Inefficient splicing of segment 7 and 8 mRNAs is an inherent property of influenza virus A/Brevig Mission/1918/1 (H1N1) that causes elevated expression of NS1 protein

Ellenor Backström Winquist a,b, Samir Abdurahman b, Anna Tranell b, Sofia Lindström b, Susanne Tingsborg b, Stefan Schwartz a,b,*

a Department of Laboratory Medicine, Section of Medical Microbiology, Lund University, 221 84 Lund, Sweden
b Department of Medical Biochemistry and Microbiology, Uppsala University, Biomedical Center, Husargatan 3, Box 582, 751 23 Uppsala, Sweden

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ABSTRACT

Influenza A virus encodes two segments (7 and 8) that produce mRNAs that can be spliced. We have investigated if naturally occurring sequence polymorphisms in the influenza A virus family affects splicing of these viral mRNAs, as that could potentially alter the NS1/NS2- and/or M1/M2-protein ratios. We compared splicing efficiency of segment 7 and 8 mRNAs of A/Brevig Mission/1918/1 (H1N1) and A/Netherlands/178/95 (H3N2), as well as various H5N1 avian strains. Results revealed that both segment 7 and 8 mRNAs of A/Brevig Mission/1918/1 (H1N1) were inefficiently spliced compared to other influenza virus segment 7 and 8 mRNAs. This resulted in production of higher levels of functional NS1 protein, which could potentially contribute to the pathogenic properties of the A/Brevig Mission/1918/1 (H1N1). We also show that A/Brevig Mission/1918/1 (H1N1) segment 8 mRNAs responded differently to overexpression of SR proteins than A/Netherlands/178/95 (H3N2).

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Introduction

Properties that determine the pathogenicity of influenza viruses are of major interest (de Wit and Fouchier, 2008). In general, it appears that the ability of an influenza virus subtype to replicate to high levels in its host, to cause infection also in the lower respiratory tract including alveoli, to induce high cytokine production, as well as antagonising innate immune responses correlate with high virulence (Basler and Aguilar, 2008). While mutations in the polymerase genes appear to confer high replicative capacity and sequence variation in the HA and NA open reading frames affect viral tropism, the NS1 protein antagonises the innate immune response to the viral infection, more specifically the interferon response (Basler and Aguilar, 2008). Although the primary protein sequence determines the pathogenic properties of these viruses, the expression levels of NS1 protein could potentially also affect the ability of a particular influenza virus subtype to cope with the innate immune response during the infection of the host.

Influenza viruses replicate in the nucleus of the infected cell (Palese and Shaw, 2006). There, the eight RNA segments of the viral genome are transcribed into mRNAs encoding at least one viral protein each. Two of the segments, segments 7 and 8, give rise to at least one protein each. Two of the segments, segments 7 and 8, give rise to at least two protein products each, one of which is produced from the unspliced mRNA transcribed directly off the viral RNA segment, and one that is produced from a spliced mRNA generated from the same viral RNA segment. Segment 7 encodes M1 from the unspliced mRNA and M2 from the spliced mRNA, while segment 8 encodes NS1 from the unspliced mRNA and NS2 from the spliced mRNA. The ratio between unspliced versus spliced mRNAs may determine the relative concentrations of the NS1 to NS2 protein and M1 to M2 protein that are synthesised in the infected cell. Splicing efficiency of these mRNAs may therefore affect the replication and pathogenesis of the influenza A virus. In particular the NS1 protein has been implicated in the pathogenesis of influenza virus (Billharz et al., 2009).

Splicing efficiency of the influenza virus segments changes during the course of the viral infection and splicing is affected by both viral and cellular proteins (Valcarcel et al., 1991). The influenza virus NS1 protein has been shown to affect splicing and transport of mRNAs (Fortes et al., 1994; Garaigorta and Ortin, 2007; Lu et al., 1994; Muraki...
Many properties of the NS1 protein point to an important role of this protein in the regulation of viral and cellular RNA processing, e.g., NS1 binds RNA directly (Hatada et al., 1992), it affects the subcellular localisation of splicing factors (Fortes et al., 1995), it inhibits polyadenylation by interacting with the canonical polyadenylation factor CPSF-30 (Nemeroff et al., 1998), and it inhibits translation (Aragon et al., 2000). The cellular splicing factor ASF/SF2 has been shown to regulate influenza virus RNA splicing (Shih and Krug, 1996). Moreover, knockdown of CLK1, a cellular kinase that phosphorylates ASF/SF2, by siRNA, or inhibition of CLK1 with TG003, strongly reduced viral replication (Karlas et al., 2010). Splicing efficiency of cellular mRNAs is regulated by cis-acting RNA elements termed splicing enhancers and splicing silencers that interact with cellular factors (Blencowe, 2000; Cartegni et al., 2002). RNA sequences on influenza virus mRNAs exert a cis-acting effect on influenza virus mRNA splicing (Nemeroff et al., 1992). The intracellular concentrations of RNA-binding proteins, the number of RNA elements, or binding sites for cellular factors at each splice site, and the affinity of each factor for a particular RNA element will determine the outcome of the splicing reaction. The enhancer and silencer RNA elements are not well conserved and their existence on various mRNA is difficult to predict due to the large sequence heterogeneity of these RNA elements. As RNA viruses often evolve very quickly as a result of the error prone RNA polymerase, it is reasonable to speculate that naturally occurring sequence polymorphism within the influenza virus A pool of viruses may affect the splicing efficiency of the influenza virus segment 7 and 8 mRNAs. Here we have monitored the splicing efficiency of influenza virus segment 7 and 8 mRNAs derived from A/Brevig Mission/1918/1 (H1N1) (Tumpey et al., 2005), A/Netherlands/178/95 (H3N2) (Rimmelzwaan et al., 2007), A/Goose/ Guangdong/1/96 (H5N1) (Xu et al., 1999), and A/Hong Kong/483/97 (H5N1) (C. Centers for disease control, prevention, 1997) viruses. Our results demonstrate that influenza virus segment 7 and 8 mRNAs derived from different influenza A viruses are spliced with different efficiency. Segment 7 and 8 mRNAs produced from influenza virus A/Brevig Mission/1918/1 (H1N1) stood out as being very inefficiently spliced, thereby producing more unspliced mRNA. We show that this results in a higher production of functional NS1 protein from A/Brevig Mission/1918/1 (H1N1) RNAs. Consequently, we speculate that differences in splicing efficiency may affect the viral replication levels in the host and/or pathogenic properties of influenza viruses.

