Splicing of influenza A virus NS1 mRNA is independent of the viral NS1 protein

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RNA segment 8 (NS) of influenza A virus encodes two proteins. The NS1 protein is translated from the unspliced primary mRNA transcript, whereas the second protein encoded by this segment, NS2/NEP, is translated from a spliced mRNA. Splicing of influenza NS1 mRNA is thought to be regulated so that the levels of NS2 spliced transcripts are approximately 10% of total NS mRNA. Regulation of splicing of the NS1 mRNA has been studied at length, and a number of often-contradictory control mechanisms have been proposed. In this study, we used 32P-labelled gene-specific primers to investigate influenza A NS1 mRNA splicing regulation. It was found that the efficiency of splicing of NS1 mRNA was maintained at similar levels in both virus infection and ribonucleoprotein-reconstitution assays, and NS2 mRNA comprised approximately 15% of total NS mRNA in both assays. The effect of NS1 protein expression on the accumulation of viral NS2 mRNA and spliced cellular β-globin mRNA was analysed, and it was found that NS1 protein expression reduced spliced β-globin mRNA levels, but had no effect on the accumulation of NS2 mRNA. We conclude that the NS1 protein specifically inhibits the accumulation of cellular RNA polymerase II-driven mRNAs, but does not affect the splicing of its own viral NS1 mRNA.

INTRODUCTION

Influenza A viruses belong to the family Orthomyxoviridae and have a segmented, negative-sense, single-stranded RNA genome. There are eight RNA segments in total, which encode up to 12 viral proteins (Chen et al., 2001; Wise et al., 2009). The influenza virus encodes its own RNA-dependent RNA polymerase (RdRp), which is responsible for both transcription and replication of the genomic viral RNA (vRNA). Both of these processes take place in the nuclei of infected cells (reviewed by Engelhardt & Fodor, 2006). Upon virus infection, negative-sense vRNA enters the nucleus, where it is transcribed into positive-sense mRNA by a primer-dependent mechanism (Krug, 1981). The viral RdRp generates short, capped RNA primers of approximately 10–15 nt by cleaving cellular pre-mRNAs (Dias et al., 2009; Yuan et al., 2009). Virus replication occurs via a two-step primer-independent process; a positive-sense copy of the vRNA known as complementary RNA (cRNA) is made first, and this in turn is used as a template to make more vRNA.

The two smallest genomic segments of influenza A virus, segments 7 (M) and 8 (NS), each encode more than one protein. The M segment encodes the M1 matrix protein from unspliced mRNA and generates at least two alternatively spliced mRNAs, one of which encodes the transmembrane ion channel M2 protein (Lamb et al., 1981; Shih et al., 1998). The NS segment encodes the NS1 protein (non-structural protein-1) from unspliced primary NS1 mRNA transcripts and the NS2/NEP protein (non-structural protein-2/nuclear export protein) from spliced transcripts. The NS1 protein functions as a suppressor of host immune responses; however, it has also been implicated in a wide variety of other activities, such as the regulation of vRNA synthesis and mRNA translation, virus particle morphogenesis, pathogenesis and the regulation of splicing (reviewed by Hale et al., 2008). The NS2/NEP protein has been shown to play a role in the nuclear export of viral ribonucleoproteins (RNP)s during the virus life cycle (O’Neill et al., 1998) and the regulation of transcription and replication (Bullido et al., 2001; Robb et al., 2009).

