Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3'-end processing machinery

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Influenza A virus NS1 protein (NS1A protein) via its effector domain targets the poly(A)-binding protein II (PABII) of the cellular 3'-end processing machinery. *In vitro* the NS1A protein binds the PABII protein, and *in vivo* causes PABII protein molecules to relocate from nuclear speckles to a uniform distribution throughout the nucleoplasm. *In vitro* the NS1A protein inhibits the ability of PABII to stimulate the processive synthesis of long poly(A) tails catalyzed by poly(A) polymerase (PAP). Such inhibition also occurs *in vivo* in influenza virus-infected cells, where the NS1A protein via its effector domain causes the nuclear accumulation of cellular pre-mRNAs which contain short (~12 nucleotide) poly(A) tails. Consequently, although the NS1A protein also binds the 30 kDa subunit of the cleavage and polyadenylation specificity factor (CPSF), 3' cleavage of some cellular pre-mRNAs still occurs in virus-infected cells, followed by the PAP-catalyzed addition of short poly(A) tails. Subsequent elongation of these short poly(A) tails is blocked because the NS1A protein inhibits PABII function. Nuclear-cytoplasmic shuttling of PABII, an activity implicating this protein in the nuclear export of cellular mRNAs, is also inhibited by the NS1A protein. *In vitro* assays suggest that the 30 kDa CPSF and PABII proteins bind to non-overlapping regions of the NS1A protein effector domain and indicate that these two 3' processing proteins also directly bind to each other.

Keywords: influenza virus NS1 protein/PABII/poly(A) elongation/pre-mRNA 3'-end processing

Introduction

Animal viruses often exploit the machinery that is used for cellular gene expression to promote the selective expression of viral genes. Such exploitations can be mediated by direct interactions between viral and cellular proteins, and these interactions can provide insights into cellular as well as viral functions. A prime example of a viral protein that undergoes functional interactions with host cell proteins is the NS1 protein of influenza A virus (NS1A protein).

The NS1 protein of all naturally occurring influenza A viruses is ~230 amino acids in length (Buonagurio *et al.*, 1986; Norton *et al.*, 1987; Suarez and Perdue, 1998). Two

functional domains have been identified in the NS1A protein: an RNA-binding/dimerization domain at the N-terminus, and an effector domain in the carboxy half (Qian *et al.*, 1994; Nemeroff *et al.*, 1995). One of the functions of the NS1A protein is the inhibition of the nuclear export of mRNAs which contain 3'-poly(A) ends (Alonso-Caplen *et al.*, 1992; Fortes *et al.*, 1994; Qian *et al.*, 1994; Qiu and Krug, 1994; Nemeroff *et al.*, 1998). This inhibition results, at least in part, from the interaction of the effector domain of the NS1A protein with a protein component of the cellular 3'-end processing machinery, the 30 kDa protein subunit of the cleavage and polyadenylation specificity factor (CPSF) (Nemeroff *et al.*, 1998). This mammalian factor, which contains three other protein subunits (160, 100 and 70 kDa), binds to the conserved AAUAAA hexamer which is located 10–30 nucleotides upstream of the cleavage site in cellular pre-mRNAs (reviewed in Wahle and Keller, 1996; Colgan and Manley, 1997; Wahle and Kuhn, 1997).

Five other cellular factors are required for efficient 3'-end processing of cellular pre-mRNAs *in vitro* (reviewed in Wahle and Keller, 1996; Colgan and Manley, 1997; Wahle and Kuhn, 1997). Processing occurs in two steps that are coupled: endonucleolytic cleavage of the pre-mRNA followed by poly(A) addition to the upstream cleavage product. In addition to CPSF, cleavage requires three other factors: the cleavage stimulation factor (CstF), which binds to the U- or G/U-rich element downstream of the cleavage site; and cleavage factors I and II (CFI and CFII). Also, poly(A) polymerase (PAP) is usually required for cleavage. After cleavage, CstF, CFI and CFII leave the processing complex, and PAP catalyzes the addition of a short (~10 nucleotide) poly(A) sequence in a reaction that requires CPSF. Subsequent elongation of this short sequence requires a poly(A)-binding protein, PABII, which joins the 3'-processing complex after the short poly(A) sequence has been added (Bienroth *et al.*, 1993; Wahle, 1995). PABII along with CPSF tethers PAP to the RNA substrate, thereby facilitating rapid, processive poly(A) addition. PABII may also play a role in the nuclear export of mRNA because it shuttles between the nucleus and cytoplasm (M. Carmo-Fonseca, personal communication).

In the present study we demonstrate that the effector domain of the NS1A protein targets another factor of the cellular 3'-end processing machinery: PABII, which joins the 3'-end processing complex after cleavage of the pre-mRNA substrate. As a consequence of this interaction, cellular pre-mRNAs that contain short (~12 nucleotide) poly(A) tails accumulate in the nuclei of influenza virus-infected cells. Our results thus indicate that 3' cleavage of some cellular pre-mRNAs still occurs in infected cells despite the binding of the NS1 protein to the 30 kDa subunit of CPSF (Nemeroff *et al.*, 1998), and that by inhibiting PABII function the NS1A protein blocks the

subsequent elongation of the 3'-short poly(A) tails of these cleaved pre-mRNAs. Thus, the two-pronged attack by the NS1A protein against PABII as well as CPSF is employed to effectively block the 3'-end processing of cellular pre-mRNAs in influenza virus-infected cells.

Results

The cellular PABII protein binds specifically to the effector domain of the influenza virus NS1A protein

Our previous experiments using the yeast two-hybrid interaction trap identified the human 30 kDa CPSF protein as one of the HeLa cell proteins which interacts with the effector domain of the influenza virus NS1A protein (Nemeroff *et al.*, 1998). Continuation of this screen led to the identification of a second human cell protein that interacts with the effector domain of the NS1A protein. Thus, a human cDNA of 750 bp expresses a protein that interacts with the full-length NS1A protein, as shown in β -galactosidase assays (Figure 1A). No detectable interaction of this human protein was observed with an NS1A protein containing a deletion of its effector domain (ΔE , deletion of amino acids 134–161 described in Qian *et al.*, 1994), or with the NS1 protein encoded by influenza B virus (NS1B protein), which lacks a functional effector domain (Wang and Krug, 1996). Sequence analysis of this human cDNA indicated that it encodes the carboxyl region of the human PABII (Braiss *et al.*, 1998). In all subsequent experiments we used the cDNA encoding the entirety of the human PABII protein. This cDNA was obtained by RT-PCR of HeLa cell RNA using PABII-specific primers.

