Stabilisation of influenza virus replication intermediates is dependent on the RNA-binding but not the homo-oligomerisation activity of the viral nucleoprotein

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Running Title: The role of influenza virus NP in stabilising cRNA

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Abstract Word Count: 100
Main Text Word Count: 1977
Number of Figures: 3
The influenza virus nucleoprotein (NP) is believed to play a central role in directing a switch from RNA genome transcription to replication by the viral RNA polymerase. However, this role has recently been disputed with the proposal of alternative regulatory mechanisms. It has been suggested that expression of viral polymerase and NP allows genome replication by stabilisation of cRNA replication intermediates and complementary ribonucleoprotein (cRNP) assembly. Here we demonstrate that the RNA-binding activity of NP is necessary for stabilisation of cRNA whereas, surprisingly, homo-oligomerisation of NP is not essential. However, both RNA-binding and homo-oligomerisation activities are essential for genome replication.

Influenza virus, a member of the Orthomyxoviridae family, contains a segmented negative sense single-stranded RNA genome. Each viral RNA (vRNA) segment is bound by the viral RNA-dependent RNA polymerase (RdRp) and nucleoprotein (NP) to form viral ribonucleoprotein (vRNP) complexes. Within the vRNP, the RdRp binds to the corkscrew structure formed by the partially complementary conserved 5' and 3' ends of the vRNA representing the vRNA promoter. The rest of the vRNA interacts with multiple copies of NP, each molecule covering approximately 24 nucleotides (reviewed in (17)). NP binds ssRNA with high affinity but little or no sequence specificity (2, 9, 22) and has been shown to homo-oligomerise and interact with the PB1 and PB2 subunits of the viral RdRp (4, 8, 14, 15, 23). Although cryo-EM reconstitution images of a mini-RNP containing nine NP molecules have been obtained (6), a detailed high-resolution structure of the vRNP is still lacking.

During the viral life cycle, the vRNA is transcribed into mRNA and replicated through a complementary RNA (cRNA) intermediate into more copies of vRNA by the viral RdRp (reviewed in (10, 17, 18)). Viral mRNAs associate with cellular factors normally associating...
with host mRNAs, e.g. nuclear and cytoplasmic cap-binding proteins, leading to the
stabilisation of viral mRNAs, their processing, nuclear export and translation (3, 16, 21). In
contrast, cRNAs are stabilised by the association of viral factors, i.e. the viral RdRp and NP,
which together from a cRNP structure similar to that of vRNPs. During infection, viral
mRNAs can be detected initially while cRNAs become detectable only after the onset of viral
protein synthesis (12). This has been interpreted to mean that a switch of polymerase function
from a transcriptase to a replicase is required for cRNA synthesis and various models have
been proposed that implicate viral and host factors as well as small viral RNAs (svRNA) in
the switching (reviewed in (17)). However, an alternative interpretation is that both mRNA
and cRNA are synthesised from early on in a stochastic manner, but cRNA is degraded by
host nucleases until sufficient amounts of viral RdRp and NP accumulate to stabilise it (20).
In agreement with this interpretation, over-expression of catalytically inactive RdRp and NP
prior to viral infection allows the accumulation of cRNA in the presence of cycloheximide,
an inhibitor of protein translation (19, 20). Expression of RdRp is absolutely essential for the
stabilisation of cRNA, while NP, although not essential, greatly increases the amounts of
accumulated cRNA. In this study we address the question of which properties of NP play a
role in the stabilisation of cRNA and the replication of cRNA to vRNA.

In order to address these questions, we set up an assay to analyse the effect of mutations in
NP on the accumulation of cRNA and its replication in viral infection. 293T cells were
infected with influenza A/WSN/33 virus in the presence of actinomycin D, an inhibitor of
DNA-templated RNA synthesis. As the viral RdRp requires host pre-mRNAs as a source of
5' capped primers for initiation of viral mRNA synthesis, treatment with actinomycin D
resulted in the inhibition of the accumulation of all three types of viral RNAs (Fig. 1A), as
expected (1, 13). Only vRNAs introduced into the cell by the infecting virions could be
detected. However, pre-expression of NP, together with a catalytically inactive viral RdRp
containing D445A/D446A mutant PB1) resulted in the linear accumulation of cRNA (Fig. 1B). These results are in agreement with previous findings that actinomycin D does not affect viral RNA replication directly and that accumulation of viral replication products is dependent on viral protein expression (1, 12, 13). Pre-expression of NP and wild-type catalytically active viral RdRp resulted not only in cRNA accumulation but also in genomic replication (linear accumulation of vRNA) (Fig. 1B). No cRNA and no replication into vRNA were detected if NP was pre-expressed without trimeric RdRp (Fig. 1B). Therefore, the presence of NP alone is not sufficient for cRNA stabilisation. These results are in agreement with the previously proposed stabilisation model for replication (20). Intriguingly, cRNA accumulated to higher levels in the presence of pre-expressed inactive RdRp compared to the plateau of accumulating cRNA in the presence of pre-expressed wild-type RdRp or during viral infection (Fig. 1A). We speculate that this may relate to instability of functional cRNPs (7), possibly by the cRNA template being degraded following replication, whereas non-functional cRNPs are presumably more stable and form a sink for accumulating cRNA.