Splicing of influenza mRNA has been demonstrated in the absence of other viral proteins, indicating that the virus uses the cellular splicing machinery in the nucleus (Lamb & Lai, 1982, 1984). Splicing of eukaryotic pre-mRNAs is extremely efficient and usually only the spliced mRNA products are found in the cytoplasm. Influenza virus deviates from this, as both spliced and unspliced mRNAs encode proteins; thus, only a portion of the mRNA is
spliced, whilst the remaining transcripts are transported to the cytoplasm without undergoing processing by the splicing machinery. Splicing of both NS1 and M1 mRNAs in influenza virus-infected cells is therefore regulated so that the level of spliced viral transcripts is thought to be approximately 10% of that of unspliced viral transcripts (Lamb et al., 1980, 1981). Early work suggested that virus-specific products, in particular the NS1 protein, were able to regulate the production of spliced viral mRNA (Inglis & Brown, 1984; Lamb et al., 1978; Skehel, 1972; Smith & Inglis, 1985). The extent of splicing was also suggested to differ between avian and mammalian cell lines (Inglis & Brown, 1984). It was reported that NS1 mRNA was not spliced detectably in vitro (Plotch & Krug, 1986), and further studies showed that this was due to cis-acting sequences in the mRNA itself, which caused a block in splicing after formation of atypically sedimenting pre-spooling complexes (Agris et al., 1989; Nemeroff et al., 1992).

In vivo studies in which NS1 mRNA was expressed from a variety of RNA polymerase II (pol II)-driven cDNA constructs implicated the NS1 protein in the retention of both spliced NS2/NEP and unspliced NS1 mRNAs in the nucleus (Alonso-Caplen & Krug, 1991; Alonso-Caplen et al., 1992; Fortes et al., 1994). The block in the nucleocytoplasmic transport of these mRNAs was hypothesized to be a control mechanism through which the NS1 protein specifically regulated the level of splicing of NS1 mRNA; however, it was later demonstrated that NS1 binds to and inhibits the 3′-end processing and subsequent export of all poly(A)-containing mRNAs (Fortes et al., 1994; Qiu & Krug, 1994; Shimizu et al., 1999). The discovery that NS1 interacts with the cellular factor CPSF-30, preventing it from processing mRNAs, suggested a mechanism by which the NS1 protein carried out this inhibition (Nemeroff et al., 1998). According to this model, the NS1 protein would selectively block the export of cellular, but not viral, mRNAs, as the poly(A) tails of viral mRNAs are not produced by the cellular 3′-end machinery (Li et al., 2001; Nemeroff et al., 1998; Poon et al., 1999). This function of NS1 was therefore proposed to be a host shut-off mechanism by which cellular gene expression is inhibited. The NS1 protein was also shown to inhibit the splicing of cellular pre-mRNAs by interacting with stem–bulge regions in both U6 and U6atac small nuclear RNAs, thus preventing the formation of spliceosome complexes (Qiu et al., 1995; Wang & Krug, 1998).

In order to analyse the splicing regulation of NS1 mRNA in a more physiologically relevant way, a recent study made use of a transient-transfection system that allowed the NS RNP complex to be replicated by the viral polymerase (Garaigorta & Ortín, 2007). In this system, viral polymerase and nucleoprotein expressed from RNA pol II-driven vectors transcribed and replicated NS genomic RNA expressed under the control of an RNA pol I promoter, after which poly(A)-containing RNA was analysed by RNase-protection assay. This investigation showed that the NS1 protein was able to inhibit the splicing and nuclear export of true viral NS1 mRNA transcripts. In contrast to this, another study showed that, whilst NS1 inhibited the splicing of cellular pre-mRNAs, splicing of the NS1 mRNA itself was resistant to this inhibition (Lu et al., 1994).

In this study, we set out to investigate the regulation of splicing of the influenza NS1 mRNA, and to do this we established a highly sensitive and quantitative method for the analysis of spliced viral transcripts using 32P-labelled gene-specific primers designed to detect NS RNA species in a reverse-transcription reaction. Our results show that the NS1 protein specifically inhibits the splicing of cellular RNA polymerase II-driven pre-mRNAs, but does not affect the splicing of viral NS1 mRNA.