**Results**

Poor splicing efficiency of influenza virus A/Brevig Mission/1918/1 (H1N1) segment 8 mRNA

In order to investigate if the RNA sequence polymorphism among influenza viruses could affect viral RNA processing, we investigated if naturally occurring RNA sequence polymorphisms in segment 8 affected influenza virus RNA splicing efficiency. To this end, we selected two influenza viruses with different pathogenic properties: pandemic Spanish A/Brevig Mission/1918/1 (H1N1) (Tumpey et al., 2005) and seasonal influenza virus A/Netherlands/178/95 (H3N2) (Rimmelzwaan et al., 2007). The NS1 and NS2 coding regions of these two strains are 89% identical at the RNA level. To test if segment 8 mRNA of A/Brevig Mission/1918/1 (H1N1) was spliced with different efficiency than segment 8 mRNAs from A/Netherlands/178/95 (H3N2), sequences from the common ATG to the stop codon of NS2 were PCR-amplified and inserted into a CMV-promoter driven expression plasmid named pCL0806 (Collier et al., 2002), thereby creating plasmids pCNH1N1 and pCNH3N2 (Fig. 1A), respectively. These plasmids were transfected into HeLa cells and cytoplasmic RNA was extracted. Northern blot analysis revealed striking differences in NS RNA levels as well as in the splicing efficiency between these two viral sequences (Fig. 1B). While H3N2 produced high mRNA levels that were predominantly spliced, the H1N1 mRNA levels were low and predominantly unspliced (Fig. 1B). Quantiﬁcation revealed that 92% of the H3N2 mRNAs were spliced, whereas only 41% of the H1N1 mRNAs were spliced. These results suggested that the function of cis-acting, viral RNA elements were affected by the sequence differences between the two viruses.

To further support the observation that H1N1 segment 8 mRNAs were inefficiently spliced compared to H3N2 segment 8 mRNAs, we expressed segment 8 of both viruses in the RNA polymerase I-based expression system (Neumann and Kawaoka, 2001; Neumann et al., 2000). In this expression system, a negative segment 8 RNA is produced by cellular RNA polymerase I in transfected cells, and the positive mRNA strand is produced by the influenza virus polymerase expressed from cotransfected plasmids. In this case, the polymerase genes were derived from influenza virus A/WSN/33 (H1N1) (Neumann and Kawaoka, 2001; Neumann et al., 2000). Polyadenylated cytoplasmic RNA from 293T cells transiently transfected with plasmids encoding the NS segment from A/Brevig Mission/1918/1 (H1N1) (pH1N1) or A/Netherlands/178/95 (H3N2) (Rimmelzwaan et al., 2007) viruses was extracted from HeLa cells and cytoplasmic RNA was extracted. Northern blot analysis revealed striking differences in NS RNA levels as well as in the splicing efficiency between these two viral sequences (Fig. 1B). While H3N2 produced high mRNA levels that were predominantly spliced, the H1N1 mRNA levels were low and predominantly unspliced (Fig. 1B). Quantiﬁcation revealed that 92% of the H3N2 mRNAs were spliced, whereas only 41% of the H1N1 mRNAs were spliced. These results suggested that the function of cis-acting, viral RNA elements were affected by the sequence differences between the two viruses.

![Fig. 1](image-url) (A) Schematic representations of CMV-promoter-driven plasmids pCNH3N2 and pCNH1N1 encoding the NS protein coding region of A/Netherlands/178/95 (H3N2) (Rimmelzwaan et al., 2007) and A/Brevig Mission/1918/1 (H1N1) (Tumpey et al., 2005), respectively. NS1 and NS2 open reading frames are indicated. Positions of 5′-splice (SD54) sites, 3′-splice sites (SA527), and the HPV-16 late polyA signal (pA) are indicated. Structures of mRNAs produced by the plasmids are indicated. (B) Northern blot of cytoplasmic RNA extracted from HeLa cells transfected in duplicates with pCNH3N2 or pCNH1N1. 18S ribosomal RNA is shown as a loading control.
(H3N2) (pH3N2) was analysed by Northern blot (Fig. 2A). Similarly to the results described above, differences in splicing efficiency were observed. While pH1N1 primarily produced unspliced NS1 mRNA, pH3N2 gave rise to high levels of spliced NS2 mRNAs in addition to unspliced NS1 mRNA (Fig. 2B, left panel). The RNAs were also analysed by RT-PCR with primers that detected both NS1 and NS2 mRNAs. Similar results were obtained by the RT-PCR analysis (Fig. 2B, right panel). Detection of the unspliced and spliced influenza virus mRNAs in the transfected cells by Northern blotting or RT-PCR was totally dependent on the transfection of the various expression plasmids as these mRNAs were undetectable in untransfected cells (Fig. 2B). These results supported the conclusion that naturally occurring sequence variations in influenza virus segment 8 could affect the efficiency of segment 8 pre-mRNA splicing.