In vitro binding experiments demonstrate that the human PABII protein binds directly to the effector domain of the NS1A protein (Figure 1B). Thus, ^{35}S -labeled NS1A protein (synthesized *in vitro*) binds to a glutathione-S-transferase (GST)–PABII fusion protein immobilized on glutathione agarose beads (lane 2). In contrast, only a very small amount of a labeled NS1A protein containing a deletion of the effector domain (NS1 ΔE) binds to the immobilized GST–PABII fusion protein (lane 3). Similarly, in the complementary experiment, ^{35}S -labeled PABII protein binds to an immobilized GST–NS1A fusion protein (lane 5), but binds very poorly to an immobilized GST–NS1 ΔE fusion protein (lane 6). Micrococcal nuclease digestion has no effect on these binding results (data not shown), indicating that the identified protein–protein interactions are direct and not mediated by RNA.

The NS1A protein via its effector domain inhibits PABII-stimulated poly(A) addition *in vitro*

One function of PABII is the stimulation of the processive synthesis of long poly(A) tails catalyzed by PAP (Bienroth *et al.*, 1993; Wahle, 1995; Wahle and Kuhn, 1997). Although optimal stimulation of PAP by PABII occurs *in vitro* in the presence of CPSF, significant stimulation also occurs in its absence. Of necessity, we assayed PABII-mediated stimulation of poly(A) addition in the absence of CPSF to eliminate any effects due to the interaction of the NS1A protein with the 30 kDa subunit of CPSF.

The substrate for polyadenylation was a precleaved L3 pre-mRNA containing a poly(A) tail of ~30–35 nucleotides. PAP alone adds only a small number of A residues

Fig. 1. Identification of a second human protein that binds specifically to the effector domain of the influenza virus NS1 protein. (A) Yeast two-hybrid results. The indicated NS1 DNA in the Gal4 DNA-binding domain vector (target protein) was cotransformed into yeast with the 750 bp human cDNA in the Gal4 activation domain vector, and transformants were assayed for β -galactosidase activity in liquid assays using Miller units (Miller, 1972). (B) The PABII protein binds directly to the effector domain of the NS1 protein *in vitro*. GST (1 μg) (lane 1) or GST–PABII (1 μg) (lanes 2 and 3) was incubated with 5 μl of ^{35}S -labeled wild-type NS1 protein (20 000 c.p.m.) (lanes 1 and 2) or with 5 μl of ^{35}S -labeled NS1 ΔE protein (20 000 c.p.m.) (lane 3). Conversely, GST (1 μg) (lane 4), GST–NS1 (1 μg) (lane 5), or GST–NS1 ΔE (1 μg) (lane 6) was incubated with 10 μl of ^{35}S -labeled PABII protein (lanes 4–6). All the incubations were carried out in the presence of 20 μl of glutathione–Sepharose 4B beads. The labeled proteins eluted from the beads were analyzed by electrophoresis on a 14% SDS–polyacrylamide gel. The positions of protein molecular weight markers (MW) are shown on the left.

to the 3' ends of this pre-mRNA substrate (Figure 2, compare lanes 1 and 2). In contrast, when the reaction is supplemented with PABII, PAP then synthesizes longer poly(A) tails, heterogeneous in length (lane 3). Addition of increasing amounts of wild-type NS1A protein causes an inhibition of the synthesis of these longer poly(A) chains (lanes 4–8). In contrast, the addition of increasing amounts of NS1A protein molecules containing a deletion of the effector domain (NS1 ΔE) does not significantly inhibit the synthesis of longer poly(A) chains (lanes 9–12). To rule out a direct interaction between the wild-type NS1A protein and PAP, we carried out non-specific poly(A) addition reactions catalyzed by PAP in the presence of MnCl_2 in the absence of PABII (Christofori and Keller, 1989): no inhibition by the NS1A protein was detected (data not shown). Based on these results, we conclude that the interaction of the effector domain of the NS1A

Fig. 2. The NS1 protein via its effector domain inhibits PABII-mediated stimulation of poly(A) addition catalyzed by PAP. PABII (0.3 pmol) was pre-incubated for 10 min at 30°C with the amounts indicated of either the NS1 protein (lanes 3–8) or the NS1ΔE protein (lanes 9–12). After addition of PAP and the RNA substrate (lane 1, S) (pre-cleaved L3 mRNAs containing 30–35 A residues), polyadenylation was initiated by the subsequent addition of ATP. After 4 min of incubation at 37°C, the RNA products were resolved by electrophoresis on 8% polyacrylamide gels containing 7 M urea. Lane 2, the RNA product in the absence of both PABII and the NS1 protein. The positions of marker double-stranded DNAs (M) are shown on the left.

protein with PABII causes the inhibition of PABII-mediated stimulation of poly(A) addition catalyzed by PAP.

The NS1A protein via its effector domain inhibits the nuclear–cytoplasmic shuttling of PABII

The PABII protein is located in the nucleus but shuttles between the nucleus and cytoplasm (M.Carmo-Fonseca, personal communication). To determine whether the NS1A protein inhibits the nuclear–cytoplasmic shuttling of PABII, we employed the interspecies heterokaryon assay (Borer and Lehner, 1989; Guiochon-Mantel *et al.*, 1991; Pinol-Roma and Dreyfuss, 1992; Meyer and Malim, 1994). Transfected Cos (monkey) cells, which either express only PABII containing an influenza virus hemagglutinin (HA) epitope at its C-terminus (PABIIHA) (Figure 3A) or express both PABIIHA and wild-type NS1A protein (Figure 3B), were fused with non-expressing NIH 3T3 (mouse) cells. The nuclei of the NIH 3T3 cells in the resulting heterokaryons could be identified by the presence of characteristic mouse satellite DNA detected by Hoechst 33258 staining (Borer and Lehner, 1989). Any PABIIHA protein that is exported from the Cos (monkey) nuclei would be reimported into the NIH 3T3 nuclei as well as the Cos nuclei (Figure 3, NIH 3T3 nuclei indicated by arrows). Consequently, export of PABIIHA from Cos cell nuclei was assayed by its reimport into the NIH 3T3 nuclei. Reimport, which occurs in the presence of PABII alone (Figure 3A), is blocked by the NS1A protein (Figure 3B). Such inhibition of the nuclear export of PABII by the NS1A protein requires its effector domain (data not shown). We conclude that the interaction of the effector domain of the NS1A protein with PABII blocks its nuclear–cytoplasmic shuttling.

