A small, but significant, gain in influenza A polymerase activity with BNP+AL1 or BNP+AL2 when analysed on an intermediate-length template versus a full-length template points towards the loops playing some role in compatibility between ANP and the influenza A polymerase. It may be that these two sequence elements L1 and L2 are required in combination for compatibility with the viral polymerase. Indeed, molecular dynamics studies proposed that a salt-bridge could form between R208 (L2) and E80 (L1), which may stabilise viral RNA in the RNA binding-groove (Tarus et al., 2012b). If this interaction between L1 and L2 was essential for NP to stabilise RNA binding and support RNA polymerase activity then the swapping of the loops may disrupt this interaction in certain combinations. Therefore, the replacement of L1 or L2 in ANP with the L1 or L2

from BNP may prevent this interaction occurring between the two loops inhibiting RNA synthesis (figure 6.4). Further work is required to broaden the understanding of these loops in RNA polymerase activity.

When the N-terminus of ANP and BNP were replaced with the sequence from the opposite influenza type this did not result in compatibility between the NP chimera and the heterotypic viral polymerase. The N-termini from the two influenza types are very different in length, with the ANP N-terminus consisting of 23 amino and the BNP N-terminus consisting of 70 amino acids. Interestingly, the data show that the N-terminus of ANP can carry out the function of the BNP N-terminus and vice versa in supporting viral RNA polymerase activity (figure 6.6). This therefore suggests that it is not the N-terminus of NP that plays a role in the compatibility between NP and the RNA polymerase. In addition, the data presented agree with previous studies demonstrating that the N-termini of both ANP and BNP contain a NLS used for the import of NP into the nucleus (Sherry et al., 2014; Wang et al., 1997; Wanitchang et al., 2013) (figure 6.6b). The very low level of ANP in the nucleus when the N-terminus was deleted, suggests that the two other NLSs identified in ANP are not sufficient for import of soluble NP into the nucleus to support transcription and replication (Bullido et al., 2000; Neumann et al., 1997). This data, however, does not determine whether the function of the N-terminus in RNA polymerase activity is solely for the import of NP into the nucleus or if the N-terminus carries out a function in supporting RNA polymerase activity, that is conserved in both ANP and BNP (Arranz et al., 2012).

Overall further work is required to decipher the determining factor(s) required for compatibility between the viral polymerase and homotypic NP. The results point towards both L1 and L2 being required for compatibility between NP and the viral

polymerase, possibly in combination with other factors not studied here. In addition, the N-terminus does not seem to be playing a role in the interaction between the viral polymerase and NP, which is required for compatibility. It is difficult to determine the cause of inhibition of RNA polymerase activity when many different properties of NP are required. Future work should focus on establishing polymerase binding, RNP complex formation and RNA polymerase processivity assays within the laboratory so that the different functions of NP can be separated and studied individually. Further work is required for a more comprehensive understanding of the data presented.

6.6 Summary

- Compatibility between the viral polymerase and NP is required for NP to support viral transcription and replication
- L1 and L2 play an important role in supporting RNA polymerase activity in ANP which cannot be carried out by the loops of BNP
- The N-terminus does not play a role in determining compatibility between NP and the viral polymerase
- The NLS present within the N-terminus of ANP and BNP plays a role in localising NP to the nucleus

7 Summary and Discussion

This thesis examines the role of NP in viral transcription and replication of the influenza virus genome. NP is a major component of the RNP complex, binding along the length of the viral RNA that is not bound by the RNA polymerase, essential for the RNA polymerase to carry out transcription and replication on a full-length genomic RNA template. This chapter is a summary of how findings presented in this thesis contribute to the overall understanding of the role and regulation of NP in RNA polymerase activity during the viral life-cycle. Each stage of the viral replication cycle will be discussed below in light of the findings presented.