Analysis of segment 8 mRNA splicing may be complicated by previously described effects of the NS1 protein on RNA processing e.g., inhibition or activation of splicing (Fortes et al., 1994; Garaigorta and Ortin, 2007; Hale et al., 2008; Lu et al., 1994; Muraki et al., 2010). We converted the NS ATG to TAG in plasmids pH1N1 and pH3N2 to prevent expression of the NS1 and NS2 proteins. This resulted in plasmids pH1N1dns and pH3N2dns (Fig. 2A). Analysis of mRNA produced from these plasmids revealed that H3N2 mRNAs were more efficiently spliced than H1N1 mRNAs. Comparing mRNAs produced from plasmids pH1N1 and pH3N2 with the mRNAs produced from the pH1N1dns and

![Fig. 2.](image-url)
To investigate if there were differences in splicing efficiency of segment 8 mRNAs derived from various avian H5N1 isolates, we also expressed segment 8 from two H5N1 isolates from the same CMV-promoter driven expression vector pCL0806 (Collier et al., 2002). mRNA levels and splicing efficiency were monitored from plasmids expressing influenza virus A/Goose/Guangdong/1/96 (H5N1) (Xu et al., 1999) (plasmid pGsGd), or influenza virus A/Hong Kong/483/97 (H5N1) (C, Centers for disease control, prevention, 1997) (plasmid pHn) (Fig. 3A). While A/Goose/Guangdong/1/96 (H5N1) is of avian origin (Xu et al., 1999), A/Hong Kong/483/97 was isolated from a human individual that had been infected with highly pathogenic avian influenza virus (C, Centers for disease control, prevention, 1997). Analysis of cytoplasmic RNA isolated from 293T cells transfected with either pHn or pGsGd revealed that high levels of mRNAs were produced from pHn, whereas low levels of mRNAs were expressed from pGsGd (Fig. 3B). The mRNAs produced from pGsGd were less efficiently spliced than mRNAs produced from pHn. In addition, H5N1 segment 8 pre-mRNA derived from A/Hong Kong/483/97 was more efficiently spliced than H1N1 segment 8 mRNAs in the RNA polymerase I expression system (Fig. 2B, right panel). We concluded that differences in mRNA levels and splicing efficiencies were present also among various H5N1 influenza A virus isolates.

We observed that a large number of nucleotide differences (8-nucleotide positions) between pHn (A/Hong Kong/483/97) and pGsGd (A/Goose/Guangdong/1/96) were located in a short region (27 nucleotides) between the NS ATG and the GT-dinucleotide of the 5′-splice site (SD54) (Fig. 3B). As these nucleotide differences were positioned close to the major 5′-splice site of the NS segment, we investigated if this sequence-heterogeneity affected splicing efficiency. A hybrid between pHn and pGsGd was generated in which the 27-nucleotide sequence from pGsGd replaced the corresponding sequence in the pHn plasmid, thereby creating pGs (Fig. 3A). Analysis of several experiments of hybrid plasmid pGs in parallel with pHn and pGsGd plasmid by Northern blotting, revealed that insertion of the 27 nucleotides from pGsGd into pHn resulted in an intermediate phenotype (see pGs, Fig. 3C). These results further supported the conclusion that natural sequence variation in influenza virus strains could affect mRNA levels and splicing efficiency of influenza A virus segment 8-derived mRNAs.

**Inefficient splicing of A/Brevig Mission/1918/1 (H1N1) segment 8 mRNAs yielded higher levels of functionally active NS1 protein**

We wished to investigate if poor splicing of the A/Brevig Mission/1918/1 (H1N1) derived segment 8 mRNAs gave rise to more NS1 protein than segment 8 mRNAs from H3N2/Netherlands/1995 as NS1 has been implicated in A/Brevig Mission/1918/1 (H1N1) pathogenesis (Billharz et al., 2009). Western blot analysis failed to reproducibly detect NS1 protein from both A/Brevig Mission/1918/1 (pCNH1N1) and H3N2/Netherlands/1995 (pCNH3N2), probably due to low expression levels (not shown). We therefore developed a more sensitive, functional assay for NS1 expression, which monitors the previously described inhibitory effect of NS1 protein on polyadenylation (Nemeroff et al., 1998). The pE5-Luc expression plasmid contains two HPV-16 polyA signals and is efficiently polyadenylated at the upstream pAE, resulting in E5 mRNA (Fig. 4A). If polyadenylation is inhibited, read-through into the luciferase gene occurs and luciferase is expressed. To demonstrate that this assay detects functional NS1 protein, we first cotransfected the pE5-Luc expression plasmid (Fig. 4A) with CMV-driven influenza virus NS1 expression plasmids.
designed not to splice and to produce high levels of FLAG-tagged NS1 protein, as detected by Western blotting (Fig. 4B). Luciferase production was induced in a dose dependent manner up to 12.5 ng of transfected NS1 plasmid per 35 mm plate (Fig. 4C). Analysis of the luciferase levels from cells transfected with pE5Luc and the FLAG-tagged NS1 expression plasmids revealed that A/Brevig Mission/1918/1 (pFNS1-H1N1) enhanced luciferase expression 18-fold, A/Netherlands/178/95 (pFNS1-H3N2) 17-fold, while A/Hong Kong/483/97 (pFNS1-H5N1) type failed to inhibit polyadenylation and induce luciferase expression (Fig. 4D). The failure of pFNS1-H5N1 to enhance luciferase activity was expected, as it has previously been shown not to interact with polyadenylation factor CPSF-30, and not to inhibit polyadenylation (Nemeroff et al., 1998). Overexpression of CPSF-30 in addition to NS1 protein, restored luciferase levels, confirming an effect of NS1 on polyadenylation (data not shown). We concluded that the polyadenylation assay was specific for NS1-mediated inhibition of polyadenylation and that the ability of NS1 from A/Brevig Mission/1918/1 (H1N1) and from A/Netherlands/178/95 (H3N2) to inhibit polyadenylation was similar.