RESULTS

Ratio of unspliced NS1 mRNA and spliced NS2 mRNA remains constant during virus infection

To analyse the accumulation of spliced and unspliced mRNAs, we designed primers to detect all viral NS RNA species by primer extension (described in Fig. 1). 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 post-infection. We initially analysed NS RNA species by using the NS-734 and NS-708 primers. The NS-708 primer was appropriate for the detection of NS2 mRNA; however, the primer-extension products from the NS1 mRNA and NS cRNA species could not be resolved (Fig. 2a). The samples were therefore also analysed by using the NS-226 primer, which produces an

![Fig. 1. Diagram of the RNA species of the A/WSN/33 NS gene. The NS vRNA is replicated via an NS cRNA intermediate and is also transcribed into NS1 mRNA and its spliced product NS2 mRNA. The 5′ donor splice site of the NS1 mRNA is at position 57 and the 3′ acceptor splice site is at position 528. Squares at the 5′ end of the mRNA species represent the 5′ cap, and AAA at the 3′ end represents the poly(A) tail. A forward primer annealing at position 734 detects NS vRNA (product size 156 nt), a reverse primer annealing at position 226 detects both NS1 mRNA and NS cRNA (product sizes approx. 240 and 226 nt, respectively), and a second reverse primer annealing at position 708 detects NS1 mRNA, NS2 mRNA and NS cRNA (product sizes approx. 721, approx. 250 and 708 nt, respectively).](image-url)
NS1 mRNA-specific product that can be resolved from the NS cRNA-specific band on a gel (Fig. 2b). Following quantification, the percentage of NS cRNA signal was deducted from the unresolved NS1 mRNA and NS cRNA band, giving a value for the NS1 mRNA alone. The spliced NS2 mRNA was quantified and found to be approximately 17% of the total NS mRNA, averaged from three independent experiments (Fig. 2c). This ratio remained constant during the entire course of the infection, suggesting that the efficiency of splicing remained constant. In order to verify that the primer-extension reaction used was carried out in the linear range, a single RNA sample harvested at 8 h post-infection was analysed by using the NS-734 and NS-708 primers. Despite increasing volumes of RNA being added to the primer-extension mixture, the ratio of unspliced NS2 mRNA to total NS mRNA and cRNA did not change (see Supplementary Fig. S1, available in JGV Online).

As the NS1 protein has previously been implicated in the regulation of splicing, protein levels in infected cell lysates were analysed by immunoblotting (Fig. 2d). It was found that the ratios of unspliced NS1 mRNA and spliced NS2 mRNA remained constant, despite levels of the NS1 protein increasing over the course of the infection. This suggests that the increasing concentrations of the NS1 protein have no effect on the splicing of the NS1 mRNA.

The accumulation of NS1 and NS2 mRNA species was also evaluated for a second virus strain, A/Udorn/72, in human lung epithelial A549 cells and for A/WSN/33 in chicken fibroblast DF-1 cells (see Supplementary Figs S2 and S3 respectively, available in JGV Online). During A/Udorn/72 virus infection, the spliced NS2 mRNA was approximately 15% of the total NS mRNA, averaged from three independent experiments (Supplementary Fig. S2c). For A/WSN/33 virus infection in DF-1 cells, the spliced NS2 mRNA was approximately 14% of the total mRNA, averaged from three independent experiments (Supplementary Fig. S3c). The ratio of NS2 spliced and NS1 unspliced transcripts remained at similar levels for both viruses over the entire course of the infection. Therefore, the efficiency of splicing of the influenza NS1 mRNA was very similar in two different virus strains and was independent of cell type.