During the first stages of viral infection, vRNP complexes are released into the cytoplasm and imported into the nucleus of the host cell via NLSs on NP (Wu et al., 2007). Within the nucleus, both viral transcription and replication occur. The viral RNA polymerase carries out both viral transcription and replication, even though their mechanisms, to generate mRNA and cRNA, respectively, are very distinct (reviewed in (Fodor, 2013)). Transcription occurs via a primer dependent mechanism using a capped primer acquired by host mRNA cap-snatching, and the synthesis of a poly(A) tail through stuttering on a stretch of uracil residues, generating a viral mRNA product. Contrary to this, replication occurs via a primer independent mechanism in two stages. Firstly a full-length copy of the vRNA template, cRNA, is synthesised by reading through the stretch of uracil residues in the template, secondly cRNA is used as a template and copied to generate progeny

vRNA. It was widely accepted in the literature that NP played a role in determining whether the viral polymerase carried out transcription versus replication (Beaton and Krug, 1986; Klumpp et al., 1997; Krug et al., 1975; Medcalf et al., 1999; Newcomb et al., 2009; Shapiro and Krug, 1988). Early evidence to support the view that the availability of viral NP plays a role in determining the initiation mode of the polymerase included that cRNA but not mRNA synthesis was affected in the presence of NP temperature-sensitive mutants at non-permissive temperatures (Krug et al., 1975; Medcalf et al., 1999; Shapiro and Krug, 1988). Identification of NP polymerase binding led to the proposal that the direct interaction between soluble NP (non-RNP associated NP) and the viral polymerase caused the RNA polymerase to switch from a transcriptase to a replicase (Biswas et al., 1998; Newcomb et al., 2009). Despite the strong consensus in the field that NP does play a role in determining the mode of the viral polymerase evidence has been gathering that suggests that this is not the case. NP is not required for the viral polymerase to carry out initiation of transcription or replication *in vitro* (Honda et al., 1988) and replication of a 46-nucleotide template could occur in the absence of NP *in vivo* (Resa-Infante et al., 2010). The work presented in this thesis supports these findings suggesting that NP is not required for the viral polymerase to carry out initiation or termination of transcription or replication. RNA templates up to 76-nucletoides in length are replicated and transcribed in the absence of NP, producing viral mRNA and vRNA which accumulate to the same level and at the same ratio when compared to in the presence of NP (figure 3.1). Therefore, NP is not required to determine the mode of initiation or termination by the viral RNA polymerase (figure 7.1[1]). NP is, however, required for the viral RNA polymerase to carry out efficient