We next tested the ability to inhibit polyadenylation and induce luciferase expression of plasmids that produced segment 8 mRNAs that could be spliced. The results revealed that H3N2/Netherlands/178/95 (pCNH3N2) induced luciferase five-fold, whereas A/Brevig Mission/1918/1 (pCNH1N1) caused an 11-fold increase in luciferase expression (Fig. 4D). This effect was seen over a range of concentrations of transfected NS1 plasmids (Fig. 4E). Segment 8 of A/Hong Kong/483/97 (pPhn) failed to induce luciferase as expected (Fig. 4D). We concluded that the poorly spliced segment 8 mRNAs from A/Brevig Mission/1918/
As it has been shown previously that SR proteins are involved in the regulation of influenza virus RNA splicing (Shih and Krug, 1996), we wished to investigate if SR proteins differently affected splicing of the various influenza viruses analysed here. Plasmids expressing ASF/SF2, SRp30c and SRp40 were cotransfected with plasmid pGs that expresses H5N1 mRNAs. The levels of unspliced and spliced mRNAs were monitored by Northern blotting of cytoplasmic RNA extracted from transfected HeLa cells. Surprisingly, overexpression of all three SR proteins individually inhibited splicing of the NS mRNAs (Fig. 5A). In parallel experiments in the lab, we have shown that SRp40 enhances HIV-1 tat mRNA splicing (Tranell et al., 2010), that SRp55 inhibits HIV-1 vpr splicing (Tranell et al., 2010) and that SRp30c enhances HPV-16L1 mRNA splicing (Somberg et al., 2011) (data not shown). The inhibitory effect of these SR proteins on influenza virus H5N1 segment 8 mRNA splicing was therefore striking.

Overexpression of SRp40 and ASF/SF2 appeared to inhibit splicing and to reduce the overall levels of influenza virus mRNAs. This is likely an effect of less efficient nuclear export of unspliced viral mRNAs. In contrast, SRp30c increased the levels of unspliced influenza virus mRNAs in the cytoplasm, suggesting that SRp30c might aid in nuclear export of influenza virus mRNAs (Fig. 5A and B). As the function of SR proteins in nuclear mRNA export requires an intact RS-domain (Cazalla et al., 2002; Huang and Steitz, 2001), we investigated the effect on the influenza virus mRNAs of a mutant SRp30c, named SRp30cDRS, that lacks the RS-domain but contains intact RNA-binding domains. The results revealed that similarly to wt SRp30c, SRp30cDRS inhibited influenza virus mRNA splicing (Fig. 5B), but in contrast to SRp30c, SRp30cDRS failed to induce high levels of influenza virus mRNAs in the cytoplasm (Fig. 5B). These results suggested that SRp30c may regulate influenza virus mRNA splicing as well as assisting in further processing or transport of the unspliced influenza virus mRNAs to the cytoplasm.

Next we investigated if H1N1 and H3N2 influenza viruses responded in a similar manner to H5N1 to the overexpression of SR proteins. We first analysed the number of predicted ASF/SF2, SRp40, SC35 and SRp55 binding sites in segments 7 and 8 of A/H3N2/Netherlands/178/95 (H3N2), A/Brevig Mission/1918/1 (H1N1) and A/Hong Kong/483/97 (H5N1) using the ESE-finder program (Cartegni et al., 2003). While H3N2/Netherlands/178/95 (H3N2) stood out by having fewer predicted ASF/SF2 sites in segment 7 than A/Brevig Mission/1918/1 (H1N1) and A/Hong Kong/483/97 (H5N1), A/Hong Kong/483/97 (H5N1) had more potential SRp40 sites than A/Brevig Mission/1918/1 (H1N1) and A/H3N2/Netherlands/178/95 (H3N2) in segment 8. However, differences in number of potential SR-protein binding sites that correlated with splicing efficiency could not be detected.

To experimentally investigate the effect of SR proteins on the splicing of segment 8, we overexpressed ASF/SF2 or SRp40 with segment 8 mRNAs from H5N1, H1N1 and H3N2. Results revealed that H5N1 and H3N2 derived mRNAs responded to SR protein overexpression in a similar manner, both produced less spliced mRNA and the levels of mRNA accumulated were lower. However, H1N1 appeared unresponsive to ASF/SF2 and SRp40 (Fig. 5 C and D). Similar levels of unspliced H1N1 NS mRNAs were detected in the absence or presence of ASF/SF2 or SRp40 (Fig. 5 C and D). We concluded that inefficient splicing of H1N1 mRNAs could not be further inhibited by overexpression of the SR proteins, suggesting that H1N1 mRNA splicing was under control of other cellular factors, or that H1N1 mRNAs lack one or more RNA elements that might be targeted by ASF/SF2 and SRp40. These results further supported the conclusion that splicing of influenza virus H1N1 mRNAs is differently regulated compared to splicing of H5N1 and H3N2 mRNAs.
Poor splicing efficiency of influenza virus segment 7 mRNAs derived from A/Brevig Mission/1918/1 (H1N1)

If poor splicing efficiency was an inherent property of A/Brevig Mission/1918/1 (H1N1) mRNAs, segment 7 and 8 mRNAs should both be poorly spliced. To test this we constructed CMV-driven segment 7 expression plasmids. To this end, we first PCR amplified segment 7 from A/Brevig Mission/1918/1 (H1N1) (Reid et al., 2002; Tumpey et al., 2005) and from A/Netherlands/178/95 (H3N2) (H3N2) (Rimmelzwaan et al., 2007), from the common M1- and M2-ATG in the 5′-end of the segment to the stop codon of M2 (Fig. 6A). The PCR products were inserted into the CMV-driven pCL086 plasmid vector (Collier et al., 2002), and the plasmids were named pCMH1N1 and pCMH3N2 (Fig. 6A).

Northern blot analysis of cytoplasmic RNA extracted from mock-transfected 293T cells revealed that segment 7 mRNAs from all three influenza virus isolates are poorly spliced (Fig. 7). In contrast, H3N2 segment 7 mRNAs were highly expressed and were predominantly in the form of spliced mRNA (Fig. 6B, lanes 5 and 6). These results were similar to those obtained with segment 8 mRNAs (Fig. 6B, lanes 1–4). We concluded that inefficient splicing is an inherent property of mRNAs derived from A/Brevig Mission/1918/1 (H1N1).