Having established an assay to evaluate the functional role of NP in the context of viral infection, we next examined the effect of mutations in the pre-expressed NP on the accumulation and replication of cRNA. The high-resolution structures of H1N1 and H5N1 NP revealed potential RNA-binding sites involving a large number of basic amino acid residues (14, 23). We initially analysed the effect of previously characterised RNA-binding mutants of H5N1 NP (14) on the accumulation and replication of cRNA in the presence of catalytically inactive mutant or wild-type H1N1 RdRp (Fig. 2A and B, respectively). Pre-expression of viral RdRp and the NP-G1 mutant (including amino acid changes R74A, R75A, R174A, R175A and R221A) yielded low but detectable levels of cRNA similar to that found in the absence of NP (Fig. 2A and B); no replication of cRNA to vRNA by the wild-type RdRp could be detected (Fig. 2B). The other mutant NPs examined (NP-G2, NP-G3, NP-G4...
and NP-Δ74-88) yielded detectable levels of cRNA accumulation and replication (Fig. 2A and B), with efficiencies broadly corresponding to the reported RNA binding affinities of the respective NP mutants (14). The exception was NP-G3 which inhibited cRNA accumulation and replication compared to wild type despite only a negligible effect on RNA binding. Preliminary data suggest that this is not due to the lower than wild type expression levels of NP-G3 (results not shown) but this is currently under further investigation. Although expression of NP-G1 was also reduced compared to wild type, increasing the expression to wild type levels did not yield increased levels of detectable cRNA (Fig. 2C).

Next we aimed to identify the critical residues in NP-G1 responsible for the inhibitory phenotype while simultaneously confirming their inhibitory effect in an H1N1 NP. We therefore introduced the mutations from H5N1 NP-G1 singly (R74A, R175A, R221A) or doubly (R74A/R75A, R174A/R175A) into an H1N1 NP and analysed them for cRNA stabilisation and replication. Single mutations showed little effect and double mutations only partially inhibited accumulation and replication of cRNA compared to wild type (Fig. 2D and E). Therefore, the two double mutations (R74A/R75A and R174A/R175A) were combined to generate a quadruple mutant NP-G1(4). When NP-G1(4) was co-expressed with inactive or wild-type RdRp, the levels of cRNA accumulation and replication were similar to levels detected in the absence of NP (Fig. 2D and E). These four mutations were found to reduce transcription and replication activity to less than 10% in RNP reconstitution experiments (Fig. 2F and G). Finally, the RNA-binding properties of NP-wt and NP-G1(4) proteins expressed in E. coli and purified to homogeneity were studied by surface plasmon resonance (14). NP-wt showed a high RNA-binding affinity, with a dissociation constant (K_D) of 16.5 nM (Fig. 2H), which is similar to that of H5N1 NP (14). The RNA-binding affinity of NP-G1(4), however, was too low to be determined. Only a weak response was observed even when a high concentration (6.25 µM) of purified NP-G1(4) protein was applied to the RNA-
immobilized chip surface (Fig. 2I). Therefore, these data are in agreement with the results obtained with H5N1 NP-G1 above and underline the importance of the RNA binding activity of NP in cRNA expression and replication. Moreover, they support a model of RNA binding involving a number of positively charged residues in the proposed G1 region of NP (14).

Overall, these results show that RdRp and NP competent in RNA binding are crucial for the accumulation and replication of cRNA.