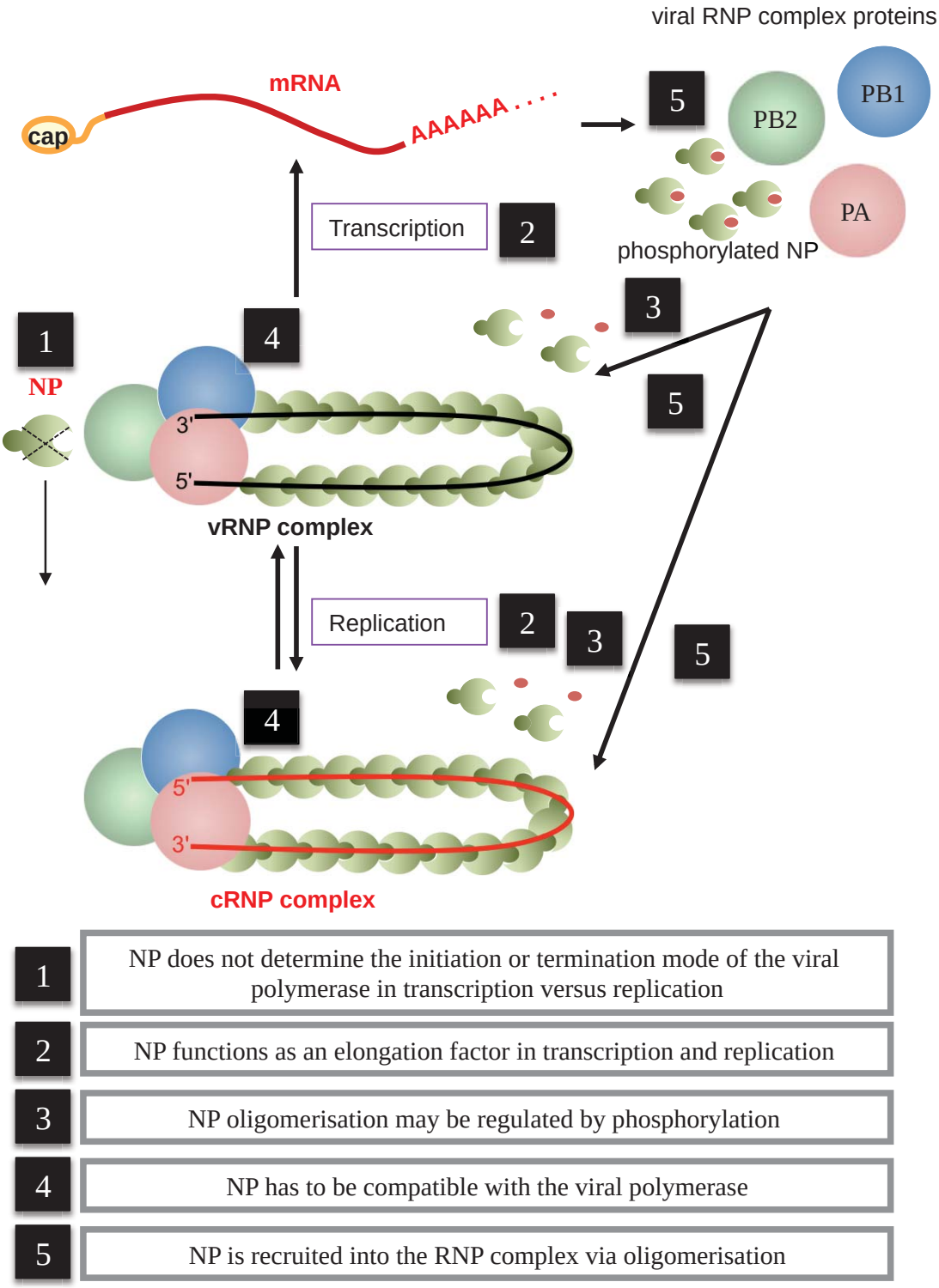


Figure 7.1. Summary of findings during the investigation of the role of NP in viral transcription and replication.

synthesis of viral transcription and replication products when the RNA template is longer than approximately 76-nucleotides in length (figure 3.1). In agreement with this the addition of NP *in vitro* facilitated the escape of the viral polymerase from the promoter, leading to the proposal that NP functioned as an elongation factor (Honda et al., 1988). The requirement for NP as the length of the template increases supports the proposal that one of NP's functions is to support viral transcription and replication during the elongation phases of both of these processes carried out by the viral RNA polymerase (figure 7.1[2]). As the length of the RNA template increases, so does its potential to form secondary structures; therefore, NP may be required to bind the RNA, stabilising it in a conformation that is compatible with RNA polymerase processivity. Indeed, NP-RNA binding is essential for NP to support RNA polymerase activity, on all length RNA templates that require NP (figure 3.12). In addition, NP binding to the RNA may facilitate the recruitment of a host-factor required for the elongation phase of transcription and replication, such as the minichromosome maintenance replicative helicase complex (Kawaguchi et al., 2011; Kawaguchi and Nagata, 2007) or UAP56 (Momose et al., 2001; Naito et al., 2007; Wisskirchen et al., 2011). For NP to function in the elongation phase of transcription and replication, on a full-length RNA template, NP is required to oligomerise along the length of the template; NP-RNA binding is not sufficient (Chan et al., 2010; Vreede et al., 2011). For the RNA polymerase to carry out transcription and replication of RNA templates longer than approximately 246 nucleotides, it requires NP to oligomerise along the RNA template (Chapter 3). It may be that monomeric NP is sufficient to stabilise the RNA in a conformation that is compatible with RNA polymerase processivity for intermediate length templates; however, as the length

