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NS2/NEP protein regulates transcription and replication of the influenza virus RNA genome

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The influenza virus RNA polymerase transcribes the negative-sense viral RNA segments (vRNA) into mRNA and replicates them via complementary RNA (cRNA) intermediates into more copies of vRNA. It is not clear how the relative amounts of the three RNA products, mRNA, cRNA and vRNA, are regulated during the viral life cycle. We found that in viral ribonucleoprotein (vRNP) reconstitution assays involving only the minimal components required for viral transcription and replication (the RNA polymerase, the nucleoprotein and a vRNA template), the relative levels of accumulation of RNA products differed from those observed in infected cells, suggesting a regulatory role for additional viral proteins. Expression of the viral NS2/NEP protein in RNP reconstitution assays affected viral RNA levels by reducing the accumulation of transcription products and increasing the accumulation of replication products to more closely resemble those found during viral infection. This effect was functionally conserved in influenza A and B viruses and was influenza-virus-type-specific, demonstrating that the NS2/NEP protein changes RNA levels by specific alteration of the viral transcription and replication machinery, rather than through an indirect effect on the host cell. Although NS2/NEP has been shown previously to play a role in the nucleocytoplasmic export of viral RNPs, deletion of the nuclear export sequence region that is required for its transport function did not affect the ability of the protein to regulate RNA levels. A role for the NS2/NEP protein in the regulation of influenza virus transcription and replication that is independent of its viral RNP export function is proposed.

INTRODUCTION

Influenza viruses are enveloped viruses with a segmented, negative-sense, single-stranded RNA genome. Both type A and B viruses have eight negative-sense viral RNA (vRNA) segments which are packaged into viral ribonucleoprotein (vRNP) complexes (Palese, 1977). In the vRNPs, each vRNA segment is associated with the nucleoprotein (NP) and the RNA-dependent RNA polymerase (Portela & Digard, 2002). The viral RNA polymerase is a heterotrimeric complex of polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA) (Braam *et al.*, 1983); this polymerase complex is responsible for both transcription and replication of the vRNA genome. During the viral life cycle, vRNPs enter the host cell nucleus where they are transcribed into capped and polyadenylated mRNA by a primer-dependent mechanism (Krug, 1981). Replication of the viral genome occurs via a two-step process. A positive-sense copy of the vRNA, known as complementary RNA (cRNA), is produced and this in turn is used as a template to make more vRNA. The vRNA therefore serves as a template for the production of both mRNA and cRNA; however, the mechanisms for the generation of these two molecules are very different and it is not yet clear how the RNA polymerase complex is committed to either transcription or replication on a given vRNA template.

The eighth vRNA segment of the influenza A virus directs the synthesis of two mRNAs. The first of these encodes the non-structural (NS) protein, NS1, while the other is derived from splicing of the NS1 mRNA and is translated into a protein of 121 aa (Lamb & Lai, 1980). This protein localizes to the cell nucleus and was originally named NS2 (Inglis *et al.*, 1979; Lamb & Choppin, 1979). However, it was demonstrated later that NS2 is present in purified virions, where it interacts with the virus matrix (M1) protein (Richardson & Akkina, 1991; Yasuda *et al.*, 1993). Both M1 and NS2 are directly involved in the nuclear export of vRNPs during the viral life cycle, therefore it has been proposed that NS2 be renamed the nuclear export protein (NEP) (O'Neill *et al.*, 1998). The nuclear export of vRNPs is carried out by the cellular export protein Crm1 (Elton *et al.*, 2001) and there is evidence that NS2/NEP

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Two supplementary tables of primer sequences are available with the online version of this paper.

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mediates the association between the two. The current model involves an export complex in which NS2/NEP has an N-terminal association with Crm1 and a C-terminal association with the viral M1 protein, which in turn is bound to vRNPs (Akarsu *et al.*, 2003). The NS2/NEP protein also binds several nucleoporins and is thought to be responsible for recruiting the export machinery and directing the export of the complex (O'Neill *et al.*, 1998).

In addition to the role of NS2/NEP in the export of vRNPs, it has been shown that a point mutation at position 32 in the NS2/NEP protein of the A/WSN/33 influenza virus Wa-182 results in the production of defective interfering (DI) particles lacking an intact PA gene after a single high-multiplicity cycle of infection (Odagiri & Tobita, 1990; Odagiri *et al.*, 1994). Furthermore, a study in which an influenza A chloramphenicol acetyltransferase (CAT) reporter gene was used to assess RNA synthesis showed that the NS2/NEP protein inhibited RNA synthesis by reducing the levels of vRNA, cRNA and mRNA (Bullido *et al.*, 2001).

The eighth vRNA segment of the influenza B virus genome also encodes both NS1 and NS2/NEP proteins, although less is known about the function of NS2/NEP in influenza B viruses compared with type A viruses. There is evidence that the protein plays a role in vRNP export (Paragas *et al.*, 2001), although it has been suggested that the complex formation between NS2/NEP, M1 and the vRNPs of influenza B is different from that of influenza A, in that the type B NS2 protein associates directly with vRNPs independently of M1 (Imai *et al.*, 2003).

In this study, we investigated the effect of the NS2/NEP protein on viral transcription and replication by using ribonucleoprotein (RNP) reconstitution assays. We found that co-expression of NS2/NEP affects viral RNA levels, resulting in reduced accumulation of transcription products and an increase in the accumulation of viral replication products. This property of NS2/NEP appears to be independent of its previously suggested function in vRNP export. Therefore, we propose a second role for the influenza NS2/NEP protein during the viral life cycle in the regulation of viral transcription and replication.

METHODS

Cell strains and culture conditions. Influenza A/WSN/33 virus stocks were prepared in MDBK cell monolayers maintained in minimal essential medium (MEM) with 10 % fetal calf serum (FCS) at 37 °C with 5 % CO_2. Viral infection and RNP reconstitution assays were carried out using human kidney 293T cells in similar culture conditions.