Segment 7 mRNAs derived from highly pathogenic avian flu H5N1 are more efficiently spliced than segment 7 mRNAs derived from A/Brevig Mission/1918/1 (H1N1)

We also PCR amplified and subcloned segment 7 sequences derived from the avian influenza virus A/Hong Kong/483/97 (H5N1) (C. Centers for disease control, prevention. 1997), resulting in plasmid pCMH5N1 (Fig. 6A). This plasmid was transfected into 293T cells in parallel with pCMH1N1 and pCMH3N2 and cytoplasmic mRNA levels and splicing efficiency of the influenza virus mRNAs were determined by Northern blotting (Fig. 6C). The RNAs derived from A/Hong Kong/483/97 (pCMH5N1) were more efficiently spliced than RNAs derived from A/Brevig Mission/1918/1 (pCMH1N1), but less efficiently spliced than those from A/Netherlands/178/95 (pCMH3N2) (Fig. 6C). We found that segment 7 mRNAs from all three influenza virus isolates were spliced with different efficiency. Interestingly, mRNAs from pCMH1N1 were the most poorly spliced mRNAs. Therefore, both segment 7 and segment 8 mRNAs derived from A/Brevig Mission/1918/1 (H1N1) are less efficiently spliced than mRNAs from all other influenza viruses tested, further supporting the conclusion that inefficient splicing is an inherent property of A/Brevig Mission/1918/1 (H1N1).

Segment 7 mRNAs derived from the A/Brevig Mission/1918/1 (H1N1) isolate are poorly spliced in both human and avian cells

If the A/Brevig Mission/1918/1 (H1N1) virus is derived from an avian host, it might be better adapted to avian cells (Reid et al., 2004), producing higher mRNA levels that are more efficiently spliced in these cells than in human cells, perhaps displaying a phenotype resembling A/Netherlands/178/95 (H3N2) in human cells. We therefore transfected the avian QT6 cell line, which originates from Japanese quail (Lee et al., 2008; Moscovici et al., 1977), with plasmids expressing segment 7 mRNAs of A/Brevig Mission/1918/1 (pCMH1N1), A/Netherlands/178/95 (pCMH3N2) or A/Hong Kong/483/97 (pCMH5N1). Analysis of cytoplasmic RNA extracted from these cells revealed that A/Netherlands/178/95 (H3N2) segment 7 mRNAs were efficiently spliced, whereas A/Brevig Mission/1918/1 (H1N1) segment 7 mRNAs were not (Fig. 7). Like in HeLa cells and 293T cells, H5N1 displayed an intermediate
Virus RNA levels, revealed that high levels of H1N1 unspliced mRNAs are less abundant and less efficient than those produced with segment 8 mRNAs (data not shown). The A/Brevig Mission/1918/1 (H1N1) derived mRNAs were both less abundant and less efficiently spliced than the A/Netherlands/178/95 (H3N2) derived RNAs, we wished to determine if differences in mRNA levels could be explained by subcellular mRNA distribution. Fractionation of 293T cells transfected with pCMH1N1, pCMH3N2, or pCMH5N1 followed by analysis of influenza virus mRNA levels in the nuclear and in the cytoplasmic fractions compared to total influenza virus RNA levels, revealed that high levels of H1N1 unspliced mRNAs were trapped in the nucleus (Fig. 8). Analysis of total RNA from cells transfected with plasmids expressing segment 8 mRNA yielded similar results to those obtained with segment 7 mRNAs (data not shown). The low levels of cytoplasmic mRNAs produced by the influenza virus A/Brevig Mission/1918/1 expression plasmid pCMH1N1 could therefore be explained by the entrapment of the poorly spliced mRNAs in the nucleus.

Discussion

The NS1 protein has been implicated in influenza virus pathogenesis as it is an inhibitor of interferon synthesis at an early stage of the viral infection (Hale et al., 2008). As such, NS1 may pave the way for efficient replication and quick transmission of virus between cells at the initial phase of the infection. Although it was shown that insertion of A/Brevig Mission/1918/1 (H1N1) segment 8 in the background of another H1N1 virus attenuated the parental virus in mice (Basler et al., 2001), it has also been shown that NS1 from A/Brevig Mission/1918/1 (H1N1) is an efficient inhibitor of interferon responses in human cells. One may therefore speculate that inefficient splicing of segment 8 mRNAs would result in production of high levels of NS1 protein after infection with A/Brevig Mission/1918/1 (H1N1), and that these high levels initially would offer this virus a longer time-frame of replication prior to debilitating antiviral responses of the host.

It has been estimated that only a small fraction of the influenza virus mRNAs are spliced (10%) (Palese and Shaw, 2006), and that influenza virus segment 7 and 8 mRNAs produced from cDNA expression plasmids are spliced with a different efficiency than mRNAs produced during infection (Lamb and Lai, 1980; Lamb and Lai, 1982; Lamb et al., 1981). If that is a true estimate of the ratio of spliced to unspliced, translationally competent influenza virus mRNAs in the cytoplasm in vivo in an infected individual, inefficient splicing would have a higher impact on the production of the proteins produced from spliced mRNAs, the M2 and NS2 proteins, than on the expression levels of NS1 and M1. We show in this paper that the splicing efficiency of segment 7 and 8 mRNAs from different influenza viruses is different. This could potentially affect virus replication and/or pathogenesis. The plausibility that viruses with great differences in the splicing efficiency still would
be viable, is supported by the observation that influenza viruses in which the 5′-splice site of segment 7 has been mutationally inactivated failed to produce M2 protein, but replicated in vitro (Cheung et al., 2005). M2 production is therefore not essential for virus replication in cell culture, but the mutant viruses displayed an attenuated phenotype (Cheung et al., 2005). NS2 is involved in the nuclear export of vRNAs (O’Neil et al., 1998). Influenza virus replication may therefore be sensitive to splicing efficiency of segment 8 mRNAs and the levels of NS2 expression (Wolschek et al., 2011). Although we have shown that cis-acting RNA sequences determine the splicing efficiency of influenza virus segment 7 and 8 mRNAs, it has been previously reported that the viral polymerase may act in trans to regulate splicing of the influenza virus mRNAs (Shih et al., 1995). Therefore, splicing of influenza virus segment 7 and 8 mRNAs are regulated by both cis-acting elements and trans-acting factors. In addition, segment 7 mRNAs encode a second 5′-splice site generating an mRNA named mRNA3 (Jackson and Lamb, 2008; Lamb and Lai, 1980; Lamb and Lai, 1982; Lamb et al., 1981; Shih et al., 1995). However, mRNA3 does not appear to be required for replication of influenza virus in vitro (Jackson and Lamb, 2008).