Fig. 3. Interspecies heterokaryon assay: the nuclear export leg of PABII protein shuttling is inhibited by the NS1 protein. Cos (monkey) cells were transfected with the indicated pBC12 plasmids encoding PABIIHA (A) or both PABIIHA and wild-type NS1 protein (B). At 40 h post-transfection, an equal number of mouse NIH 3T3 cells was added. After a 3 h incubation in the presence of CM, cell fusion was induced with 50% PEG. PABIIHA was detected by indirect immunofluorescence, and Hoechst 33258 fluorescence was used to visualize DNA (Borer and Lehner, 1989). The corresponding phase-contrast pictures are presented for the two cultures. NIH 3T3 nuclei in heterokaryons are indicated by arrows. In the cells transfected with the NS1 gene, the NS1 protein was located in the nuclei of only the Cos cells and not the NIH 3T3 cells (data not shown).

The NS1A protein via its effector domain causes the nuclear relocation of the PABII protein

The ability of the NS1A protein to inhibit the nuclear–cytoplasmic shuttling of PABII suggests that the NS1A protein interacts with the PABII protein *in vivo*. To determine whether the NS1A protein and PABII have similar distributions in the cell nucleus, we used indirect immunofluorescence and confocal microscopy (Figure 4). In the absence of the NS1A protein the PABII protein is concentrated in discrete, variably sized regions (Figure 4A, panel 1) (Krause *et al.*, 1994), which presumably correspond to the speckles where splicing factors have been shown to accumulate (Spector, 1993; Singer and Green, 1997). When the NS1A gene is introduced into the cells by transfection, the PABII protein undergoes a dramatic nuclear redistribution: it is no longer concentrated in speckles, but rather spreads out more evenly throughout the nucleoplasm (Figure 4A, panel 2). This distribution is similar to that of the NS1A protein itself, as shown by

Fig. 4. The NS1 protein via its effector domain causes the nuclear relocation of the PABII protein. (A) Cos cells were either not transfected (Control, panel 1), were transfected with a pBC12 plasmid expressing the wild-type NS1 protein (panels 2–4), or were infected with influenza A/Udorn virus for 3.5 h (panels 5–7). Indirect immunofluorescence was used to detect either PABII (panels 1, 2 and 5), the NS1 protein (panels 3 and 6), or both PABII and the NS1 protein (panels 4 and 8), and the cells were examined by confocal microscopy. (B) Cos cells were either transfected with a pBC12 plasmid expressing the NS1ΔE protein (panels 1–3), or were not transfected (Control, panel 4), or were transfected with a pBC12 plasmid expressing the wild-type NS1 protein (panels 5–7). Indirect immunofluorescence was used to detect either PABII (panel 1), the NS1ΔE protein (panel 2), both PABII and the NS1ΔE protein (panel 3), the SC35 splicing factor (panels 4 and 5), the NS1 protein (panel 6), or both SC35 and the NS1 protein (panel 7).

immunostaining with anti-NS1 antiserum (Figure 4A, panel 3) and by overlay of the two confocal images (Figure 4A, panel 4). In addition, in influenza virus-infected cells the PABII protein undergoes a similar

dramatic nuclear redistribution: it is spread out evenly throughout the nucleoplasm rather than localizing in speckles (Figure 4A, panel 5), resulting in a distribution similar to that of the nuclear NS1A protein (Figure 4A, panels 6 and 7).

To determine whether this action of the NS1A protein requires its effector domain, cells were transfected with an NS1 gene encoding the NS1ΔE protein (Figure 4B, panels 1–3). In the presence of the NS1ΔE protein the PABII protein remains in speckles (Figure 4B, panel 1). This distribution differs from that of the NS1ΔE protein itself (Figure 4B, panel 2), as dramatically demonstrated by double immunostaining (Figure 4B, panel 3). We conclude that the NS1 protein via its effector domain causes cellular PABII proteins which are in nuclear speckles to relocate throughout the nucleoplasm.

To determine whether the NS1A protein non-specifically causes the redistribution of other nuclear proteins which are concentrated in speckles, we chose the SC35 splicing factor as a representative speckle-associated cellular protein (Spector, 1993; Singer and Green, 1997). In both the absence (Figure 4B, panel 4) and the presence (Figure 4B, panel 5) of the NS1A protein the SC35 protein remains in speckles. The NS1A protein spreads out more evenly throughout the nucleoplasm (Figure 4B, panel 6), and as shown by overlaying the two confocal images, the distribution of the SC35 protein differs from that of the NS1A protein (Figure 4B, panel 7). In infected cells, SC35 also remains in speckles (data not shown; Wolff *et al.*, 1998). We conclude that the NS1A protein does not disrupt speckles *per se* and does not cause the nuclear redistribution of all speckle-associated nuclear proteins. Conversely, the NS1A protein can interact with cellular nuclear proteins that are not localized in speckles. The 30 kDa CPSF protein that interacts with the NS1A protein (Nemeroff *et al.*, 1998) is not concentrated in speckles in the absence of the NS1 protein, but rather is spread out evenly throughout the nucleoplasm (Figure 4B, panel 8).