Plasmids. The protein expression plasmids pcDNA-PB1, pcDNA-PB2, pcDNA-PA, pcDNA-NP and pcDNA-3A have been described previously (Fodor *et al.*, 2002), as have the pPOLI-PB1-RT, pPOLI-PB2-RT, pPOLI-PA-RT, pPOLI-HA-RT, pPOLI-NP-RT, pPOLI-NA-RT, pPOLI-M-RT and pPOLI-NS-RT vRNA transcription plasmids (Fodor *et al.*, 1999). To generate the pPOLI-NS1-RT plasmid, site-directed mutagenesis was carried out on the pPOLI-NS-RT plasmid, whereby a stop codon was introduced into the NS2 ORF after 15 aa residues and 2 nt mutations were introduced into the acceptor splice site. These plasmids have coding sequences derived from the A/WSN/33 virus. The pcDNA-NS2 (A/WSN) plasmid was constructed by PCR amplifying the NS2 ORF using pPOLI-NS-RT as template (primers 5′-ATATGAATTCATAATGGATCCAAACACTGTGTCAAGCTTTC-AGGACATACTGATGAGGATGT-3′ and 5′-ATGCCTCGAGTTAA-ATAAGCTGAAACGAGA-3′). The PCR product was digested with *Eco*RI and *Xho*I and cloned into pcDNA-3A digested with the same restriction enzymes. The pcDNA-NS2 (A/Vic) and pcDNA-NS2 (B/Beij) plasmids containing the NS2 protein ORFs from the A/Victoria/3/75 and B/Beijing/1/87 viruses, respectively, were constructed using a similar strategy. The pcDNA-TAP-NS2 plasmid was created by replacing the CTD sequence in the pcDNA-TAP-CTD construct (Engelhardt *et al.*, 2005), using the *Not*I and *Xba*I restriction sites, with the NS2 ORF PCR-amplified from pcDNA-NS2 (A/WSN). The 53–121, 1–53, 21–121 and 1–90 NS2 deletion mutants were constructed by PCR amplification of the pcDNA-TAP-NS2 template (primers given in Supplementary Table S1, available in JGV Online). Following amplification, 53–121 and 21–121 PCR products were digested with *Not*I and self-ligated, and 1–53 and 1–90 were digested with *Xho*I and cloned into pcDNA-TAP-NS2 digested with the same restriction enzyme. All constructs were confirmed by sequencing and protein expression was confirmed by Western blot analysis. Plasmids encoding influenza B virus sequences were kindly provided by Wendy Barclay (Imperial College, London) and have been described previously (Jackson *et al.*, 2002). The pCIPB1, pCIPB2, pCIPA and pCINP plasmids express RNA polymerase subunits and NP of the influenza B/Panama/45/90 virus, while the pPRPA and pPRNA plasmids express PA and NA vRNA, respectively, of the influenza B/Beijing/1/87 virus.

Transfections and infections. Transfections for RNP reconstitution assays were performed in human kidney 293T cells in suspension in 35 mm dishes (approx. 10^6 cells) by using 10 μl Lipofectamine 2000 (Invitrogen) and 1 μg of each of the relevant plasmids in 1.5 ml MEM with 10 % FCS. Cells were harvested 48 h post-transfection (p.t.) or at the time points indicated. Human kidney 293T cells were infected with A/WSN/33 virus at an m.o.i. of either 2.5 or 10 in a total of 1 ml MEM with 0.5 % FCS per 35 mm dish. Cells were harvested at the time points indicated. Where required, cells were treated with 5 μg actinomycin D ml^{-1} at 37 °C for 1 h at 22 h p.t. and then infected with A/WSN/33 virus at an m.o.i. of 10. Cells were harvested at 4 h post-infection (p.i.).

RNA isolation and primer extension analysis. Total RNA was extracted from 293T cells in 35 mm dishes using 1 ml TRIzol Reagent (Invitrogen). 1/30th of each of the RNA samples was analysed by primer extension. RNA was mixed with an excess of two ^{32}P-labelled DNA primers in 5 μl and denatured by heating at 95 °C for 3 min. The mixture was cooled on ice and transferred to 45 °C for 2 min prior to the addition of 5 μl 2 × transcription mix [2 × First Strand Buffer (Invitrogen), 20 mM DTT, 1 mM dNTP mix and 50 U SuperScript II RNase H^- reverse transcriptase (Invitrogen)] also heated to 45 °C. The reaction was stopped after 1 h by the addition of 8 μl 90 % formamide, and heating at 95 °C for 3 min. Transcription products were analysed on 6 % polyacrylamide gels containing 7 M urea in TBE buffer and detected by autoradiography. The viral-gene-specific primers used are given in Supplementary Table S2, available in JGV Online. A primer detecting cellular 5S rRNA (5′-TCCCAGGCGGTCTCCCATCC-3′) was included as an internal control where required.

Western blotting. Cell lysates were analysed by SDS-PAGE followed by Western blotting. The antibodies used were anti-NS2 (Akarsu *et al.*, 2003) and anti-rabbit horseradish peroxidase-conjugated IgG

(Sigma); these were detected using an Immobilon chemiluminescent Western detection kit (Millipore).

RESULTS

Regulation of viral RNA accumulation during infection and in RNP reconstitution assays

Over the last decade, reconstitution of recombinant vRNP complexes in transfected cells from co-expressed PB1, PB2 and PA polymerase subunits, NP and vRNA, followed by the analysis of reporter gene expression/activity or a direct analysis of accumulating viral RNAs by a primer extension assay, has become the method of choice for studying influenza virus polymerase activity *in vivo* (Deng *et al.*, 2006; Fodor *et al.*, 2002; Gabriel *et al.*, 2005; Labadie *et al.*, 2007; Mullin *et al.*, 2004; Pleschka *et al.*, 1996; Salomon *et al.*, 2006; Vreede *et al.*, 2004). However, as the RNP reconstitution assay involves only the minimal components required for viral transcription and replication, it might not reflect all of the regulatory events that occur during transcription and replication in virus-infected cells. In