The origin of the A/Brevig Mission/1918/1 (H1N1) virus is still debated (Reid et al., 2004). The cloned and sequenced virus was obtained from the second wave of the pandemic in the autumn of 1918 and is therefore

Fig. 9. (A) Schematic representations of pCMH1N1, pCMH3N2, and of the hybrids between segment 7 sequences from H1N1/Brevig Mission/1918/1 (Tumpey et al., 2005) and A/Netherlands/178/95 (H3N2) (Rimmelzwaan et al., 2007). The names of the CMV-promoter-driven plasmids encoding the hybrids are indicated to the left. pCMH1N1 and pCMH3N2 encode the M protein coding region of A/Brevig Mission/1918/1 (H1N1) (Tumpey et al., 2005) and A/Netherlands/178/95 (H3N2) (Rimmelzwaan et al., 2007), respectively. Percentage spliced M mRNA in the blot was calculated as described in Materials and methods. Percentage spliced mRNA product is indicated to the right of each plasmid hybrid. (B) Northern blot on cytoplasmic RNA extracted from HeLa cells transfected with pCMH3N2, pCMH1N1, or the various segment 7 hybrid plasmids. 18S ribosomal RNA is shown as a loading control. (C) Percentage spliced mRNA produced from the various segment 7 hybrids plotted against percentage of sequences derived from H1N1.
believed to represent the more pathogenic virus that arose after the summer in 1918 (Tumpey et al., 2005), as a result molecular changes of the virus as it replicated in its human host. The sequence of the less pathogenic 1918 virus of the first wave is unknown, as is its true origin. Our observation that segments 7 and 8 from A/Brevig Mission/1918/1 (H1N1) are both inefficiently spliced suggests a common origin of these two segments. It appears that segment 7 has properties that suggest an avian origin (Reid et al., 2002), but with signs of adaptation to a human host, whereas the origin of segment 8 has been more difficult to deduce (Basler et al., 2001). However, the splicing efficiency of the two A/Brevig Mission/1918/1 (H1N1) derived segments was equally inefficient in avian QT6 cells as in human HeLa cells. In addition, splicing of the A/Hong Kong/483/97 (H5N1) virus of avian origin (C, Centers for disease control, prevention, 1997), although isolated from a human patient, was equally efficient in HeLa and QT6 cells. Inefficient splicing of both segment 7 and 8 mRNAs of influenza virus H1N1-1918, and efficient splicing of the corresponding H3N2-1995 mRNAs, may reflect an adaptation of each virus to different intracellular environments, or may be purely incidental. However, there are properties of avian species that are not taken into account in these experiments, such as the higher body temperature of birds and the replication of avian viruses in mucosal cells of the gut in their natural host (Kalthoff et al., 2010).

Poor splicing efficiency of the RNA segments derived from A/Brevig Mission/1918/1 (H1N1) is best described as the absence or presence of RNA elements, or binding sites for cellular RNA-binding proteins that regulate mRNA splicing (Blencowe, 2000; Cartegni et al., 2002). The SR-protein- and the hnRNP-families represent well-known splicing factors (Graveley, 2000; Long and Caceres, 2009; Shepard and Hertel, 2009; Tautenberger, 2006; Zhong et al., 2009). Although not studied in great detail, it is established that the relative concentrations of these factors differ between tissues (de la Grange et al., 2010; Grosso et al., 2008)[www.proteinatlas.org], as well as between healthy and diseased tissue (Fackenthal and Godley, 2008: Fay et al., 2009) (www.proteinatlas.org). Autopsies of patients who succumbed to the second wave of the Spanish flu pandemic showed no signs of systemic spread of the infection, or virus in any other tissue than the airways. It is reasonable to speculate that various cells in the upper or lower respiratory tract may display different SR-protein or hnRNP-profiles, or other RNA-binding protein expression profiles. Consequently, these cells would differ in their ability to accommodate influenza viruses at the different compartments of the airways. High levels of both ASF/FS2 and SRp55 are detected in the respiratory epithelial cells in nasopharynx and bronchus, as well as in pneumocytes and macrophages in the lung (www.proteinatlas.org). In contrast, SRp40 is undetectable in the pneumocytes and macrophages of the lung, whereas high expression is observed in nasopharynx and bronchus (www.proteinatlas.org). The only antiserializer available to SRp30c, to our knowledge, does not work in immunohistochemistry and we have not seen stainings of airway epithelia for SRp30c expression published. However, our findings reported here, show that splicing of A/Brevig Mission/1918/1 (H1N1)- and A/Netherlands/178/95 (H3N2)-mRNAs respond differently to ASF/FS2, SRp30c and SRp40 overexpression, and the observation that ASF/FS2 is highly expressed in the lower respiratory tract, while SRp40 is not, indicates that the interactions between viral RNA and cellular splicing factors may affect replication of influenza viruses differently. More importantly, it may determine where in the airways various influenza viruses would be able to replicate.

A total of 37 nucleotide positions, relatively evenly distributed between positions +250 and +690 (where A in the M ATG is +1) in segment 7, differ between H1N1 and H3N2. One or more of these changes are likely to affect binding sites for cellular splicing factors. The analysis of potential binding sites for different SR proteins in the ESE finder program (Cartegni et al., 2003), did not uncover consistent differences among the various influenza viruses, although H3N2 segment 7 was predicted to contain fewer ASF/FS2 sites than H1N1. This was not the case for segment 8. We also subjected radiolabelled segment 7 RNA from position 250 to 690 from A/Brevig Mission/1918/1 (H1N1) influenza virus and A/Netherlands/178/95 (H3N2) to UV cross-linking to nuclear extract, but we did not identify differences in profiles of proteins cross-linking to these RNAs (data not shown). However, it should be said that UV-cross-linking is better suited to detect differences in short RNA segments, and cross-linking of these segment 7 RNAs of 440 nucleotides may therefore have failed to detect differences in interactions with RNA binding proteins. Furthermore, the structure of the influenza virus RNA may change as a result of the sequence polymorphism (Gultyaev et al., 2007). Such changes may also affect the functionality of the viral RNA. The various influenza virus segment 7 and 8 expression plasmids lack the UTR sequences and we cannot exclude that they affect splicing efficiency as previously reported (Shih et al., 1995).