Next we addressed the role of oligomerisation of NP in cRNA stabilisation and replication. Homo-oligomerisation of NP in which the tail loop of one NP is inserted into a groove of the neighbouring NP molecule is an important factor in maintaining RNP structure. The amino acid residues involved in these interactions have been identified and well characterised (6, 8, 23), including in our laboratory (5, 14). The expected inhibitory effect of substituting amino acid residues involved in the intra- and inter-chain interactions of the tail loop (E339A, V408S/P410S, R416A and L418S/P419S) on viral replication and transcription (5) were confirmed by vRNP (Fig. 3A) and cRNP (Fig. 3B) reconstitution experiments. To determine whether homo-oligomerisation is important for the accumulation of cRNA, NP mutants were pre-expressed with viral RdRp in actinomycin D-treated cells infected with influenza virus (Fig. 3C and D). Surprisingly, substantial (50-80%) accumulation of cRNA could be detected in the presence of catalytically inactive viral RdRp and mutant NPs compared to wild-type NP (Fig. 3C). However, replication of cRNA in the presence of wild-type viral RdRp and mutant NPs was severely inhibited (Fig. 3D). The reduced relative accumulation of cRNA (25-45%) in the presence of wild-type RdRp and mutant NPs compared to wild-type NP (Fig. 3D) is presumably due to the inability of the mutant NP to fully support replicative synthesis of vRNA and cRNA. As oligomerisation NP mutants have previously been found to possess reduced RNA binding affinity compared to wild type in vitro (5, 9), probably due to their reduced oligomeric state, we analysed the association of oligomerisation NP mutants with...
cRNA. Consistent with the model that cRNA stabilisation requires NP to bind RNA, the cRNA detected in the presence of inactive RdRp (Fig. 3C) was shown to be bound by the wild-type or mutant NP through primer extension analyses of RNA co-immunoprecipitated with NP (Fig. 3E). Overall, we conclude that homo-oligomerisation of NP, although essential for replication, is not essential for stabilisation of cRNA.

It is not currently known what regulates the relative rates and levels of vRNA and cRNA accumulation during viral infection (reviewed in (17)). Whereas vRNA continues to accumulate at late time points and to high levels, cRNA accumulation flattens out at low levels early during replication (Fig. 1A). As discussed earlier, our data (Fig. 1B) suggest that cRNA may achieve equilibrium between vRNA-templated synthesis and degradation of the cRNA template following replication to vRNA. We speculate that in RNPs assembled with wild-type polymerase and NP oligomerisation mutants (Fig. 3D), this state of equilibrium would be disrupted due to both the reduction in cRNA stabilisation compared to wild-type NP (Fig. 3C) and the replication-deficient phenotype (Fig. 3A and B). According to this model, both the rate of cRNA degradation (following cRNA-templated replication to vRNA) and vRNA-templated synthesis of cRNA would be reduced compared to wild-type RNPs.

In summary, our results show that the viral RdRp is essential while NP plays an important role in the stabilisation of cRNA replication intermediates. The RNA-binding activity of NP was found to be essential for stabilising cRNA; surprisingly, however, the oligomerisation activity was not critical. This raises the question of how NP enhances cRNA stability. We envisage that single NP molecules bound to cRNA and the RdRp near the cRNA termini could stabilise the association of the RdRp with the promoter sequences comprising the 5' and 3' ends of cRNA. This might be sufficient for enhancing cRNP stability. Additionally or alternatively, non-oligomeric NP molecules may be bound along the length of cRNA, contributing to cRNP stability. An NP mutant unable to bind RdRp would help to distinguish
between these possibilities. In contrast to stabilisation of cRNA, both the RNA-binding and homo-oligomerisation activities of NP were found to be essential for the assembly of replication-competent cRNPs.

We thank Otto Haller for antibodies and Koyu Hara for the 5S rRNA-specific primer. This work was supported by the MRC (G0700848) to E. Fodor and a Hong Kong Research Grants Council General Research Fund (CUHK 472808) to P.C. Shaw.

REFERENCES


FIGURE LEGENDS

Fig. 1. Actinomycin D-mediated inhibition of influenza A/WSN/33 virus RNA replication can be rescued by the expression of PB1, PB2, PA, and NP. (A) Time course of viral infection (multiplicity of infection = 5) in the absence (untreated) or presence (Act D treated) of 5 µg of actinomycin D/ml. The lower panel is a longer exposure of the upper panel showing the presence of vRNA introduced by the infecting virions in actinomycin D-treated cells. (B) Time course of viral infection (multiplicity of infection = 5) in the presence of 5 µg of actinomycin D/ml after prior (15 to 16 h) transfection of plasmids (11) expressing viral PB2, PA, NP and wild-type PB1 (w), D445A/D446A mutant PB1 (m) or no PB1 (-). Viral RNA species were analyzed by NA gene-specific primer extension assays (20). Weak mRNA-specific signals detected are due to pre-expressed wild-type RdRp being able to carry out residual mRNA synthesis in the presence actinomycin D. A primer specific for 5S rRNA (TCCCAGGCGGTCTCCCATCC) was used as an internal control. * indicates an unidentified band possibly related to vRNA. Quantitation was by phosphorimage analysis using the Aida software. It should be noted that the intensities of bands between gels from different experiments are not comparable due to variations in labelling intensities of the primers used.