of the template increases so does its potential to form secondary structure and oligomerisation of NP along the length of the RNA may become essential to stabilise the RNA in a conformation that is compatible with polymerase processivity. In agreement with this, dimeric NP could better support transcription and replication of intermediate length templates when compared to monomeric NP (figure 3.11). Despite NP being required for the elongation phase of both viral transcription and replication, results indicated that the presence of NP-RNA binding mutants and oligomerisation mutants affected the accumulation of mRNA but not vRNA by mechanisms unknown (figure 3.6, 2.12 and 4.8). In RNP reconstitutions on intermediate length templates and in competition experiments, the presence of NP oligomerisation mutants caused a decrease in mRNA to vRNA ratios whereas the presence of NP-RNA binding mutants resulted in an increase in the mRNA to vRNA ratio. Further work is required to understand what causes these effects on the mRNA to vRNA ratio and whether it is dependent on the NP mutants being incorporated into the RNP complex.

When the vRNP complexes are imported into the nucleus, transcription can occur without prior expression of viral proteins (Vreede et al., 2011). Viral mRNA, transcribed by the viral RNA polymerase, is packaged into mRNP complexes and exported from the nucleus, where it is translated by the host-cell machinery (reviewed in (York and Fodor, 2013)). The newly translated RNP complex viral proteins are then imported into the nucleus and bind to progeny RNA synthesised in replication. For import of NP into the nucleus, NLSs are used which are present on NP. Three NLSs have been identified in NP: an unconventional NLS located in the N-terminus, a bipartite NLS between residues 198-216 (Neumann et al., 1997) and a NLS between residues 320-400 (Bullido et al., 2000). The data presented in

this thesis suggest that the N-terminus within both ANP and BNP plays a major role in the import of NP into the nucleus, as when the N-terminus was removed this resulted in NP having a cytoplasmic localisation (figure 6.6). Once NP is in the nucleus, it is required to bind to the progeny RNA and support viral transcription and replication. In addition, soluble NP, that is not part of the RNP complex, may play a role in the stabilisation of the RNA polymerase, as in the presence of increasing concentrations of NP that cannot bind RNA, transcription and replication of a short RNA template increased (figure 3.4 and 3.5). For newly translated NP to enter the nucleus and bind progeny RNA, it has to be maintained as a monomer, for it to be encapsidation competent (Chenavas et al., 2013; Tarus et al., 2012a). Phosphorylation site S165 within the insertion-groove oligomerisation domain was identified in two independent studies and found to play a role in regulating NP oligomerisation and hence NP's ability to support viral transcription and replication (Chenavas et al., 2013; Hutchinson et al., 2012). The data presented in this thesis show that this site plays a critical role in the viral life-cycle, with both un-regulated NP oligomerisation and inhibited oligomerisation being detrimental to viral growth (figure 7.1[3]). This is in agreement with a recent study supporting the role of phosphorylation within the insertion-groove at position S165 in the regulation of NP oligomerisation (Mondal et al., 2015). This study also identified phosphorylation of a residue within the tail-loop of NP, which also appears to play a role in the regulation of NP oligomerisation. If newly translated NP is phosphorylated to maintain it in a RNA encapsidation competent form, while it is imported into the nucleus, it must then be dephosphorylated once in the vicinity of the viral polymerase, for it to assemble onto the RNA and form a RNP complex. This dephosphorylation may

be carried by a phosphatase that is associated with the RNA polymerase, for example protein phosphatase 6, shown to interact with the RNA polymerase and to affect the accumulation of viral transcription and replication products (York et al., 2014). Further work to identify both the host kinase and phosphatase responsible for NP phosphorylation and dephosphorylation could allow the regulation of NP oligomerisation to be targeted therapeutically.