**Materials and methods**

**Plasmids**

To construct pCMH1N1, primers H1N1MS and H1N1MA were used to PCR-amplify segment 7 coding sequences from A/Brevig Mission/1/1918 (Reid et al., 2002; Tumpey et al., 2005). To construct pCMH3N2, primers H3N2MS and H3N2MA were used to PCR-amplify segment 7 coding sequences from A/Netherlands/178/95 (Rimmelzwaan et al., 2007). To construct pCMH5N1, primers H5N1MS and H5N1MA were used to PCR-amplify segment 7 coding sequences from A/Hong Kong/483/97. To construct pCNH1N1, primers H1N1NS and H1N1NA were used to PCR-amplify segment 8 coding sequences from A/Brevig Mission/1/1918 (H1N1) (Basler et al., 2001; Tumpey et al., 2005). To construct pCNH3N2, primers H3N2NS and H3N2NA were used to PCR-amplify segment 8 coding sequences from A/Netherlands/178/95 (H3N2) (Rimmelzwaan et al., 2007). To construct pGs, primers H5N1NS and H5N1NA were used to PCR-amplify segment 8 coding sequences from A/Hong Kong/483/97 (H5N1) (C, Centers for disease control, prevention, 1997). To construct pfn, primers H5N1NHns and H5N1NHnna were used to PCR-amplify segment 8 coding sequences of A/Hong Kong/483/97 (H5N1) (C, Centers for disease control, prevention, 1997). The PCR-amplified sequences listed above were separately digested with BssHII and Xhol and inserted into pCL0806 (Collier et al., 2002). To construct pGSD, the coding region of A/Goose/Guangdong/1/96 (H5N1) (Xu et al., 1999) was first synthesised in its entirety by GenScript, followed by digestion with BssHII and Xhol and transfer to pCL0806 (Collier et al., 2002). The segment 7 H1N1–H3N2 hybrids were generated by PCR-mutagenesis using the following primers for each plasmid: p1011 (H1N1MS, FMA1, FMA2 and H3N2MA), p1213 (H1N1MS, FMA2, FMA2 and H3N2MA), p1415 (H3N2MS, FMS3, FMA3 and H1N1MA), p1617 (H3N2MS, FMS4, FMA4 and H1N1MA). The resulting PCR-fragments were digested with BssHII and Xhol and transferred to plasmid pCL0806 (Collier et al., 2002).

A Quick Change II PCR-mutagenesis kit (Stratagene) was used to convert the NS ATG to TAA in pH3N2 and pH1N1, thereby creating plasmids pH3N2ds and pH1N1ds, respectively. pNS1F-H5N1, pNS1F-H1N1 and pNS1F-H3N2 were made by introducing mutations at the 3′ splice site (splice acceptor), and mutations to destroy the pyrimidine tract to avoid NS2 splicing using the Quick Change II PCR mutagenesis method (Stratagene) with primers H1N1d3pSSrev and H1N1d3pSSforw and H3N2d3pSSrev and H3N2d3pSSforw (Table 1). The resulting plasmids were subjected to further PCR-mutagenesis to insert a FLAG-tag at the C-terminus of NS1 using primers CMVs in combination with either H1N1NS1Frev or H3N2NS1Frev.

To construct pE5Luc, the HPV-16 E5 coding sequence, early UTR and the early polya signal pAE were PCR amplified with primers Sense1 and ESAs and inserted into pBELM (Zhao et al., 2004) digested...
with Sall and BamHl. A PCR fragment containing poliovirus internal ribosome entry site followed by the luciferase coding sequence was inserted as a BamHI–XhoI fragment, resulting in pE5Luc.

Plasmids encoding influenza virus segment 7 and 8 sequences were generously provided by the following researchers: Dr. R. Fouchier, Erasmus Medical Center, The Netherlands, (A/Netherlands-178-95 (H3N2)) [Rimmelzwaan et al., 2007], Dr. A. Garcia-Sastre, Mount Sinai School of Medicine, USA (A/Brevig Mission/1/1918 (H1N1)) [Tumpey et al., 2005], Dr. Y. Kawaoka, University of Wisconsin-Madison, USA (A/Hong Kong/483/97 (H5N1)) [C, Centers for disease control, prevention, 1997]. The coding region of influenza virus A/Goose/Guangdong/1/96 (H5N1) (Xu et al., 1997) segment 8, from the NS ATG to the stop codon of NS2, was synthesised by GenScript. Primer sequences were: forward (5′-ATATGACTTCACATCTGCTGTCCTGTTTGGAATACCTTGTGAGCCTTTGCAGC-3′) and reverse (5′-ATATGACTTCACATCTGCTGTCCTGTTTGGAATACCTTGTGAGCCTTTGCAGC-3′) of NS2. Primer sequences for NS1 were: forward (5′-ATATGACTTCACATCTGCTGTCCTGTTTGGAATACCTTGTGAGCCTTTGCAGC-3′) and reverse (5′-ATATGACTTCACATCTGCTGTCCTGTTTGGAATACCTTGTGAGCCTTTGCAGC-3′) of NS1.