In influenza virus-infected cells the NS1A protein blocks PABII function: elongation of the short poly(A) chains of cellular pre-mRNAs is inhibited

Previously we have shown that in cells co-transfected with two plasmids, one encoding the NS1A protein and one encoding a target pre-mRNA, the 3'-cleavage of this pre-mRNA is efficiently inhibited (Nemeroff *et al.*, 1998). These results lead to an apparent puzzle: if 3'-cleavage is already inhibited, then why would the NS1A protein also need to inhibit the function of the PABII protein, which functions in 3'-end processing after cleavage? One possibility is that in influenza virus-infected cells, the 3'-cleavage of some cellular pre-mRNA molecules still occurs because CPSF binds to the AAUAAA sequence of the pre-mRNA despite the binding of the NS1A protein to the 30 kDa subunit of CPSF. Cleavage would be followed by the addition of short (~10 nucleotide) poly(A) sequences to the 3'-ends of these pre-mRNAs. This addition would be catalyzed by PAP in a reaction that requires the interaction of CPSF with PAP (reviewed in Wahle and Keller, 1996; Colgan and Manley, 1997; Wahle and Kuhn, 1997). Subsequent elongation of these short poly(A) sequences requires PABII as well as CPSF (Bienroth *et al.*, 1993; Wahle, 1995; Wahle and Kuhn,

Fig. 5. (A) Schematic of the PCR Poly(A) Test assay for measuring the lengths of the poly(A) sequences on mRNAs and pre-mRNAs. See text for detailed discussion. (B) Influenza virus infection causes the nuclear accumulation of cellular pre-mRNAs containing a short (~12 nucleotide) poly(A) sequence because of the inhibition of PABII function by the NS1 protein. 293 cells were infected with influenza A WSN virus (40 p.f.u./cell) for the indicated times (lanes 1–5 and lanes 7–11), or were infected with influenza A/Turkey/Oregon/71 virus (40 p.f.u./cell) for 6 h (lanes 6 and 12). The cells were fractionated into nucleus (lanes 1–6) and cytoplasm (lanes 7–12), and the lengths of the poly(A) tails on β-actin mRNA and pre-mRNA were determined using the PCR Poly(A) Test assay. The PCR products were analyzed by gel electrophoresis. The lengths of the poly(A) tails on the β-actin mRNAs and pre-mRNAs was estimated by subtracting the molecular weight of the β-actin-specific PCR sequence [lacking poly(A)] from the molecular weight of the double-strand DNA markers run on the same gel. The arrow denotes the position of the short (~12 nucleotide) poly(A) chains on the nuclear β-actin pre-mRNAs in cells infected by influenza A WSN virus.

1997) and hence would be inhibited if the NS1A protein also inhibits the function of PABII.

Our postulate predicts that the NS1A protein in influenza A virus-infected cells would cause the nuclear accumulation of cellular pre-mRNAs containing a short (~10 nucleotide) poly(A) sequence that cannot be elongated. To test this prediction, we determined the lengths of the poly(A) tails on a specific cellular mRNA (β-actin mRNA) in the cytoplasm and on its nuclear pre-mRNA at various times after influenza virus infection.

The length of these poly(A) tails was measured using the PCR Poly(A) Test assay (Salles and Strickland, 1995) (Figure 5A). In this assay, phosphorylated oligo(dT)₁₂₋₁₈ chains that are annealed to the poly(A) tails of the mRNA (and the pre-mRNA) at 42°C are ligated together. The resulting oligo(dT)–poly(A) duplex would be expected to contain a 3'-overhang of oligo(A) that is not annealed to oligo(dT). In the next step this overhang is annealed at a low temperature to an oligo(dT)₁₂ sequence which is linked to an 18 nucleotide anchor (random) sequence. Specifically, a large molar excess of the oligo(dT)₁₂–anchor sequence is annealed to the 3'-oligo(A) overhang at 12°C, and is ligated to the extended oligo(dT) sequence which is already annealed to the poly(A) of the mRNA. The extended oligo(dT) sequence is then used as the primer for the reverse transcriptase copying of the mRNA (and pre-mRNA). This DNA copy is amplified by PCR using as primers both the oligo(dT)₁₂–anchor sequence and an oligonucleotide primer that is identical to an internal β-actin mRNA sequence, and the PCR products are then analyzed by gel electrophoresis.

In uninfected cells (0 time), both cytoplasmic β-actin mRNA and its nuclear pre-mRNA contain heterogeneous poly(A) tails (Figure 5B, lanes 1 and 7). After influenza A virus infection (starting at 2 h post-infection), the lengths of the majority of the poly(A) tails of β-actin pre-mRNAs in the nucleus dramatically decrease, and a predominant poly(A) species that is ~12 nucleotides in length accumu-

lates (Figure 5B, lanes 3–5, denoted by the arrow). The length of these poly(A) tails was verified by sequencing the cloned DNA copy of the predominant β-actin PCR product. Similar short poly(A) tails were found on another cellular pre-mRNA in the nucleus, β-tubulin pre-mRNA, whereas the poly(A) tails on viral mRNAs in the nucleus remain heterogeneous and of large size (data not shown). In contrast to the nuclear β-actin pre-mRNAs, the poly(A) tails of cytoplasmic β-actin mRNAs remain heterogeneous in length during infection (Figure 5B, lanes 8–11). The cytoplasmic poly(A) tails decrease in average size during infection, as would be expected for cytoplasmic mRNAs that are not replenished with new molecules. However, mRNA molecules containing a distinct poly(A) species of ~12 nucleotides in length were not detected in the cytoplasm, indicating that the nuclear pre-mRNAs with such short poly(A) tails are not exported to the cytoplasm.

To establish that the effector domain of the NS1A protein is responsible for the accumulation of short poly(A) tails on nuclear β-actin pre-mRNAs, we infected cells with the laboratory-generated isolate of the influenza A/Turkey/Oregon/71 virus strain that encodes a truncated NS1A protein lacking an effector domain (Norton *et al.*, 1987; Suarez and Perdue, 1998). No detectable accumulation of short (~12 nucleotide) poly(A) tails on nuclear β-actin pre-mRNAs occurs (Figure 5B, lane 6), indicating that such accumulation requires the effector domain of the NS1A protein. We conclude that these short (~12 nucleotide) poly(A) sequences cannot be elongated because the NS1A protein via its effector domain inhibits the PABII protein.