Fig. 2. Effect of RNA-binding mutations in NP on cRNA stabilisation and replication. (A to E) Cells were infected in the presence of 5 µg of actinomycin D/ml after prior (15 to 16 h) transfection of plasmids (11) expressing wild-type or mutant viral proteins as indicated. (A and B) Wild-type (wt), none (-) or mutant H5N1 NPs (14) were co-expressed with or without...
(-3P) catalytically inactive (A) or wild-type (B) RNA polymerase. (C) Wild-type (wt), none (-) or increasing amounts of G1 mutant H5N1 NP were co-expressed with or without (-3P) catalytically inactive RNA polymerase. (D and E) Wild-type, none (-) or mutant H1N1 NPs were co-expressed with catalytically inactive (D) or wild-type (E) RNA polymerase. H5N1 NP-G1 (14) was included as a control. Weak mRNA-specific signals detected (B and E) are due to pre-expressed wild-type RdRp being able to carry out residual mRNA synthesis in the presence actinomycin D. (F and G) Ribonucleoprotein reconstitution assays were performed in 293T cells transfected with expression plasmids for the RNA polymerase, wild-type (wt), none (-) or G1(4) mutant H1N1 NP and an NA-gene specific vRNA (F) or cRNA (G) template. Viral RNA species were analyzed by NA gene-specific primer extension assays (20) and the accumulation relative to wild type was quantitated by phosphorimage analysis using the Aida software. The vRNA (F) and cRNA (G) values obtained in the absence of NP (-), corresponding to input RNA generated by RNA polymerase I transcription, were subtracted from the values obtained in the presence of wild-type (wt) or G1(4) mutant NP. The results shown with NP-G1(4) represent the average of two independent experiments with range. A primer specific for 5S rRNA was used as an internal control. Expression of NP was analysed by Western blot using a rabbit polyclonal antibody (a gift of O. Haller, University of Freiburg). RanBP5 detected with a rabbit polyclonal antibody (Santa Cruz) was used as a loading control. (H and I) Surface plasmon resonance of wild-type (H) or G1(4) mutant (I) H1N1 NP against immobilized RNA suitable for binding one NP molecule (24 nucleotides in length) (14). Different concentrations of purified proteins were injected as shown and the signal (RU) was plotted over time. Diamonds (H) and the grey line (I) are experimental data while black lines (H) are fitted curves.

Fig. 3. Effect of homo-oligomerisation mutations in NP on cRNA stabilisation and replication. (A and B) Ribonucleoprotein reconstitution assays were performed in 293T cells
transfected with expression plasmids for the RNA polymerase, wild-type (wt), none (-) or
G1(4) mutant H1N1 NP and an NA-gene specific vRNA (A) or cRNA (B) template. Viral
RNA species were analyzed by NA gene-specific primer extension assays (20) and the
accumulation relative to wild type was quantitated by phosphorimage analysis using the Aida
software. The vRNA (A) and cRNA (B) values obtained in the absence of NP (-),
corresponding to input RNA generated by RNA polymerase I transcription, were subtracted
from the values obtained in the presence of wild-type (wt) or mutant NPs. The results shown
represent the average of two independent experiments with range. (C and D) Cells were
infected in the presence of 5 µg of actinomycin D/ml after prior (15 to 16 h) transfection of
plasmids (11) expressing wild-type (wt) , none (-) or mutant NPs together with catalytically
inactive (C) or wild-type (D) RNA polymerase. Viral RNA species were analyzed and
quantitated as above. Weak mRNA-specific signals detected (D) are due to pre-expressed
wild-type RdRp being able to carry out residual mRNA synthesis in the presence actinomycin
D. A primer specific for 5S rRNA was used as an internal control. (E) Immunoprecipitations
of cell lysates from (C) were performed with or without antibodies specific for NP. RNA was
isolated from cell lysates or immunoprecipitates (IP) and analysed by NA gene-specific
primer extension. Expression or immunoprecipitation of NP was analysed by Western blot.
RanBP5 was used as a loading control where relevant.
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Stabilization of Influenza Virus Replication Intermediates Is Dependent on the RNA-Binding but Not the Homo-Oligomerization Activity of the Viral Nucleoprotein

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Volume 85, no. 22, p. 12073–12078, 2011. Page 12076, Fig. 3C and E should appear as shown below.