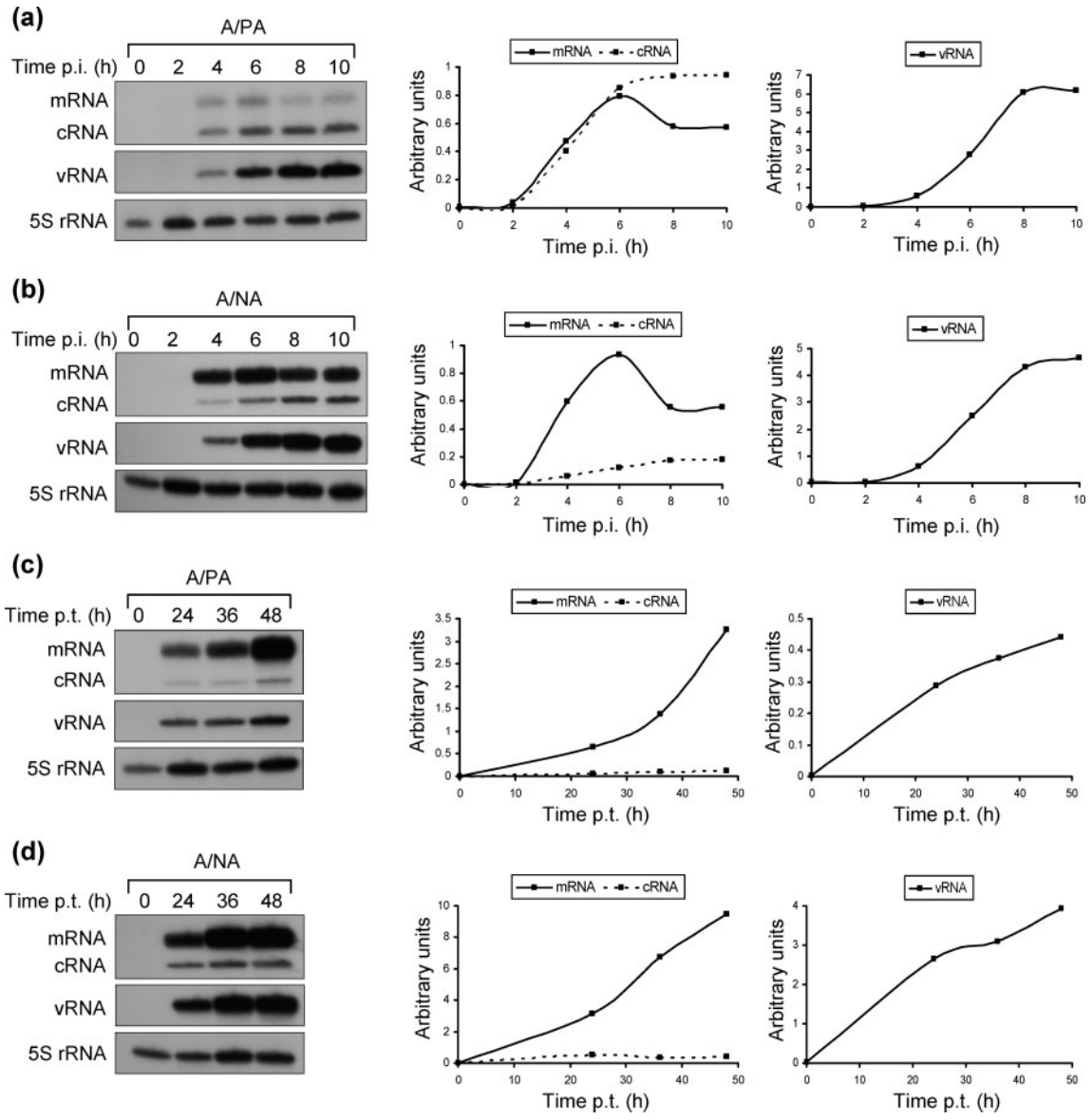

Fig. 1. Differential accumulation of viral RNAs during infection and RNP reconstitution assays. (a and b) 293T cells were infected with A/WSN/33 at an m.o.i. of 2.5 and RNA was harvested at 0, 2, 4, 6, 8 and 10 h p.i.; viral RNAs were analysed by either NA or PA gene-specific primer extension as described in Methods. (c and d) 293T cells were transfected with plasmids expressing the PB1, PB2, PA and NP viral proteins and either NA or PA vRNA; RNA was harvested at 0, 24, 36 and 48 h post-transfection (p.t.). The figures and quantitative analyses shown are single representatives of three independent experiments.

order to investigate this, we compared the accumulation of viral RNAs derived from the PA and NA gene segments during a time-course of viral infection and RNP reconstitution (Fig. 1). Two major differences were apparent. (i) During infection, a dramatic difference in the mRNA : cRNA ratios between the PA and NA genes was observed (Fig. 1a and b), while this difference was not apparent in the RNP reconstitution assay (Fig. 1c and d). (ii) During infection, viral mRNA levels peaked at 6 h post-infection for both the PA and NA segments, while vRNA continued to accumulate; however, no such temporal regulation was observed during the RNP reconstitution assay in which mRNA and vRNA showed a continuous increase in accumulation until the latest time point analysed. These differences indicate that the regulation of viral RNA accumulation in RNP reconstitution assays differs from that during viral infection in both the relative ratios of RNA products and their temporal regulation.

Expression of the viral NS2/NEP protein affects the accumulation of viral RNAs in RNP reconstitution

The results shown in Fig. 1 suggest that a factor present during viral infection that is absent in the RNP reconstitution assay is responsible for the observed differences in the accumulation of viral RNAs. It is unlikely that the regulation observed during infection is due to a cellular protein(s), as the same cell type was used for both infections and RNP reconstitutions, thus suggesting that it is the result of a viral protein(s) not present in the RNP reconstitution assay. Indeed, following RNP reconstitutions in which either the NA, M1 and M2, NS1 or NS2/NEP viral proteins were co-expressed, it was found that the presence of the NS2/NEP protein altered the mRNA and cRNA levels by downregulating mRNA and upregulating cRNA accumulation to more closely resemble the situation found during viral infection (compare lane 6 with lanes 1 and 2 in Fig. 2a and b). There was also an apparent increase in the accumulation of vRNA in the presence of NS2/NEP. In contrast, the NA, M1 and M2, and NS1 proteins had little or no effect on the RNA ratios. The presence of each of the co-expressed viral proteins was confirmed by Western blot analysis or a neuraminidase assay in the case of NA (data not shown).

Next, we extended our analysis of the effect of NS2/NEP on the accumulation of viral RNAs derived from other viral gene segments (Fig. 3). Statistical analysis of three independent experiments demonstrated that the cRNA levels of all gene segments were significantly increased in the presence of NS2/NEP, while mRNA levels were significantly decreased for all genes with the exception of the NP and NA genes (Fig. 3b). The vRNA levels of all the genes increased or remained unchanged with the exception of the NP gene, which showed a decrease in the accumulation of vRNA in the presence of NS2/NEP. We conclude that the co-expression of the NS2/NEP protein in the RNP reconstitution assay results in a general trend of increased accumulation of the replication products and decreased accumulation of the transcription products.

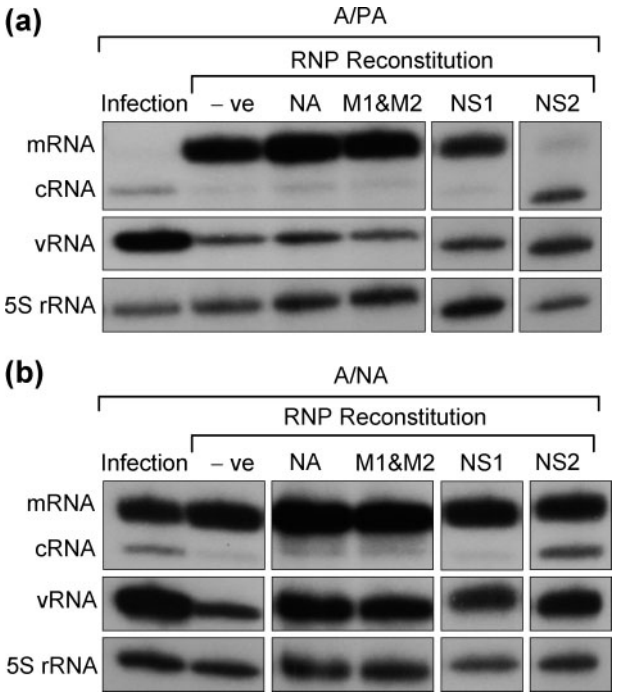

Fig. 2. The viral NS2/NEP protein alters mRNA : cRNA ratios during RNP reconstitution assays. (a) Comparison of PA gene-specific RNA levels by primer extension analysis following either infection or RNP reconstitution. 293T cells were infected with A/WSN/33 virus at an m.o.i. of 10 and total RNA was harvested at 6 h p.i. Plasmids expressing the PB1, PB2, PA and NP viral proteins and PA vRNA were transfected into 293T cells, with plasmids expressing either the NA, M1 and M2, NS1 or NS2 proteins, as indicated. M1 and M2 were both expressed from the pPOLI-M-RT plasmid in the presence of the viral RNA polymerase and NP. Total RNA was harvested at 48 h p.t. and analysed by PA-gene-specific primer extension. (b) As for (a) but testing NA-gene-specific RNA levels.