PABII and 30 kDa CPSF bind to non-overlapping regions of the NS1A protein; the NS1A, PABII and 30 kDa proteins form a ternary complex

The effector domain of the NS1A protein binds two cellular proteins which are components of the cellular 3'-end processing system, PABII and 30 kDa CPSF

Fig. 6. PABII and 30 kDa CPSF bind to non-overlapping regions of the NS1 protein; the NS1, PABII and 30 kDa CPSF proteins form a ternary complex. GST–NS1 (1 μ g) was incubated with 10 μ l each of 35 S-labeled PABII and 30 kDa CPSF proteins (lane 1). GST–30 kDa CPSF (1 μ g) (lanes 2–5) was incubated either with 10 μ l of 35 S-labeled NS1 protein (lane 2), with 10 μ l of 35 S-labeled NS1 protein plus 5 μ g of unlabeled purified PABII (lane 3), with 10 μ l of 35 S-labeled PABII (lane 4), or with 10 μ l each of 35 S-labeled PABII and the NS1 proteins (lane 5). GST–PABII (1 μ g) (lanes 6–8) was incubated either with 10 μ l of 35 S-labeled 30 kDa CPSF protein (lane 6), with 10 μ l each of 35 S-labeled 30 kDa CPSF and NS1 proteins (lane 7), or with 10 μ l of 35 S-labeled 30 kDa CPSF plus 5 μ g of unlabeled purified NS1 protein (lane 8). The incubations were carried out in the presence of 20 μ l of glutathione–Sepharose 4B beads, and the labeled proteins eluted from the beads were analyzed by electrophoresis on a 14% SDS–polyacrylamide gel. The positions of protein molecular weight markers (MW) are shown on the left.

(Nemeroff *et al.*, 1998; present study). When both 35 S-labeled PABII and 35 S-labeled 30 kDa CPSF are added together to a GST–NS1A fusion protein immobilized on glutathione agarose beads, both labeled proteins bind to the GST–NS1A protein (Figure 6, lane 1). Because the GST–NS1A protein is in excess, this experiment does not establish whether these two cellular proteins are binding to non-overlapping regions on the same NS1A protein molecule, or are binding to different NS1A protein molecules. To distinguish between these two possibilities, 35 S-labeled NS1A protein was bound to immobilized GST–30 kDa CPSF in the absence (lane 2) and in the presence (lane 3) of an 8-fold molar excess of unlabeled purified PABII protein relative to the GST–30 kDa CPSF protein. This excess unlabeled PABII protein does not reduce the binding of 30 kDa CPSF to the labeled NS1A protein, suggesting that PABII and 30 kDa CPSF bind to non-overlapping regions of the NS1A protein. Individual NS1A protein molecules would then be able to bind simultaneously both PABII and 30 kDa CPSF protein molecules.

Unexpectedly, other binding assays showed that the two cellular 3'-end processing components, the PABII and 30 kDa CPSF proteins, also bind to each other. Thus, 35 S-labeled PABII binds to immobilized GST–30 kDa CPSF both in the absence (Figure 6, lane 4) and in the presence (Figure 6, lane 5) of 35 S-labeled NS1A protein. In addition, 35 S-labeled 30 kDa CPSF binds to immobilized GST–PABII both in the absence (Figure 6, lane 6) and in the presence (Figure 6, lane 7) of 35 S-labeled NS1A protein. The binding of 35 S-labeled 30 kDa CPSF to immobilized GST–PABII is not diminished by the presence of a 10 molar excess of unlabeled NS1A protein relative to the GST–PABII protein (Figure 6, compare lane 8 with lane 6), suggesting that the NS1A and 30 kDa CPSF

proteins bind to non-overlapping regions of the PABII protein. We conclude that *in vitro* the two cellular 3'-end processing factors, PABII and 30 kDa CPSF, and the NS1A protein would all bind to each other to form a ternary complex.

Discussion

We show that the effector domain of the influenza virus NS1A protein targets the PABII protein of the cellular 3'-end processing machinery. PABII functions after 3'-cleavage of the cellular pre-mRNA, and is required for the PAP-catalyzed processive synthesis of long poly(A) tails (reviewed in Wahle and Keller, 1996; Colgan and Manley, 1997; Wahle and Kuhn, 1997). We demonstrate that this activity of the PABII protein is inhibited by the NS1A protein, both *in vitro* and *in vivo*. In influenza A virus-infected cells the NS1A protein via its effector domain causes the nuclear accumulation of cellular pre-mRNAs that contain short (~12 nucleotide) poly(A) sequences. Consequently, despite the binding of the NS1A protein to the 30 kDa subunit of CPSF (Nemeroff *et al.*, 1998), these cellular pre-mRNAs undergo 3' cleavage, followed by the PAP-catalyzed addition of a short (~12 nucleotide) poly(A) sequence to their 3'-ends. However, the elongation of this short poly(A) sequence is inhibited because the activity of PABII is inhibited by the NS1A protein. Hence, the NS1A protein-mediated inhibition of PABII function is needed to efficiently block the 3'-end processing of cellular pre-mRNAs in influenza virus-infected cells. Further, the pre-mRNAs that contain these short poly(A) tails are not exported from the nucleus, indicating that elongated poly(A) chains are needed for nuclear export.

The binding of the viral NS1A protein to PABII may also ensure that cellular mRNAs are not exported to the cytoplasm. PABII protein molecules shuttle between the nucleus and the cytoplasm, an activity that implicates this protein in the nuclear export of cellular mRNAs (M.Carmo-Fonseca, personal communication; present study). In fact, it can be argued that the reason that the 3'-end processing of cellular pre-mRNAs is required for their nuclear export (Eckner *et al.*, 1991; Huang and Carmichael, 1996; Nemeroff *et al.*, 1998) is that such processing produces the poly(A) tails which bind the PABII protein molecules that play an important role in mediating the nuclear export of cellular mRNAs. We show that the NS1A protein inhibits the nuclear export of PABII protein molecules in transfected cells. We presume that this inhibition also occurs in influenza virus-infected cells, particularly in light of our demonstration that in infected cells the NS1A protein inhibits the function of PABII during 3'-end processing.

Our results indicate that the NS1A protein-mediated block in PABII as well as CPSF function is responsible for the block in the nuclear export of cellular mRNAs which occurs in infected cells (Katze and Krug, 1984; Krug *et al.*, 1989; Nemeroff *et al.*, 1998; present study). By blocking nuclear export of cellular mRNAs, influenza A virus via its NS1A protein specifically shuts off cellular gene expression and traps cellular pre-mRNAs in the nucleus, where they would be accessible to the viral cap-dependent endonuclease for the production of the capped

RNA primers that are needed for viral mRNA synthesis (Krug *et al.*, 1989). In contrast, the nuclear export of viral mRNAs in infected cells would not be blocked, because the poly(A) tails of viral mRNAs are not synthesized by the cellular 3'-end processing machinery. Rather, these poly(A) tails are synthesized by the virus-encoded transcriptase, which reiteratively copies a short stretch of U residues in the virion RNA template (Robertson *et al.*, 1981; Krug *et al.*, 1989). Consequently, the NS1A protein would selectively inhibit the nuclear export of cellular, and not viral, mRNAs in influenza A virus-infected cells.