As discussed above, for the first stage of replication to occur, soluble NP and polymerase are required to bind to the viral RNA to form progeny RNP complexes. In the RNP complex NP forms an oligomeric chain along the length of the RNA and it is thought that the interaction between the NP monomers occurs via the tail-loop of one NP monomer binding to the insertion-groove of the adjacent NP monomer (Arranz et al., 2012; Moeller et al., 2012; Ng et al., 2008; Ye et al., 2006). For this to occur NP monomers have to be oriented in one direction along the RNA and data in this thesis suggest that the NP tail-loop is oriented towards the 5' end of the RNA (figure 4.2 and 4.3).

NP has been shown to directly interact with the viral RNA polymerase (Biswas et al., 1998; Marklund et al., 2012; Poole et al., 2004) and it has therefore been proposed that recruitment of NP onto the progeny RNA may occur via NP interacting with the viral polymerase, either by binding to the RNA polymerase that is bound to the 5' end of the progeny RNA or to the replicating polymerase (Zheng et al., 2013). Compatibility is required between NP and the viral RNA polymerase in order for NP to support the RNA polymerase in transcription and replication (Chapter 6 (figure 7.1[4])). NP derived from influenza B virus is not compatible with a RNA polymerase derived from influenza A virus and vice versa. This heterotypic incompatibility is thought to be due to the absence of an

interaction between the RNA polymerase and NP (Crescenzo-Chaigne et al., 1999). The stage in viral replication when this homotypic compatibility is required is unclear; it may be required for the formation of the RNP complex or for the processivity of the viral polymerase. When the N-terminus of NP was swapped between the two influenza types this did not affect the ability of homotypic NP with a heterotypic N-terminus to support viral transcription and replication by a homotypic RNA polymerase. Therefore the N-terminal sequence element is not responsible for compatibility between NP and the viral polymerase. It appears that several sequence elements or a sequence element not studied in chapter 6 is responsible for the homotypic compatibility and further work is required for a more comprehensive understanding of the data presented.

Once the initial NP is bound to the progeny cRNA, further recruitment of NP has to occur for the formation of the cRNP complex where NP is bound along the length of the RNA that is not bound by the viral RNA polymerase. In the context of a full-length RNA gene segment the results presented in this thesis suggest that recruitment of NP onto the RNA occurs initially via the tail-loop of the recruited NP interacting with the insertion-groove of the resident NP (figure 7.1[5]). This recruitment via oligomerisation can occur independently of NP-RNA binding. It has been shown that oligomerisation of NP is important for its recruitment; however, RNP-like complexes can still form in the presence of NP oligomerisation mutants suggesting that a secondary mechanism, for example RNA binding, can be used for the recruitment of NP (figure 3.10). The requirement for a higher concentration of NP oligomerisation mutant compared to wildtype NP to support transcription and replication of intermediate length templates may be due to the less efficient recruitment of the NP oligomerisation

mutant onto the RNA compared to wildtype NP (figure 3.9). Further recruitment of NP would occur until the cRNA is fully bound by NP, forming a cRNP complex. This cRNP complex is then replicated to generate progeny vRNA (Jorba et al., 2009; York et al., 2013). This vRNA, like the cRNA, is also encapsidated by the viral polymerase and NP, as discussed above, to form progeny vRNP complexes. These vRNP complexes are thought to carry out secondary transcription and replication and are also exported from the nucleus. Once exported from the nucleus, vRNP complexes are directed to and then assembled at the cell membrane, to form progeny virus particles, budding from the apical membrane of polarised cells.

Overall this thesis contributes towards our understanding of the role of NP in transcription and replication and how its regulation allows for efficient recruitment to the RNA during the viral life-cycle.

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