In order to address the question of whether NS2/NEP has a similar effect on viral RNA levels during viral infection, an experiment was carried out in which the polymerase, NP and NS2/NEP proteins were pre-expressed in 293T cells, followed by virus infection in the presence of actinomycin D. As actinomycin D inhibits viral transcription and consequently the production of viral proteins, this system allowed us to study the effect of NS2/NEP on the accumulation of viral RNAs in the context of a viral infection, without the interference of other viral proteins. At the concentration of actinomycin D used, low levels of mRNA could be detected by primer extension, indicating that limited transcription still occurred. The results showed a similar decrease in the accumulation of mRNA and increase in the accumulation of cRNA and vRNA to that observed in the RNP reconstitution assay (Fig. 3c and d). This indicates that the NS2/NEP protein can mediate an

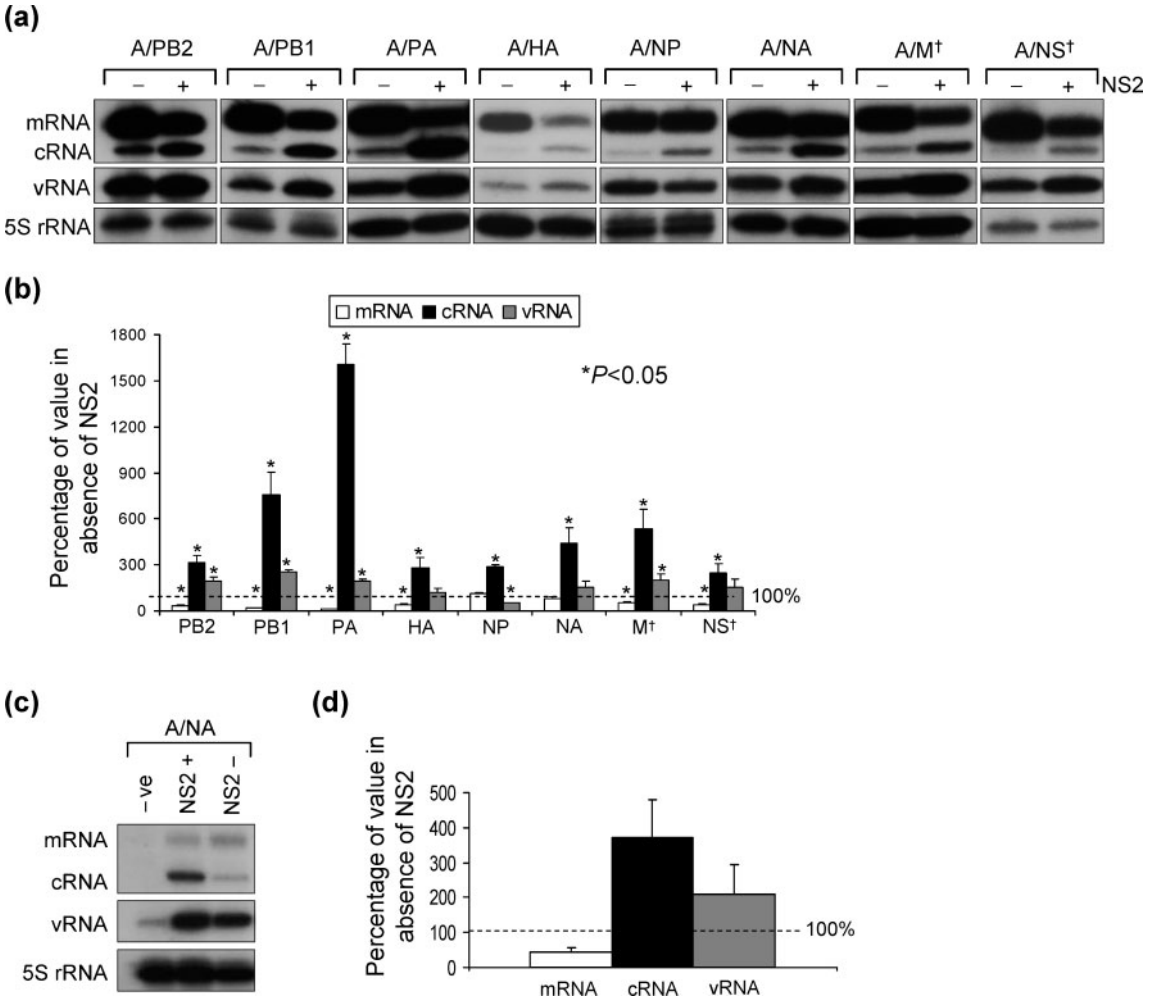

Fig. 3. The viral NS2/NEP protein regulates the levels of transcription and replication. (a) 293T cells were transfected with plasmids expressing the PB1, PB2, PA and NP proteins as well as the relevant viral gene vRNA. Either empty vector or a plasmid encoding the NS2 protein was co-transfected into the cells. RNA was harvested after 48 h and analysed by gene-specific primer extension. †Primers detect M1 or NS1 mRNA and M or NS cRNA and vRNA. (b) Quantification of RNA levels for the different viral genes following RNA reconstitution with or without NS2 co-expression. For each gene segment, mRNA, cRNA and vRNA levels in the presence of NS2 were calculated from three independent experiments and expressed as a percentage of the values in the absence of NS2 (which were set as 100 %). (c) 293T cells were transfected with plasmids expressing the PB1, PB2, PA and NP proteins and either NS2/NEP or empty vector, followed by infection with A/WSN/33 virus at an m.o.i. of 10 in the presence of 5 μg actinomycin D ml^{-1}. RNA was harvested at 4 h p.i. and analysed by primer extension. (d) Quantification of RNA levels for the NA gene expressed as a percentage of the values in the absence of NS2, averaged from two independent experiments. Bars indicate SD.

effect on the accumulation of RNA species derived from a viral infection, suggesting that the NS2/NEP protein plays a role in regulating transcription and replication during the viral life cycle.

The effect of NS2/NEP on the accumulation of viral transcription and replication products is functionally conserved in influenza B viruses

An alignment of the amino acid sequence of the A/WSN/33 and B/Beijing/1/87 virus NS2/NEP proteins shows that there is only 22 % identity between the two (Fig. 4a). Nonetheless, when the B/Beijing/1/87 NS2/NEP protein was co-expressed in an influenza B RNP reconstitution assay, a decrease in the accumulation of mRNA and an increase in the accumulation of cRNA, an effect similar to that in influenza A RNP reconstitution, was observed for both the NA and PA gene segments (Fig. 4b). No effect on the accumulation of vRNA was observed. The ability of the NS2/NEP protein to downregulate mRNA and upregulate cRNA accumulation appears to be functionally conserved between influenza A and B viruses.