Reverse transcription (RT)-PCR

200 ng of cytoplasmic RNA was reverse transcribed at 42 °C using 1 ml TRI Reagent solution (Ambion) for each 60 mm plate with cells, according to the manufacturers’ protocol. Cytoplasmic RNA was isolated after fractionation of transfected cells with isoB/NP40 buffer (10 mM Tris pH 7.9, 0.15 M NaCl, 1.5 mM MgCl2, 0.5% NP40), according to standard protocol. Nuclear RNA was isolated in 1 ml TRI Reagent solution (Ambion) from the nuclear pellet obtained in the isoB/NP40 fractionation step. Oligo-dT beads (Ambion) were used to extract polyadenylated cytoplasmic RNA according to the manufacturers protocol.

Northern blotting was carried out by size separation of 10 μg cytoplasmic RNA on 1.2% agarose gels containing 2.2 M formaldehyde followed by transfer over night to nitrockcellulose filters. Filters were hybridised to radiolabelled probe over night at room temperature. DNA probes against CMV promoter derived sequences (CMV-promoter driven plasmids) or NS coding sequences (RNA polymerase I plasmids) were radiolabelled with [α-32P]dCTP using a Decaprime kit (Ambion) or with Ready-to-lable beads (GE-Health Care). To calculate percentage splicing, we divided the amount of radioactivity (determined by phosphorimager PharoX (Biorad) using Quantity One) in the band representing spliced mRNA with the sum of the mRNA and multiplied with the factor 100.

RNA extraction, Northern blotting and radiolabelled DNA probe synthesis

Isolation of total RNA was performed at 24 h posttransfection using 1 ml TRI Reagent solution (Ambion) for each 60 mm plate with cells, according to the manufacturers’ protocol. Cytoplasmic RNA was isolated after fractionation of transfected cells with isoB/NP40 buffer (10 mM Tris pH 7.9, 0.15 M NaCl, 1.5 mM MgCl2, 0.5% NP40), according to standard protocol. Nuclear RNA was isolated in 1 ml TRI Reagent solution (Ambion) from the nuclear pellet obtained in the isoB/NP40 fractionation step. Oligo-dT beads (Ambion) were used to extract polyadenylated cytoplasmic RNA according to the manufacturers protocol.

Northern blotting was carried out by size separation of 10 μg cytoplasmic RNA on 1.2% agarose gels containing 2.2 M formaldehyde followed by transfer over night to nitrockcellulose filters. Filters were hybridised to radiolabelled probe over night at room temperature. DNA probes against CMV promoter derived sequences (CMV-promoter driven plasmids) or NS coding sequences (RNA polymerase I plasmids) were radiolabelled with [α-32P]dCTP using a Decaprime kit (Ambion) or with Ready-to-lable beads (GE-Health Care). To calculate percentage splicing, we divided the amount of radioactivity (determined by phosphorimager PharoX (Biorad) using Quantity One) in the band representing spliced mRNA with the sum of the mRNA and multiplied with the factor 100.

Reverse transcription (RT)-PCR

200 ng of cytoplasmic RNA was reverse transcribed at 42 °C using Superscript II and random hexamers according to the manufacturers protocol (Invitrogen). 2 μl of cDNA was amplified in a 100 μl reaction with primers All_NS_FP (5′-GAATTCAGACAAAGCGGTTGACA AAGACATAATGGAT-3′) and All_NS_Rev (5′-AGTAGAAACAGGGGT TTTTATCCATTAA-3′) that detect spliced and unspliced segment 8 mRNAs from various influenza virus types. GAPDH cDNA was amplified as control using previously described primers (Johansson et al., 2005).
RNA polymerase I expression system

RNA polymerase I promoter driven plasmids encoding the entire segment 8 of A/Brevig Mission/1/1918 (H1N1) (Tumpey et al., 2005) or A/Netherlands/178/95 (Rimmelzwaan et al., 2007) were cotransfected with four RNA polymerase II driven pCAGGS plasmids expressing either A/WSN/33 influenza virus NP protein, PA, PB1 or PB2, as described previously (Neumann and Kawaoka, 2001; Neumann et al., 2000). Cytoplasmic, polyadenylated RNA was extracted at 24 h post-transfection of 293T cells.

Luciferase assay

100 ng pE5Luc plasmid DNA, NS expressing plasmid as indicated in the figure, and empty pCL0806 plasmid (Collier et al., 2002) (empty vector) up to 500 ng or 1 µg were transfected in 35 mm plates. Cells were collected and washed in 1 mL PBS and lysed in 200 µL lysis buffer (25 mM Tris pH 7.9, 2 mM DTT, 10% glycerol, 1% Triton X-100). Luciferase activity was assayed on 40 µL cell lysate and 50 µL luciferin-assay solution (470 µM Beetle Luciferin-potassium salt (Promega), 530 µM ATP, 270 µM coenzyme A, 33.3 mM DTT, 0.1 mM EDTA, 2.67 mM MgSO4, 1.07 mM MgCl2, 20 mM Tricine pH 7.8).

Western blotting

Western blotting was performed according to the Odyssey protocol (Licor). In brief, cells were harvested in 250 µL RIPA (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40), 60 µg of protein lysate and protein marker Biorad precision+ was separated on Any kD mini-PROTEAN col (Licor). In brief, cells were harvested in 250 µL cell lysate and 90 µL lysis buffer (25 mM Tris pH 7.9, 2 mM DTT, 10% glycerol, 1% Triton X-100). Western blotting was performed according to the Odyssey protocol (Licor). In brief, cells were harvested in 250 µL RIPA (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40), 60 µg of protein lysate and protein marker Biorad precision+ was separated on Any kD mini-PROTEAN col (Licor). In brief, cells were harvested in 250 µL cell lysate and 90 µL lysis buffer (25 mM Tris pH 7.9, 2 mM DTT, 10% glycerol, 1% Triton X-100). Luciferase activity was assayed on 40 µL cell lysate and 50 µL luciferin-assay solution (470 µM Beetle Luciferin-potassium salt (Promega), 530 µM ATP, 270 µM coenzyme A, 33.3 mM DTT, 0.1 mM EDTA, 2.67 mM MgSO4, 1.07 mM MgCl2, 20 mM Tricine pH 7.8).

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