**Fig. 2.** Ratio of unspliced NS1 mRNA and spliced NS2 mRNA remains constant during virus infection. 293T cells were infected with A/WSN/33 at an m.o.i. of 2.5 and RNA was harvested at regular times post-infection (p.i.). (a) NS1 mRNA and cRNA (●) and NS2 mRNA (■) were analysed by using the NS-708 primer and NS vRNA (▲) was analysed by using the NS-734 primer. A primer detecting cellular 5S rRNA was included as an internal control. a.u., Arbitrary units. (b) NS1 mRNA (●) and NS cRNA (■) products were resolved by using the NS-226 primer. (c) Quantification of spliced NS2 mRNA expressed as a percentage of the total NS mRNA, averaged from three independent experiments; error bars represent SD. ND, Non-detectable. (d) Western blot analysis of NS1 using an anti-NS1 antibody [a gift from Adolfo García-Sastre (Mount Sinai School of Medicine, New York, USA)].
Ratio of unspliced NS1 mRNA and spliced NS2 mRNA in an RNP-reconstitution assay is similar to that in an infection

To investigate the regulation of splicing of the NS1 mRNA further, the accumulation of NS1 and NS2 mRNAs was evaluated in a viral RNP-reconstitution assay. 293T cells were transfected with plasmids expressing the A/WSN/33 PB1, PB2, PA and NP proteins and an NS vRNA template, and RNA was harvested at 0, 11, 24, 34 and 48 h post-transfection. These time points differ from those used during infection, as viral NS RNA species generated during the RNP-reconstitution assay are only detectable by primer extension after approximately 10 h. The NS vRNA template is transcribed into mRNA for the production of both the NS1 and NS2/NEP proteins. Primer-extension analysis from three independent experiments showed that the spliced NS2 mRNA was on average approximately 14% of the total NS mRNA (Fig. 3a–c), and remained at this level at all time points. The ratio of unspliced NS1 mRNA and spliced NS2 mRNA in an RNP-reconstitution assay was therefore very similar to that occurring during a virus infection.

When cell lysates were analysed by immunoblotting for the NS1 protein, it was found that levels of NS1 increased and reached a plateau at 24 h post-transfection (Fig. 3d). The kinetics of NS1 protein production therefore differ between virus infection (Fig. 2d) and RNP reconstitution, despite the accumulation of spliced NS2 mRNA occurring at similar levels in the two assays.

NS1 protein expression has no effect on NS1 mRNA splicing

The NS1 protein has previously been implicated in the control of the regulation of splicing (Alonso-Caplen & Krug, 1991; Alonso-Caplen et al., 1992; Fortes et al., 1994; Garaigorta & Ortin, 2007). As similar levels of spliced NS2 mRNA accumulated in cells infected with two different virus strains and an RNP-reconstitution assay, we hypothesized that the presence of the NS1 protein in these

![Graphs and images related to Fig. 3](image-url)
experiments was sufficient to control the regulation of splicing. To test this, an RNP-reconstitution assay was set up where the splicing of NS1 mRNA was analysed in either the presence or the absence of the NS1 protein. 293T cells were transfected with plasmids expressing the PB1, PB2, PA and NP proteins and either an NS vRNA or a ΔNS1 vRNA with a stop codon in the NS1 open reading frame (ORF), and the accumulation of viral mRNA was analysed by primer extension (Fig. 4a, b). Both NS1 and NS2/NEP proteins were produced from the NS vRNA, whereas only NS2/NEP could be detected when the ΔNS1 vRNA was used (Fig. 4c). Although the accumulation of all viral NS RNA species was reduced in the absence of the NS1 protein (Fig. 4a, b), the ratio of spliced and unspliced NS mRNAs remained unaffected (Fig. 4d). Overexpression of the NS1 protein with the ΔNS1 vRNA also had no detectable effect on the ratio of spliced and unspliced RNAs. The slight variations observed in ratios between the different samples were not statistically significantly different in a two-tailed t-test (data not shown).

Although our anti-NS1 antibody did not detect any NS1 protein expression from the ΔNS1 vRNA construct, it was possible that C-terminal protein fragments were expressed using initiation codons located downstream of the introduced stop codon, and that these were sufficient for splicing regulation. In order to address this possibility, we investigated whether NS1 deletion mutants had any effect on the accumulation of NS2 mRNA. For this, we used an NS vRNA template that encoded only the RNA-binding domain of the NS1 protein (NS1 aa 1–80) and an NS vRNA template that encoded an NS1 protein with a large deletion in the effector domain (NS1 Δ134–161) (Fig. 5). NS1 and NS2/NEP expression was confirmed by Western blot analysis (Fig. 5a) and NS RNA was analysed by primer extension (Fig. 5b, c). It was found that similar levels of spliced NS2 mRNA were made in the presence of wild-type NS1 protein and the deletion mutants (Fig. 5d). We can therefore exclude the possibility that any potential NS1 protein fragments containing only the effector domain have an effect on splicing.