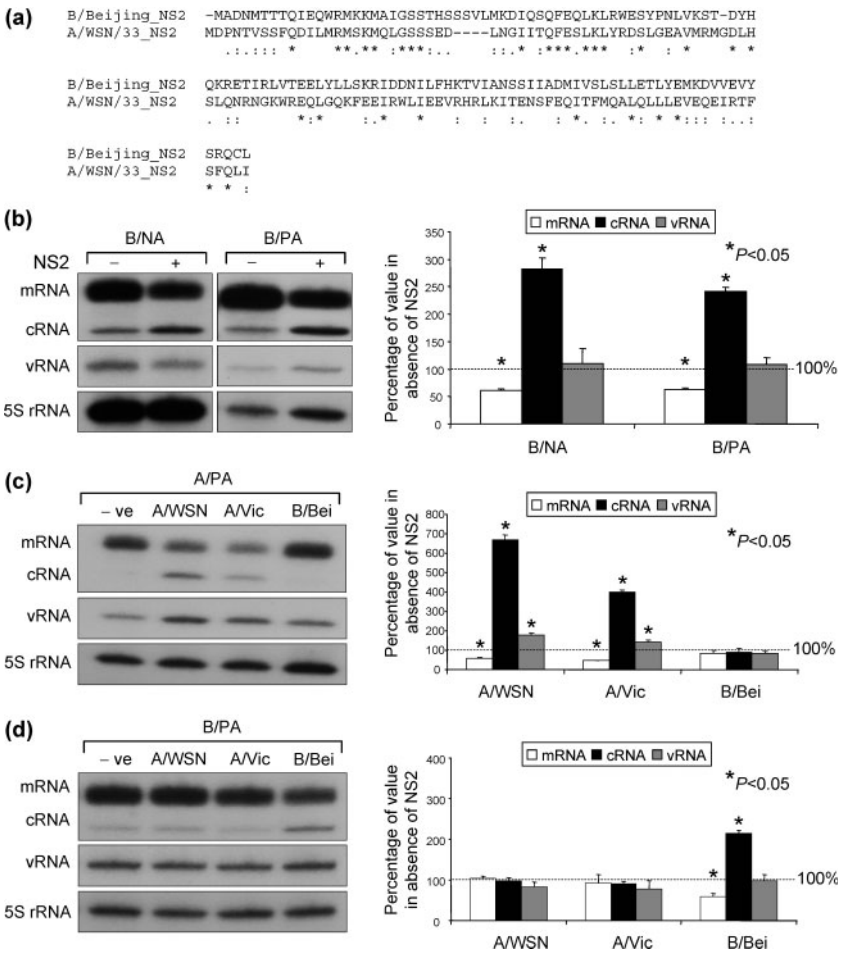

Fig. 4. The effect of NS2/NEP is conserved in influenza B viruses. (a) CLUSTAL analysis of the amino acid sequence of the A/WSN/33 virus and B/Beijing/1/87 virus NS2 proteins. Amino acid sequence identity, conserved substitutions and semi-conserved substitutions are indicated by asterisks (*), double dots (:) and dots (.), respectively. (b) Comparison of viral RNA levels from influenza B NA and PA genes in the presence or absence of NS2. 293T cells were transfected with plasmids expressing the influenza B PB1, PB2, PA and NP proteins and a NA or PA vRNA, with and without the NS2 protein. RNA was harvested after 48 h and analysed by primer extension. (c) Analysis of the effect of different NS2 proteins on influenza A RNA levels. 293T cells were transfected with plasmids expressing the influenza A PB1, PB2, PA and NP proteins, PA vRNA, and the A/WSN/33, A/Victoria/3/75 or B/Beijing/1/87 NS2 proteins as indicated. RNA was harvested after 48 h and analysed by primer extension. (d) Analysis of the effect of different NS2 proteins on influenza B RNA levels. Quantitative analysis of three independent experiments was carried out in each case. Bars indicate SD.

A mixing experiment was carried out in which the effect of influenza A NS2/NEP proteins was tested in an influenza B RNP reconstitution assay (Fig. 4c), and conversely where the effect of an influenza B NS2/NEP protein was analysed in an influenza A RNP reconstitution assay (Fig. 4d). The regulatory role of NS2/NEP was found to be type-specific, as influenza A virus NS2/NEP proteins affect RNA levels in type A RNP reconstitutions but not in type B, and similarly a type B NS2/NEP protein only has an effect in an influenza B RNP reconstitution assay. These results suggest that expression of NS2/NEP directly affects viral transcription and replication processes rather than indirectly affecting the host cell.

The effect of NS2/NEP on viral RNA levels is independent of M1 expression

The current model of vRNP nucleocytoplasmic export involves an export complex in which NS2/NEP has an N-terminal association with Crm1 and a C-terminal association with M1, which in turn is bound to vRNPs (Akarsu *et al.*, 2003). We performed RNP reconstitution assays in which NS2/NEP alone or both NS2/NEP and the M1 protein were co-expressed (Fig. 5a). The expression of M1 was confirmed by Western blot analysis (data not shown). The effect of NS2/NEP on viral RNA levels of the PA segment was similar in both the presence and absence of M1, as a significant decrease in mRNA and increase in cRNA was observed in both cases (Fig. 5b). Similar results were obtained for the PB1, PB2 and NA segments (data not shown). The NS2/NEP protein therefore has the ability to regulate viral replication and transcription independently of M1 expression.

It should be noted that, when M1 was expressed in the RNP reconstitution assays, there was a general decrease in all viral RNA species (Fig. 5a and b). Previous studies have shown that expression of the M1 protein can repress transcriptional activity of RNPs (Baudin *et al.*, 2001; Elster *et al.*, 1997; Watanabe *et al.*, 1996; Ye *et al.*, 1987, 1989), which could be an explanation for the lower RNA levels observed.

The nuclear export sequence (NES) of NS2/NEP is not required for its regulatory activity

In order to gain further information about the role of NS2/NEP in the regulation of transcription and replication, a number of NS2/NEP deletion mutants were constructed,