After transfection of cells with a plasmid expressing only the NS1A protein, PABII protein molecules relocalize from their accumulation in discrete speckles to an essentially uniform distribution throughout the nucleoplasm. Such relocalization of PABII requires the effector domain of the NS1A protein. A plausible interpretation of these results is that the NS1A protein molecules via their effector domains bind to the PABII protein molecules located in speckles and move them to multiple sites distributed throughout the nucleoplasm, where NS1A protein molecules are located. We show that nuclear relocalization of PABII also occurs in influenza virus-infected cells. Others have reported that in influenza A virus-infected cells, another cellular protein (of unknown function) relocalizes from speckles to a uniform distribution in the nucleoplasm (Wolff *et al.*, 1998). We demonstrate that in transfected cells the NS1A protein is selective: it does not cause the nuclear redistribution of the SC35 splicing factor, which remains localized in nuclear speckles. This result confirms the report that the SC35 factor remains in speckles in influenza A virus-infected cells (Wolff *et al.*, 1998). Finally, we show that the NS1A protein can also interact with a nuclear protein which is not in speckles, the 30 kDa CPSF protein that is uniformly distributed throughout the nucleoplasm even in the absence of the NS1A protein.

Our competitive binding assays suggest that the 30 kDa CPSF and PABII proteins bind to non-overlapping regions of the NS1A protein effector domain. Hence, a single NS1A protein molecule would be able to simultaneously bind, and hence inactivate, both the PABII protein and the 30 kDa CPSF protein. As a consequence, a smaller number of NS1A protein molecules would be needed to inhibit efficiently the 3'-end processing of cellular pre-mRNAs. Further, we demonstrate that the PABII and 30 kDa CPSF proteins also bind to each other *in vitro*, an interaction that has not been reported previously. We propose that such an interaction between PABII and the 30 kDa protein subunit of CPSF occurs normally during 3'-end processing. During the second step of 3'-end processing, poly(A) addition, the PABII and CPSF factors cooperate to tether PAP to the pre-mRNA (reviewed in Wahle and Keller, 1996; Colgan and Manley, 1997; Wahle and Kuhn, 1997). Based on our results, we propose that CPSF and PABII directly interact with each other during this step and that this interaction is mediated by the 30 kDa subunit of CPSF. Consistent with such a CPSF-PABII interaction, it has been reported that PABII increases the stability of CPSF-RNA complexes (Bienroth *et al.*, 1993).

The present results establish that the mechanism by which the NS1A protein of influenza A virus inhibits the cellular 3'-end processing system is more elaborate than

previously postulated (Nemeroff *et al.*, 1998). As shown in Figure 7, we propose that an individual NS1A protein in virus-infected cells forms a complex with both PABII and the 30 kDa subunit of CPSF. In this complex the two cellular 3'-processing proteins, 30 kDa CPSF and PABII, also bind directly to each other. The presence of PABII in 3'-processing complexes that are formed prior to 3'-cleavage may be attributed to the binding of PABII to the NS1A protein, because PABII has not been found in 3'-cleavage complexes formed *in vitro* (reviewed in Wahle and Keller, 1996; Colgan and Manley, 1997; Wahle and Kuhn, 1997). As a result of the formation of the complex between CPSF and the NS1A protein, the binding of CPSF to the AAUAAA sequence of some cellular pre-mRNA molecules is blocked, so that 3'-cleavage and subsequent addition of A residues do not occur (Nemeroff *et al.*, 1998) (pathway I).

However, as demonstrated in the present study, other cellular pre-mRNA molecules are in fact cleaved in influenza virus-infected cells, indicating that CPSF does bind to the AAUAAA sequence of these cellular pre-mRNAs, despite the binding of the NS1 protein and PABII to the 30 kDa CPSF subunit (pathway II). Perhaps the sequence around the 3'-cleavage sites of these pre-mRNAs strongly favors formation of a stable 3'-end processing complex. The binding of CPSF to the AAUAAA sequence of a pre-mRNA, which is unstable, is stabilized by the CstF factor, which binds to a sequence downstream of the cleavage site (reviewed in Wahle and Keller, 1996; Colgan and Manley, 1997; Wahle and Kuhn, 1997). Strong stabilization by CstF may offset the inhibition of CPSF binding caused by the binding of the NS1A protein and PABII to the 30 kDa CPSF subunit. After these pre-mRNAs are cleaved, a short poly(A) sequence is added by PAP in a reaction that requires CPSF (reviewed in Wahle and Keller, 1996; Colgan and Manley, 1997; Wahle and Kuhn, 1997). Hence, CPSF, which interacts with PAP via its 160 kDa subunit (Murthy and Manley, 1995), retains its ability to interact functionally with PAP despite the binding of both the NS1A protein and PABII to the 30 kDa subunit of CPSF. Despite the apparent ability of CPSF to interact with PAP, subsequent elongation of the short poly(A) sequence is blocked. Therefore, this blockage most likely results from the binding of the NS1A protein to PABII, which along with CPSF is needed to tether PAP to poly(A) so that it can catalyze the processive addition of A residues (Bienroth *et al.*, 1993; Wahle, 1995). Because the NS1A protein does not inhibit the binding of PABII to poly(A) (data not shown), it is likely that the binding of the NS1A protein to PABII blocks the functional interaction of PABII with PAP. This mechanism for the action of the NS1A protein is based on previous *in vitro* results which showed that PAP needs to interact with both CPSF and PABII to carry out the processive extension of poly(A) tails (Bienroth *et al.*, 1993; Wahle, 1995). Consequently, via this two-pronged attack against PABII as well as CPSF, the NS1A protein effectively blocks the 3'-end processing of cellular pre-mRNAs in influenza virus-infected cells.