In order to investigate further the effect of NS1 protein expression on the accumulation of spliced NS2 mRNA, we carried out an experiment in which splicing of the NS gene mRNA was analysed in virus-infected cells treated with cycloheximide, which inhibits protein synthesis. In agreement with previous work (Odagiri et al., 1991), it was observed that bands corresponding to spliced NS2 mRNA transcripts could be detected in the cycloheximide-treated samples (see Supplementary Fig. S4, available in JGV Online). Splicing of the NS1 mRNA in infected cells is therefore still able to proceed without the presence of newly synthesized NS1 protein.

**NS1 and NS2 mRNAs are polyadenylated**

In contradiction to our results, a previous study investigating splicing regulation showed that only NS2 mRNA could be detected if NS1 protein expression was abrogated, leading the authors to conclude that the NS1 protein inhibits splicing of the NS1 mRNA (Garaigorta & Ortin, 2007). In those experiments, poly(A)-containing RNA was selected by oligo(dT)-cellulose chromatography for analysis, and so it was possible that NS1 mRNA could not be detected as it was not polyadenylated. We therefore chose to analyse poly(A)-separated NS segment-derived RNA using our primer-extension technique. 293T cells were transfected with plasmids expressing the PB1, PB2, PA and NP proteins, either an NS or ΔNS1 vRNA, and either empty vector or a plasmid expressing the NS1 protein. Total RNA was harvested after 48 h and separated into poly(A)^− and poly(A)^+ fractions using oligo(dT)-cellulose, and NS RNAs were analysed by primer extension. No NS1 or NS2 mRNA species were present in the poly(A)^− fractions, indicating that all detectable mRNA is polyadenylated, whilst all detectable NS vRNA and cellular
5S rRNA products were found in the poly(A)$^{-}$ fraction (Fig. 6). These results confirmed our earlier observation that similar levels of unspliced NS1 and spliced NS2 mRNAs were produced in both the presence and the absence of the NS1 protein.

**NS1 protein expression reduces the accumulation of spliced β-globin mRNA**

Having determined that the NS1 protein plays no role in the regulation of splicing of the NS1 mRNA, we were interested in the effect of this protein on host cellular transcripts. To test the effect of NS1 protein expression on the splicing of a cellular spliced mRNA, we used a radioactively labelled primer to detect specifically both the spliced and unspliced mRNA species of the human β-globin gene. 293T cells were transfected with a plasmid expressing β-globin mRNA, with NS1 wild-type or NS1 G184R, a mutant defective in CPSF-30 binding (Das et al., 2008), and β-globin mRNA was analysed by primer extension (Fig. 7). Wild-type NS1 expression resulted in an overall reduction in transcription, as the accumulation of both the spliced and unspliced β-globin mRNAs was reduced (Fig. 7a). This effect appears to be due to the ability of the NS1 protein to bind and inhibit the cellular mRNA processing factor CPSF-30, as the NS1 G184R mutant did not show any reduction in transcription. In addition, by measuring the ratio of spliced β-globin mRNA to total β-globin mRNA, we found that expression of wild-type NS1 resulted in a significant reduction in the level of splicing (Fig. 7b). We also observed that expression of the NS1 G184R mutant did not result in an inhibition of β-globin splicing, confirming an earlier study showing that splicing inhibition is dependent on the effector domain of NS1 binding to CPSF-30 (Li et al., 2001). We therefore conclude that wild-type NS1 protein can specifically inhibit the splicing of a cellular RNA pol II-driven mRNA.