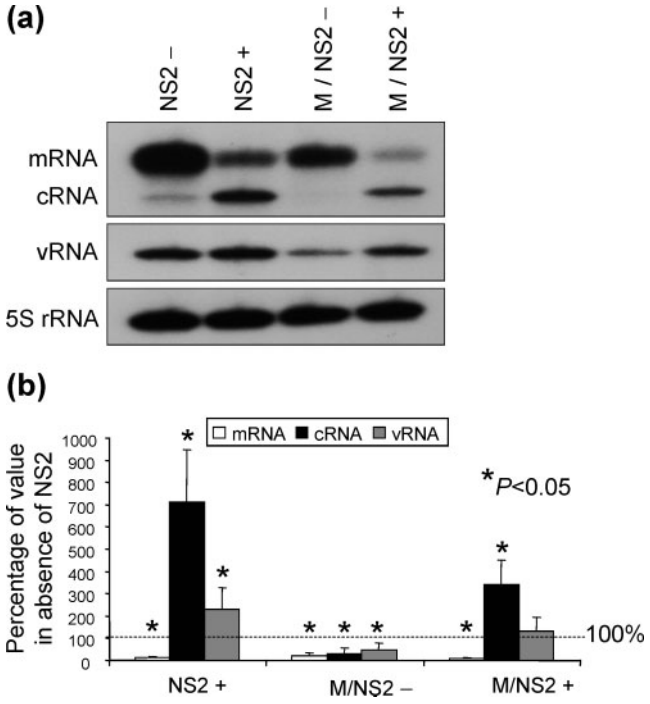

Fig. 5. Alteration of viral RNA levels by NS2/NEP expression is independent of M1 expression. (a) Comparison of the effect of NS2/NEP on PA RNA levels in the presence and absence of M1. 293T cells were transfected with plasmids expressing the influenza PB1, PB2, PA and NP proteins and PA vRNA, as well as either empty vector or a plasmid expressing NS2. M1 protein was expressed from a pPOLI-M-RT transcription plasmid which produces both M1 and M2. RNA was harvested after 48 h and analysed by primer extension. (b) Quantitative analysis of three independent experiments. Bars indicate SD.

including C-terminal and N-terminal fragments and proteins lacking either NES or the C2 alpha-helical structure of the C terminus (Fig. 6a). The deletion mutants were made using a pcDNA-TAP-NS2 protein-expression plasmid. The presence of the tag does not interfere with the ability of NS2/NEP to affect viral RNA levels in RNP reconstitution assays (data not shown). Expression of the mutant NS2/NEP proteins was verified by Western blot analysis (Fig. 6b) and shown to be similar to wild-type levels, with the exception of the NS2 1–90 construct that has slightly reduced protein expression. The localization of the NEP/NS2 deletion mutants was analysed by immunofluorescence. Preliminary data revealed no obvious differences in the localization pattern of the deletion mutants compared with the wild type (data not shown). The effect of mutant NS2/NEP proteins was tested in RNP reconstitution, followed by the analysis of viral RNAs of the PA gene segment (Fig. 6c). Viral RNA levels from three independent experiments were quantified and compared with values obtained in the absence of NS2/NEP, which were set as 100 % (Fig. 6d).

Deletion mutants 1–53 and 1–90 did not significantly affect viral RNA levels, and although expression of the NS2 53–121 mutant did result in an apparent increase in the level of cRNA, this was not statistically significant at $P<0.05$ (Fig. 6d). In contrast, the NS2 21–121 protein did produce a significant increase in cRNA levels, similar to that produced by wild-type NS2/NEP (Fig. 6d). Both of the NS2/NEP deletion mutants that resulted in an increased accumulation of cRNA contained the C2 alpha helix in the C terminus, suggesting that this region of the protein is important for the observed increase.

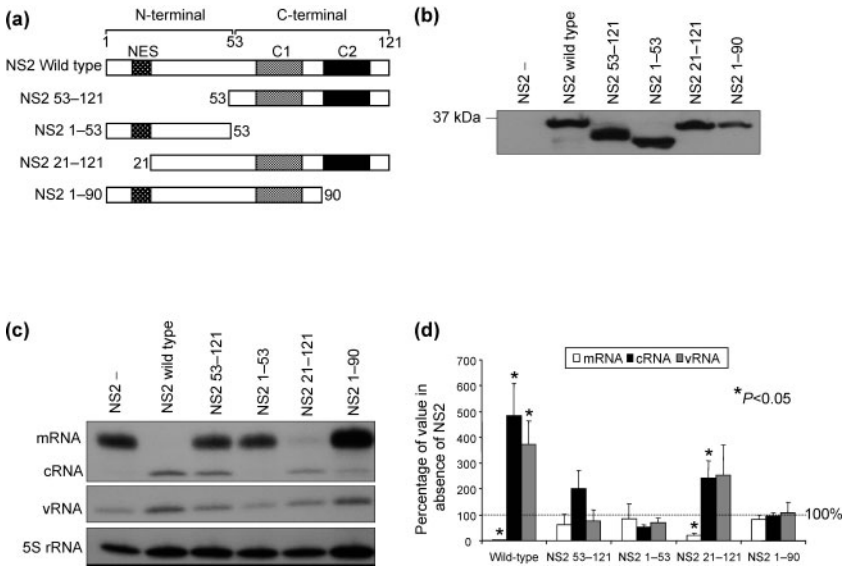

Fig. 6. Analysis of NS2/NEP deletion mutants. (a) Diagram of the NS2 deletion mutants. NES indicates the nuclear export sequence, while C1 and C2 indicate the C terminal α-helical domains (Akarsu *et al.*, 2003). (b) Western blot analysis of the NS2 deletion mutants. (c) Comparison of the effects of the NS2 deletion mutants on PA gene RNA levels by primer extension analysis. 293T cells were transfected with plasmids expressing the influenza PB1, PB2, PA and NP proteins and PA vRNA, as well as either empty vector, pcDNA-TAP-NS2 (wild-type) or the NS2 mutants. RNA was harvested after 48 h and analysed by primer extension. (d) Quantitative analysis of RNA levels in the presence of the NS2 mutants. RNA levels in the presence of wild-type NS2 or the mutants were calculated from three independent experiments and expressed as a percentage of the values in the absence of NS2 (which were set as 100 %). Bars indicate SD.

The NS2 21–121 mutant was the only protein to produce a significant decrease in mRNA similar to that produced by the wild-type protein (Fig. 6d). NS2 21–121 shows both a significant decrease in mRNA and an increase in cRNA levels, indicating that almost the full-length protein is required for an effect on both transcription and replication. As the C-terminal fragment alone (NS2 53–121) cannot cause a significant decrease in mRNA levels, the region located between aa 21 and 53 of the protein appears to be important for its effect on mRNA. This region alone cannot produce a decrease in mRNA levels, however, as demonstrated by the NS2 1–53 and NS2 1–90 constructs. Therefore, in order to produce an effect on both mRNA and cRNA, both the C2 alpha-helical region, located between aa 94 and 115, and region 21–53 of the N terminus of the NS2/NEP protein are required.

NS2 21–121 has the ability to affect viral mRNA and cRNA levels despite lacking the NES region located between aa 12 and 21 which has been implicated in Crm1 binding and shown to be vital for vRNP export (Neumann *et al.*, 2000). We therefore conclude that the NES region of NS2/NEP is not required for the regulation of transcription and replication and that this activity of NS2/NEP is likely to be independent of its transport function.