Neither the uncleaved pre-mRNA (produced in pathway I) nor the cleaved pre-mRNA containing a short poly(A) tail (produced in pathway II) is exported from the nucleus. The block in the nuclear export of these pre-mRNAs may be attributed, at least in part, to the absence

Fig. 7. Proposed two-pronged mechanism by which the NS1 protein of influenza A virus inhibits the cellular 3'-end processing system in infected cells. Pathway I: the binding of the NS1 protein (and PABII) to the 30 kDa subunit of CPSF blocks the binding of CPSF to the AAUAAA sequence of some cellular pre-mRNA molecules, thereby blocking 3'-cleavage of these pre-mRNAs. The uncleaved pre-mRNAs remain in the nucleus. Pathway II: CPSF binds to the AAUAAA sequence of other cellular pre-mRNA molecules, despite the binding of the NS1 protein and PABII to the 30 kDa CPSF subunit. A short poly(A) sequence is then added to these cleaved pre-mRNAs by PAP in a CPSF-dependent reaction. Subsequent elongation of the short poly(A) sequence is blocked by the binding of the NS1 protein to PABII, resulting in the nuclear accumulation of cleaved pre-mRNAs containing short poly(A) tails.

of elongated poly(A) tails which bind PABII molecules as part of the elongation process itself. These PABII molecules may help mediate nuclear export.

We have shown that as a consequence of its binding to the 30 kDa CPSF and PABII proteins the effector domain of the NS1A protein plays an essential role in the inhibition of 3'-end processing of cellular pre-mRNAs and in the resulting inhibition of their nuclear export (Nemeroff *et al.*, 1998; present study). Accordingly, it will be of great interest to identify the specific amino acid sequences of the effector domain which interact with the 30 kDa CPSF and PABII proteins. In addition, we have recently shown that only the effector domain, and not the RNA-binding domain, is required for the inhibition of the 3'-end processing of cellular pre-mRNAs. In our early mutagenesis experiments, which were carried out without the benefit of structural information, several alanine replacements that were introduced into the RNA-binding domain inactivated the activity of the NS1A protein in the inhibition of the nuclear export of cellular pre-mRNAs (Qian *et al.*, 1994). However, these alanine replacements have now been shown to disrupt the dimeric structure of

the NS1A protein (Wang *et al.*, 1999), a profound structural alteration that could lead to inactivation of the effector domain as well as the RNA-binding domain. Consequently, using a NS1A protein that contains alanines substituted for the only two amino acids that are required for RNA binding *per se* and not for dimerization (Wang *et al.*, 1999), we readdressed the issue of the role of the RNA-binding domain: the RNA-binding domain is *not* required for the inhibition of the 3'-end processing of cellular pre-mRNAs (W.Wang, Y.Li and R.M.Krug, unpublished experiments).

On the basis of current information, it can be concluded that all naturally occurring influenza A viruses encode a NS1A protein which contains an effector domain that functions in the inhibition of 3'-end processing of cellular pre-mRNAs and in the inhibition of their nuclear export. Thus, it has been shown that a functional effector domain is present in the NS1A proteins that exhibit the widest divergence in sequence among influenza A viruses, those encoded by the A/Udorn/72 and A/Alberta/Duck/60 viruses (Wang and Krug, 1996). At one time it was thought that one naturally occurring virus, A/Turkey/Oregon/71,

encodes a NS1A protein lacking the carboxy amino acid sequence containing the effector domain (Norton *et al.*, 1987). However, it has now been established that naturally occurring A/Turkey/Oregon/71 virus encodes a full-length NS1A protein, indicating that the previously characterized virus isolate was probably generated during multiple passages in the laboratory (Suarez and Perdue, 1998). Thus, this effector domain function for influenza A viruses is conserved in nature, indicating that it probably plays important roles in natural infections. Several roles can be envisaged. For example, by blocking the nuclear export of cellular mRNAs, the effector domain of the NS1A protein should enhance viral mRNA synthesis and hence viral gene expression, thereby enhancing efficient propagation in nature. Furthermore, the inhibition of cellular gene expression should be greatly enhanced, thereby presumably making the virus more pathogenic and virulent. Influenza A viruses that encode an NS1A protein lacking an effector domain have been generated in the laboratory. These viruses do replicate in tissue culture, although significantly lower virus titers are produced in most cell lines (Norton *et al.*, 1987; Egorov *et al.*, 1998). In addition, these viruses are attenuated in mice, and did not kill mice after intranasal inoculation, unlike influenza A viruses that encode a full-length NS1A protein (Egorov *et al.*, 1998).

Several viruses in addition to influenza virus employ multiple strategies to regulate or inhibit a specific cellular function. For example, vaccinia virus specifies three proteins that counteract the double-stranded RNA-activated PKR kinase (Beattie *et al.*, 1991; Watson *et al.*, 1991; Xiang *et al.*, 1998). Once activated, this kinase would phosphorylate the α subunit of the eIF-2 translation initiation factor, resulting in the shutdown of translation (reviewed in Katze, 1992; Mathews, 1993). One vaccinia virus protein (E3L) binds and sequesters double-stranded RNA (Watson *et al.*, 1991), a second protein (K3L) functions as a pseudosubstrate that competes with the natural eIF-2 α substrate (Beattie *et al.*, 1991), and a third protein (A18R) suppresses aberrant viral transcription that would lead to an increase in viral double-stranded RNA (Xiang *et al.*, 1998). Influenza A virus is unique in that it employs a single protein, the NS1A protein, to mount a two-pronged attack against a cellular function, the 3'-end processing of cellular pre-mRNAs.

Materials and methods

Yeast two-hybrid library screening

The open reading frame of the NS1 protein of influenza A/Udorn virus was fused to the DNA-binding domain of Gal4 in the pAS2-1 yeast plasmid (TRP1, amp^r, CYH2^S). The yeast pACT plasmid (LEU2, amp^r) containing a human B cell cDNA library was kindly provided by Stephen Elledge. The two plasmids were cotransfected into the Y190 yeast strain (Harper *et al.*, 1993) using the lithium acetate method (Gietz *et al.*, 1992). Transformants that grew on plates lacking leucine, tryptophan and histidine (in the presence of 20 mM 3-amino-1,2,4-triazole) were screened for β -galactosidase expression using X-gal as a chromogenic indicator. Expressing colonies were restreaked and again assayed for β -galactosidase expression. Cycloheximide (CM) selection was then used to select for yeast colonies that contain only the cDNA library plasmid and not the pAS2-1 plasmid containing NS1 DNA (Harper *et al.*, 1993). To determine whether a library plasmid expressed a protein that specifically interacts with the effector domain of the NS1 protein, individual colonies were transformed with a pAS2-1 plasmid containing either the NS1 Δ E sequence or the NS1 sequence of influenza B virus. Only those transformed colonies that failed to interact with these two

test plasmids were used for the isolation of plasmids that were grown in *Escherichia coli*.