**DISCUSSION**

In this study, we investigated the splicing regulation of the influenza A virus NS1 mRNA. Following either virus infection or RNP reconstitution, both NS1 and NS2 mRNA species were analysed by primer extension. The results obtained confirmed that the splicing of NS1 mRNA was maintained at low levels in both virus infection and RNP-reconstitution assays. Using this method, it was possible to quantify the accumulation of spliced NS2 mRNA, which was found to be 15% of total NS mRNA levels during virus infection (Fig. 2). This agrees with early studies estimating that spliced influenza NS2 mRNA was approximately 10%
of total mRNA (Lamb et al., 1980, 1981). However, in contrast to early studies in which it was proposed that the splicing efficiency of both the NS1 and M1 mRNAs increased over time during virus infection (Smith & Inglis, 1985; Valcarcel et al., 1991), we showed that levels of spliced NS2 mRNA remained constant at a low level over the course of an infection.

Our experiments revealed that similar levels of splicing of the NS1 mRNA occurred during infection with two different virus strains and in an RNP-reconstitution assay. This led us to investigate whether the NS1 protein was in fact required for splicing regulation. When a stop codon was inserted into the NS1 mRNA so that NS1 protein expression was abrogated, we found that splicing was unaffected (Fig. 4), thus leading us to conclude that NS1 protein expression does not regulate splicing of viral transcripts. Although the NS2/NEP protein was expressed in all samples, we speculate that it is unlikely to have an effect on the efficiency of splicing (Fig. 4). The levels of NS2/NEP expression differed greatly between the samples, i.e. there was an approximately 80% reduction between the NS vRNA and ANS1 vRNA sample (Fig. 4c). It is therefore unlikely that such different amounts of a regulatory protein would result in similar splicing efficiency between the samples. We suggest that the increase in NS2/NEP protein levels when NS1 is co-expressed may be due to the known function of NS1 to enhance selectively the translation of viral mRNAs (reviewed by Hale et al., 2008). Interestingly, we observed that, despite the ratio of spliced and unspliced RNAs being unaffected, the accumulation of all vRNA species was reduced in the absence of the NS1 protein (Fig. 4a, b). The NS1 protein has previously been shown to interact with the viral RNP (Kuo & Krug, 2009; Marion et al., 1997) and has been suggested to play a role in the regulation of virus transcription and replication (Min et al., 2007). It is therefore possible that the absence of NS1 in our experiments affected polymerase function.

Our analyses showed that similar levels of spliced NS2 mRNA were produced in the presence and absence of the NS1 protein. This is in contrast to a study by Garaigora & Ortin (2007), in which a similar transfection system was used to show that the NS1 protein appeared to inhibit the splicing and nuclear export of NS1 mRNA transcripts. In those experiments, poly(A)-containing RNA was selected by oligo(dT)-cellulose chromatography for splicing analysis, and we therefore also analysed poly(A)-separated RNA by primer extension to exclude the possibility that our analysis of total RNA was responsible for the differing results. We confirmed that all NS1 and NS2 mRNAs were polyadenylated and that similar levels of spliced NS2 mRNA were produced in both the presence and the absence of the NS1 protein (Fig. 6). Thus, the poly(A) separation of RNA does not explain the differences between the two studies; however, we cannot exclude the involvement of other methodological differences.

We also analysed the effect of NS1 protein expression on the accumulation of a spliced cellular mRNA, and found that NS1 expression reduced the accumulation of spliced β-globin mRNA (Fig. 7). It therefore appears that the NS1 protein specifically inhibits the splicing of cellular mRNAs, but does not affect the splicing of viral NS1 mRNA, in agreement with a previous study (Lu et al., 1994).