DISCUSSION

In this report, we provide experimental evidence that the influenza NS2/NEP protein plays a role in the regulation of transcription and replication of the influenza virus RNA genome. In particular, co-expression of NS2/NEP resulted in a decrease in mRNA and an increase in cRNA and vRNA accumulation in RNP reconstitution assays of most of the gene segments. For example, co-expression of NS2/NEP resulted in a dramatic reduction in PA-specific mRNA levels to resemble the low levels typically observed for the three polymerase genes during viral infection (see Fig. 2a). It is believed that the low mRNA : cRNA ratios characteristic of the three polymerase gene segments during viral infection, compared with the high mRNA : cRNA ratios of the non-polymerase gene segments, are caused by a single nucleotide change at position 4 of the 3′ end of the vRNA promoter, which is a C residue in the polymerase genes but a U residue in most of the non-polymerase gene segments (Lee & Seong, 1998; Lee *et al.*, 2003). However, in RNP reconstitution assays, we have observed high mRNA : cRNA ratios for the polymerase genes (see Figs 1 and 3), which suggests that the nature of the residue at position 4 alone is not sufficient to determine mRNA : cRNA ratios. Our results suggest that, during viral infection, NS2/NEP plays a critical role in determining mRNA : cRNA ratios.

The effect of NS2/NEP on RNA levels is influenza-virus-type-specific; influenza A virus NS2/NEP affects influenza A RNPs, while influenza B virus NS2/NEP has a regulatory effect on influenza B RNPs, but neither affects the other type. The regulation by NS2/NEP occurs independently of M1 expression and is not affected by deletion of the NES region that has been shown to be important for the nuclear export function of NS2/NEP. We therefore propose that NS2/NEP has multiple roles during the influenza virus life cycle: in addition to its previously described role in vRNP export, the protein also functions in a regulatory capacity. This regulation is likely to be a critical requirement for the viral life cycle, as it is conserved in both influenza A and B viruses.

At this time, we can only speculate on how NS2/NEP mediates its effect on the accumulation of viral RNAs. As the regulatory effect is influenza-virus-type specific, it is unlikely that NS2/NEP would mediate its effect indirectly by affecting the host cell; it is more likely that NS2/NEP mediates its activity via a direct interaction with the components of the vRNPs. Indeed, the influenza type B and type C NS2/NEP proteins have been shown to associate directly with the vRNP complex (Paragas *et al.*, 2001; Kohno *et al.*, 2009). We have performed co-purification and co-immunoprecipitation assays to investigate the possibility of an interaction between NS2/NEP and the components of influenza type A vRNPs but, in agreement with others (O'Neill *et al.*, 1998; Imai *et al.*, 2003), no interaction could be detected (data not shown). However, the possibility of a weak or transient interaction cannot be ruled out. In fact, the atypical decrease in vRNA levels observed for the NP gene when NS2/NEP is expressed (Fig. 3a and b) is suggestive of a possible role for NP. The conserved chemical nature of the amino acid residues between influenza type A and B NS2/NEP proteins suggests a common tertiary structure (Akarsu *et al.*, 2003), which supports the hypothesis that both type A and B NS2/NEP proteins are mediating the regulation of transcription and replication in a similar way.

Our results do not distinguish whether NS2/NEP has an effect on the synthesis of RNAs or on their stability. According to one possible scenario, binding of NS2/NEP to vRNPs (and possibly cRNPs) inhibits their ability to act as templates for both transcription and replication. This would inevitably lead to a reduction in the accumulation of viral mRNA levels, as observed in our experiments. It could also lead to the observed increase in the accumulation of cRNA/vRNA levels if we postulate that transcriptionally inactive RNPs are more stable as they are less likely to be subject to degradation by nucleases. During transcription, RNPs must unfold to some degree to allow transcription by the RNA polymerase; this may increase the accessibility of the RNA to nucleases, resulting in a greater degree of degradation. Intriguingly, there seems to be little, if any, correlation between the increase of cRNA and vRNA levels for the different gene segments analysed (see Fig. 3b). This might indicate that NS2/NEP could indeed act via the stabilization of RNPs, resulting in transcriptionally inactive forms. There are many possible explanations for why NS2/NEP may do this; one possibility is that by binding to RNPs, NS2/NEP, known to accumulate in the nucleus of cells late in infection, could induce an RNP conformation

that destines RNPs for packaging, rather than acting as templates for transcription and replication.

It is unlikely that the regulatory function of NS2/NEP is directly related to vRNP export. Mutation of a tryptophan at residue 78 in the C terminus of the NS2/NEP protein, previously demonstrated to be critical for binding to M1 (Akarsu *et al.*, 2003), did not affect the ability of NS2/NEP to regulate viral RNA levels (data not shown). In addition, fractionation experiments showed that the nuclear and cytoplasmic localization of viral RNA species was not altered by NS2/NEP and leptomycin B, an inhibitor of Crm1, did not alter the effect of NS2/NEP (data not shown).

In a previous study, it was suggested that the NS2/NEP protein inhibits RNA synthesis by reducing the levels of vRNA, cRNA and mRNA (Bullido *et al.*, 2001). Although this study does suggest a regulatory role for NS2/NEP, the results do not correlate entirely with our own results where only mRNA levels were reduced and cRNA levels were in fact increased. The effect observed by Bullido *et al.* (2001) was strictly dependent on the expression levels of NS2/NEP. We have therefore carried out experiments to address the question of how increasing amounts of NS2/NEP affect the accumulation of viral RNAs. We have found that the effect on viral RNA levels becomes more pronounced if more NEP/NS2 protein is expressed, i.e. a stronger decrease in the accumulation of mRNA and a stronger increase in the accumulation of cRNA is observed (data not shown). We have also confirmed that the expression levels of NS2/NEP in the RNP reconstitution system used here were comparable to those in viral infection (results not shown). We suggest that the differences between the two studies may be explained by the vaccinia helper virus system used in the previous experiments, which may itself affect influenza virus replication or the host cell.

In summary, we have shown that regulation of transcription and replication during viral infection and in RNP reconstitution assays differ. We have found that the co-expression of NS2/NEP affects the accumulation of viral RNAs in RNP reconstitutions, suggesting a novel regulatory function for NS2/NEP. We therefore propose that the NS2/NEP protein plays a role in the regulation of viral transcription and replication during the viral life cycle.

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REFERENCES

Akarsu, H., Burmeister, W. P., Petosa, C., Petit, I., Muller, C. W., Ruigrok, R. W. & Baudin, F. (2003). Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2). *EMBO J* **22**, 4646–4655.

Baudin, F., Petit, I., Weissenhorn, W. & Ruigrok, R. W. (2001). *In vitro* dissection of the membrane and RNP binding activities of influenza virus M1 protein. *Virology* **281**, 102–108.

Braam, J., Ulmanen, I. & Krug, R. M. (1983). Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. *Cell* **34**, 609–618.

Bullido, R., Gómez-Puertas, P., Saiz, M. J. & Portela, A. (2001). Influenza A virus NEP (NS2 protein) downregulates RNA synthesis of model template RNAs. *J Virol* **75**, 4912–4917.

Deng, T., Vreede, F. T. & Brownlee, G. G. (2006). Different de novo initiation strategies are used by influenza virus RNA polymerase on its cRNA and viral RNA promoters during viral RNA replication. *J Virol* **80**, 2337–2348.