Glutathione–Sephadex affinity selection

The indicated GST fusion protein, which was expressed and purified as previously described (Qiu and Krug, 1994), was combined with the indicated ³⁵S-labeled protein(s) synthesized *in vitro*, and subjected to glutathione–Sephadex affinity selection as previously described (Nemeroff *et al.*, 1995). To prepare the labeled proteins, the DNA encoding the indicated protein was subcloned into pGEM1 and translated using a Promega TnT Coupled Transcription/Translation kit in the presence of [³⁵S]methionine.

Protein purification

NS1 wild-type or mutant sequences were inserted into a pET-derived expression vector that encodes six histidine residues at the C-terminus of the encoded NS1 protein (Gunderson *et al.*, 1997). *Escherichia coli* BL21(DE3) that was transformed with the recombinant plasmid was induced with isopropyl- β -D-thiogalactoside, and cell pellets were collected. It was necessary to purify each of the NS1 proteins under denaturing conditions. The cells were disrupted in a buffer containing 6 M guanidine hydrochloride (GuHCl), and the resulting lysate was bound to a Ni²⁺–NTA resin. The resin was washed extensively, first with the loading buffer containing 6 M GuHCl, and then with a buffer lacking GuHCl and containing 25 mM Tris–HCl pH 7.4, 0.5 M KCl and 10% glycerol (buffer A). The NS1 protein was eluted from the resin with buffer A containing 0.4 M imidazole. The purity of the NS1 proteins was established by gel electrophoresis. Because the RNA-binding activity of the NS1 protein is dependent on the formation of the dimeric structure of the protein (Wang *et al.*, 1999), the refolding of the wild-type and Δ E forms of the NS1 protein was measured by assaying their RNA-binding activities, using double-stranded RNA as the RNA target. The two NS1 proteins were approximately equal in their RNA-binding activity, suggesting that the two proteins refolded to a similar extent.

PABII-mediated stimulation of polyadenylation *in vitro*

The RNA substrate used in these assays was pre-cleaved L3 mRNA containing 30–35 A residues at its 3'-terminus. To prepare this substrate, pre-cleaved L3 mRNA was transcribed from plasmid pSP6L3pre in the presence of [α -³²P]UTP (Humphrey *et al.*, 1987) and then incubated with purified PAP (kindly provided by Sam Gunderson) in the presence of unlabeled ATP and Mn²⁺, conditions that result in non-specific adenylation (Christofori and Keller, 1989). The products of the reaction were resolved by electrophoresis on 8% polyacrylamide gels containing 7 M urea. Pre-cleaved L3 mRNAs containing ~30–35 A residues were eluted from the gel, ethanol precipitated, and dissolved in water. Polyadenylation reactions containing 0.3 pmol PABII (kindly provided by Elmar Wahle) were pre-incubated for 10 min at 30°C in either the absence or the presence of the indicated amounts of the NS1 protein (containing a histidine tag). Polyadenylation was carried out as described by Wahle (1995). Briefly, after addition of PAP (100 fmol) and the RNA substrate (100 fmol, 20 000 c.p.m.), the reaction mixtures (final volume of 25 μ l) were prewarmed at 37°C, and polyadenylation was then initiated by the addition of ATP (1 mM). After 4 min of incubation at 37°C, the reactions were terminated, and the RNA products were analyzed by electrophoresis on 6% polyacrylamide gels containing 7 M urea.

Indirect immunofluorescence and confocal microscopy

Cos cells, which were grown on glass coverslips, were transfected with a pBC12 plasmid encoding either wild-type NS1 or the NS1 Δ E protein. Control cells were not transfected. Where indicated, Cos cells were not transfected, but were infected with 20 p.f.u. per cell of influenza A/Udorn virus for 3.5 h. Cells were analyzed by indirect immunofluorescence as described previously (Chen *et al.*, 1998). For the localization of the NS1 and PABII proteins, the primary antibodies were monoclonal mouse antibodies against the NS1 protein (kindly provided by Jonathan Yewdell) and rabbit anti-PABII antiserum (kindly provided by Elmar Wahle). For the localization of the NS1 and SC35 proteins, the primary antibodies were polyclonal anti-NS1 rabbit antiserum and monoclonal mouse antibodies against the SC35 protein. The secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-mouse antibody and rhodamine-conjugated goat anti-rabbit antibody. The cells were examined by confocal microscopy as described previously (Chen *et al.*, 1998).

Interspecies heterokaryon assay for nucleocytoplasmic shuttling of PABII

Cos cells were transfected either with only a pBC12 plasmid encoding PABIIHA or with two PBC12 plasmids, one encoding PABIIHA and one encoding the NS1 protein. At 40 h post-transfection the cells (on coverslips) were trypsinized and mixed with an equal number of mouse NIH 3T3 cells. The cell mixture was replated and incubated for 3 h in the presence of CM at 50 µg/ml. Because of the CM treatment, all of the PABIIHA protein in the Cos cells was localized in the nucleus. Cell fusion was induced by inverting the coverslips into a drop of prewarmed 50% polyethylene glycol (PEG) for 2 min. The cells were washed twice with prewarmed phosphate-buffered saline and were then incubated at 37°C for an additional 3 h in new medium containing 50 µg/ml CM. PABIIHA and the NS1 protein were detected by indirect immunofluorescence. To stain the DNA of the cells, Hoechst 33258 was added at the time of incubation with the secondary antibody (Borer and Lehner, 1989).

Determination of the lengths of poly(A) tails on a specific cellular mRNA in virus-infected cells

Monolayers of 293 cells were infected with influenza A virus, and at the indicated times postinfection were fractionated into nuclei and cytoplasm as previously described (Alonso-Caplen *et al.*, 1992; Lu *et al.*, 1994). RNA was extracted from these two cellular fractions using guanidinium isothiocyanate. The lengths of the poly(A) tails on the β-actin mRNAs and pre-mRNAs in these two RNAs were measured using the PCR Poly(A) Test assay (Salles and Strickland, 1995) (see text). In the PCR amplification step, the β-actin specific oligonucleotide was: 5'-CACACAGGGAGGTGATAGCAATAGCATTGC-3'. After PCR amplification, the DNA products were analyzed by electrophoresis on a 4% polyacrylamide gel containing 7 M urea.

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