There are many plausible explanations for why the NS1 protein would reduce the accumulation of cellular spliced transcripts, the most likely of which would be as part of a host shut-off mechanism. NS1 interaction with CPSF-30 and subsequent inhibition of cellular mRNA processing and nuclear export suggest a mechanism by which the NS1 protein can selectively decrease cellular, but not viral, mRNA accumulation (Li et al., 2001; Nemeroff et al., 1998; Poon et al., 1999). Indeed, we found that a mutant NS1 protein unable to bind CPSF-30 (NS1 G184R) was unable to prevent accumulation of spliced cellular transcripts (Fig. 7).

Although we have shown that splicing of the viral NS1 mRNA is not affected by NS1 protein expression, spliced transcripts are nonetheless maintained at low levels of approximately 15%, presumably to ensure that both NS1 and NS2/NEP proteins are expressed during the virus life
cycle. At this point, we can only speculate as to how the influenza virus maintains these low levels of spliced NS2 transcripts. A possible mechanism of splicing regulation may lie in the secondary structure of the mRNA itself. Early studies implicated cis-acting sequences in the NS1 mRNA that inhibited splicing by preventing the mRNA from forming a correctly folded splicing substrate (Agris et al., 1989; Nemeroff et al., 1992). In addition, a highly conserved pseudoknot structure has been identified that encompasses the 3′ splice site of the NS1 mRNA in both influenza A and B viruses, and has been suggested to be involved in the regulation of RNA-processing events such as splicing (Gultyaev et al., 2007; Gultyaev & Olsthoorn, 2010). The fact that the proportion of spliced NS2 mRNA remains constant over time supports this hypothesis, suggesting that the regulation of viral splicing is not dependent on either host or viral protein accumulation.

A second possibility for splicing regulation may be due to differential processing pathways for cellular pre-mRNAs synthesized by RNA pol II and influenza transcripts, which are synthesized by the viral RNA polymerase. There is increasing evidence to suggest that RNA pol II transcription and mRNA processing, such as splicing, are coupled in vivo (Hirose & Manley, 2000; Howe, 2002; Proudfoot et al., 2002). The viral polymerase has been shown to be associated with the C-terminal domain of RNA pol II, possibly to allow viral transcripts to be accessed by the cellular splicing machinery (Engelhardt et al., 2005). We speculate that this close association may allow splicing of influenza mRNAs to occur; however, this process is not efficient enough to ensure 100 % splicing.

In summary, we have quantified the accumulation of unspliced NS1 and spliced NS2 mRNA during virus infection. We have demonstrated that NS1 protein expression inhibits the accumulation of a spliced cellular mRNA, but does not affect the splicing of its own viral NS1 mRNA. We speculate that this could be beneficial to the virus, as the NS1 protein specifically reduces cellular transcription, whilst viral transcripts remain unaffected.

**METHODS**

**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-NS1-RT and pPOLI-NS1-RT vRNA transcription plasmids (Fodor et al., 1999; Robb et al., 2009). The pDNA-NS1 plasmid was made by PCR amplification of the NS1 ORF from the pPOLI-NS1-RT template and subcloning into AgeI-digested pcDNA-3A. The pcDNA-AN51 plasmid was made by site-directed mutagenesis of the pcDNA-NS template, whereby a stop codon was introduced into the NS1 ORF by using the PCR primers 5′-GTAGATTGGCTTTTGATGGCATGTCCG-3′ and 5′-CGGACATG-CCATCAAAAAGCAATCTAC-3′. The pPOLI-NS1, pPOLI-NS1 Δ134–161 and pPOLI-NS1 1–80 plasmids were made by Andrea Mikulásová (Sir William Dunn School of Pathology). pPOLI-AN51 was made by site-directed mutagenesis carried out on the pPOLI-NS1-RT plasmid, whereby a stop codon was introduced into the NS1 ORF after 15 codons. The pPOLI-NS1-RT Δ134–161 mutant was constructed by round-the-plasmid PCR amplification of the pPOLI-NS1-RT template, followed by self-ligation. The pPOLI-NS1-RT 1–80 mutant was made by site-directed mutagenesis carried out on the pPOLI-NS1-RT plasmid, whereby a stop codon was introduced into the NS1 ORF after 80 aa. The pCAGGS-NS1 plasmid was made by cloning the A/Udorn/72 NS1 ORF into the empty pCAGGS vector, and the pCAGGS-NS1-G184R plasmid was made by site-directed mutagenesis of pCAGGS-NS1. The CA plasmid expressing the human β-globin mRNA was a kind gift from Steven West (Sir William Dunn School of Pathology).