Elster, C., Larsen, K., Gagnon, J., Ruigrok, R. W. & Baudin, F. (1997). Influenza virus M1 protein binds to RNA through its nuclear localization signal. *J Gen Virol* **78**, 1589–1596.

Elton, D., Simpson-Holley, M., Archer, K., Medcalf, L., Hallam, R., McCauley, J. & Digard, P. (2001). Interaction of the influenza virus nucleoprotein with the cellular CRM1-mediated nuclear export pathway. *J Virol* **75**, 408–419.

Engelhardt, O. G., Smith, M. & Fodor, E. (2005). Association of the influenza A virus RNA-dependent RNA polymerase with cellular RNA polymerase II. *J Virol* **79**, 5812–5818.

Fodor, E., Devenish, L., Engelhardt, O. G., Palese, P., Brownlee, G. G. & García-Sastre, A. (1999). Rescue of influenza A virus from recombinant DNA. *J Virol* **73**, 9679–9682.

Fodor, E., Crow, M., Mingay, L. J., Deng, T., Sharps, J., Fechter, P. & Brownlee, G. G. (2002). A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase inhibits endonucleolytic cleavage of capped RNAs. *J Virol* **76**, 8989–9001.

Gabriel, G., Dauber, B., Wolff, T., Planz, O., Klenk, H. D. & Stech, J. (2005). The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. *Proc Natl Acad Sci U S A* **102**, 18590–18595.

Imai, M., Watanabe, S. & Odagiri, T. (2003). Influenza B virus NS2, a nuclear export protein, directly associates with the viral ribonucleoprotein complex. *Arch Virol* **148**, 1873–1884.

Inglis, S. C., Barrett, T., Brown, C. M. & Almond, J. W. (1979). The smallest genome RNA segment of influenza virus contains two genes that may overlap. *Proc Natl Acad Sci U S A* **76**, 3790–3794.

Jackson, D., Cadman, A., Zurcher, T. & Barclay, W. S. (2002). A reverse genetics approach for recovery of recombinant influenza B viruses entirely from cDNA. *J Virol* **76**, 11744–11747.

Kohno, Y., Muraki, Y., Matsuzaki, Y., Takashita, E., Sugawara, K. & Hongo, S. (2009). Intracellular localization of influenza C virus NS2 protein (NEP) in infected cells and its incorporation into virions. *Arch Virol* **154**, 235–243.

Krug, R. M. (1981). Priming of influenza viral RNA transcription by capped heterologous RNAs. *Curr Top Microbiol Immunol* **93**, 125–149.

Labadie, K., Dos Santos Afonso, E., Rameix-Welti, M. A., van der Werf, S. & Naffakh, N. (2007). Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells. *Virology* **362**, 271–282.

Lamb, R. A. & Choppin, P. W. (1979). Segment 8 of the influenza virus genome is unique in coding for two polypeptides. *Proc Natl Acad Sci U S A* **76**, 4908–4912.

Lamb, R. A. & Lai, C. J. (1980). Sequence of interrupted and uninterrupted mRNAs and cloned DNA coding for the two overlapping nonstructural proteins of influenza virus. *Cell* **21**, 475–485.

Lee, K. H. & Seong, B. L. (1998). The position 4 nucleotide at the 3′ end of the influenza virus neuraminidase vRNA is involved in temporal regulation of transcription and replication of neuraminidase RNAs and affects the repertoire of influenza virus surface antigens. *J Gen Virol* **79**, 1923–1934.

Lee, M. K., Bae, S. H., Park, C. J., Cheong, H. K., Cheong, C. & Choi, B. S. (2003). A single-nucleotide natural variation (U4 to C4) in an influenza A virus promoter exhibits a large structural change: implications for differential viral RNA synthesis by RNA-dependent RNA polymerase. *Nucleic Acids Res* **31**, 1216–1223.

Mullin, A. E., Dalton, R. M., Amorim, M. J., Elton, D. & Digard, P. (2004). Increased amounts of the influenza virus nucleoprotein do not promote higher levels of viral genome replication. *J Gen Virol* **85**, 3689–3698.

Neumann, G., Hughes, M. T. & Kawaoka, Y. (2000). Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. *EMBO J* **19**, 6751–6758.

Odagiri, T. & Tobita, K. (1990). Mutation in NS2, a nonstructural protein of influenza A virus, extragenically causes aberrant replication and expression of the PA gene and leads to generation of defective interfering particles. *Proc Natl Acad Sci U S A* **87**, 5988–5992.

Odagiri, T., Tominaga, K., Tobita, K. & Ohta, S. (1994). An amino acid change in the non-structural NS2 protein of an influenza A virus mutant is responsible for the generation of defective interfering (DI) particles by amplifying DI RNAs and suppressing complementary RNA synthesis. *J Gen Virol* **75**, 43–53.

O'Neill, R. E., Talon, J. & Palese, P. (1998). The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *EMBO J* **17**, 288–296.

Palese, P. (1977). The genes of influenza virus. *Cell* **10**, 1–10.

Paragas, J., Talon, J., O'Neill, R. E., Anderson, D. K., García-Sastre, A. & Palese, P. (2001). Influenza B and C virus NEP (NS2) proteins possess nuclear export activities. *J Virol* **75**, 7375–7383.

Pleschka, S., Jaskunas, R., Engelhardt, O. G., Zurcher, T., Palese, P. & García-Sastre, A. (1996). A plasmid-based reverse genetics system for influenza A virus. *J Virol* **70**, 4188–4192.

Portela, A. & Digard, P. (2002). The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. *J Gen Virol* **83**, 723–734.

Richardson, J. C. & Akkina, R. K. (1991). NS2 protein of influenza virus is found in purified virus and phosphorylated in infected cells. *Arch Virol* **116**, 69–80.

Salomon, R., Franks, J., Govorkova, E. A., Ilyushina, N. A., Yen, H. L., Hulse-Post, D. J., Humberd, J., Trichet, M., Rehg, J. E. & other authors (2006). The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. *J Exp Med* **203**, 689–697.

Vreede, F. T., Jung, T. E. & Brownlee, G. G. (2004). Model suggesting that replication of influenza virus is regulated by stabilization of replicative intermediates. *J Virol* **78**, 9568–9572.

Watanabe, K., Handa, H., Mizumoto, K. & Nagata, K. (1996). Mechanism for inhibition of influenza virus RNA polymerase activity by matrix protein. *J Virol* **70**, 241–247.

Yasuda, J., Nakada, S., Kato, A., Toyoda, T. & Ishihama, A. (1993). Molecular assembly of influenza virus: association of the NS2 protein with virion matrix. *Virology* **196**, 249–255.

Ye, Z. P., Pal, R., Fox, J. W. & Wagner, R. R. (1987). Functional and antigenic domains of the matrix (M1) protein of influenza A virus. *J Virol* **61**, 239–246.

Ye, Z. P., Baylor, N. W. & Wagner, R. R. (1989). Transcription-inhibition and RNA-binding domains of influenza A virus matrix protein mapped with anti-idiotypic antibodies and synthetic peptides. *J Virol* **63**, 3586–3594.