**Cells and viruses.** Human kidney 293T cells were obtained from the Cell Bank of the Sir William Dunn School of Pathology, and chicken fibroblast DF-1 cells were provided by Munir Iqbal of the Institute for Animal Health, Compton, UK. Human lung epithelial A549 cells and the A/Udorn/72 virus were from the University of St Andrews. A/WSN/33 virus was provided by Peter Palese (Mount Sinai School of Medicine, New York, USA).

**Transfections and infections.** Transfections for RNP-reconstitution assays were performed in 293T cells in suspension in 35 mm dishes (approx. 10⁶ cells) by using 10 μl Lipofectamine 2000 (Invitrogen) and 1 μg of each of the relevant plasmids in 1.5 ml minimal essential medium (MEM) with 10 % fetal calf serum (FCS) (both from PAA Laboratories). 293T, A549 or DF-1 cells were infected with A/WSN/33 or A/Udorn/72 virus at an m.o.i. of 2.5 in 1 ml MEM with 0.5 % FCS per 35 mm dish. When cycloheximide was used, the cells were infected with A/WSN/33 virus at an m.o.i. of 5 in 1 ml MEM containing 0.5 % FCS and 100 μg cycloheximide ml⁻¹ (Sigma).

**Primer design.** The online program Primer3 (Rozen & Skaletsky, 2000) was used to design gene-specific primers to detect both positive- and negative-sense viral RNA species. The primers used to detect NS1-specific A/WSN/33 positive-sense RNA were NS-226 (5′-CGCTCCACTATTTGCTTTCC-3′) (Vreede et al., 2004) and NS-708 (5′-TCCATTCAAGTCCTCCGATG-3′). The primers used to detect A/Udorn/72 positive-sense RNA were NS-199 (5′-TGGCTGCTTGG-ATTTGA-3′) and NS-648 (5′-GTTGGAGTCTCCAACTTCTCA-3′). NS-734 (5′-TGATGAAGAAGTGGACACAG-3′) was used to detect both A/WSN/33 and A/Udorn/72 negative-sense RNA (Vreede et al., 2004). The primer β-globin 342 (5′-CAGGATCAAGTGCTGG-ACAGA-3′) was used to detect spliced β-globin mRNA. A primer detecting cellular 5S rRNA (5′-TCCACAGGCGGTCATCC-3′) was included as a control where required.

**RNA isolation and primer-extension analysis.** Total RNA was extracted from 293T cells (approx. 10⁶ cells) using 1 ml TRIzol reagent (Invitrogen), and one-thirtieth of each of the samples was analysed by primer extension as described previously (Robb et al., 2009). Note that the signals for mRNA and cRNA are directly comparable, as they are both detected by the same positive-sense RNA-specific primer. However, vRNA is detected by a different, negative-sense RNA-specific primer and therefore these signal intensities cannot be compared directly with those of mRNA and cRNA.

**Poly(A) separation.** Total RNA was extracted from transfected 293T cells (approx. 10⁶ cells) using 1 ml TRIzol reagent (Invitrogen) and dissolved in 50 μl water. The total RNA sample was denatured by heating to 95 °C for 10 min before being added to the oligo(dT)-cellulose. A Micro-Fast Track mRNA isolation kit (Invitrogen) was used, the cells were infected with A/WSN/33 virus at an m.o.i. of 5 in 1 ml MEM containing 0.5 % FCS and 100 μg cycloheximide ml⁻¹ (Sigma).
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