Influenza virus NS1 protein interacts with viral transcription–replication complexes in vivo

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The interaction of influenza virus NS1 protein with other viral products in the infected cell was analysed by co-immunoprecipitation studies. The three subunits of the polymerase and the nucleoprotein, but not M1 protein, were co-immunoprecipitated by NS1-specific serum but not when control serum was used. Such co-immunoprecipitation was not sensitive to RNase treatment of the immunoprecipitates. Co-immunoprecipitation was also obtained when the viral transcription–replication system was reconstituted in vivo by transfection of cDNAs and model vRNA template into vaccinia virus–T7-infected cells. Analysis of the RNA pulled-down in the NS1-specific precipitates indicated the presence of both vRNA and mRNA. These results are discussed in the context of the phenotype of virus temperature-sensitive mutants affected in the NS1 gene.

The influenza A viruses are the prototype of the family Orthomyxoviridae. The influenza A virions contain eight different single-stranded RNA segments of negative polarity (vRNAs) which encode a total of ten proteins (for a review see Lamb, 1989). The transcription and replication of the viral genome is carried out by the RNA polymerase, a complex made up by the PB1, PB2 and PA subunits (Honda et al., 1990; Kato et al., 1985). In the infection cycle, the incoming viral RNA is first transcribed into mRNAs that contain 5′-cap and terminal sequences of cellular origin (reviewed in Krug et al., 1989). Replication of the vRNAs proceeds by the synthesis of positive-polarity copies of the vRNAs (cRNAs) (Hay, 1982). The switch from transcription to replication is regulated by the synthesis of new nucleoprotein (NP) molecules, since cRNA, as well as vRNA, are encapsidated with NP in the form of ribonucleoprotein complexes (RNP).

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The NS1 protein is the only virus protein not present in the virus particle (Lamb, 1989; Richardson & Akkina, 1991). It accumulates in the nucleus of the infected cell at early times during infection and is also present in the cytoplasm at later times (Nieto et al., 1992). The expression of NS1 protein from cDNA leads to the alteration of a number of processes involving cellular RNA, such as mRNA nucleo-cytoplasmic transport, and pre-mRNA splicing (Fortes et al., 1994, 1995), as well as to enhancement of viral mRNA translation (de la Luna et al., 1995; Enami et al., 1994). It has been shown that NS1 protein binds various types of RNA: virion RNA (Hatada et al., 1997), poly(A) and U6 snRNA (Qiu & Krug, 1994; Qiu et al., 1995) and viral mRNA (Park & Katze, 1995). However, most of the evidence presented so far for the RNA-binding properties of NS1 protein has arisen from experiments in vitro and little is known about the situation in the influenza virus-infected cell. In this report, we analysed directly the viral proteins and RNAs that associate with NS1 protein during infection.

Cultures of COS-1 cells were infected with influenza virus (A/Victoria/3/75 strain) at a multiplicity of 5–10 p.f.u. per cell or mock-infected, and then labelled with [35S]Met-Cys from 5 to 6h post infection (p.i.). Cell extracts were prepared in TNE–1% NP40 buffer (see Fig. 1 legend) and used in immunoprecipitation assays with either control or NS1-specific hyperimmune rat sera. The precipitates were washed five times with TNE–1% NP40 buffer, five times with RIPA buffer and were separated by electrophoresis in a 7.5–20% polyacrylamide gradient gel. After transfer to an Immobilon membrane, the samples were analysed by either autoradiography or Western blot. The results are presented in Fig. 1. The NS1-specific serum brought down NS1 protein and two additional radiolabelled protein bands in the 80–90 kDa range (Fig. 1A, asterisks). Since these bands have the same mobility as the presumptive P protein bands present in the total extracts obtained from the influenza virus-infected cells, these results suggested that NS1 protein is associated with the virus polymerase complex. To ascertain whether each of the polymerase subunits can be co-immunoprecipitated with NS1 protein, the proteins co-immunoprecipitated were assayed by Western blot using rabbit sera specific for NS1 protein, each of the polymerase subunits, the NP or M1 protein. The results are
Fig. 1. Co-immunoprecipitation of viral polymerase and NP from influenza virus-infected cells by anti-NS1 serum. Cultures of COS-1 cells (about 10^7 cells) were infected with influenza virus at an m.o.i. of 5–10 p.f.u. per cell or mock-infected. At 5 h p.i. the cells were labelled with ^35^S-Met-Cys (A) or were not labelled (B, C). Extracts were prepared at 6 h p.i. in 100 mM NaCl–5 mM EDTA–1% NP40–50 mM Tris–HCl pH 7±5 (TNE–1% NP40). For immunoprecipitation, control or NS1-specific rat sera were bound to protein G–Sepharose and the resins were further incubated with the extracts (equivalent to about 2 ± 5 x 10^6 cells) for 1 h at room temperature. After washing five times with TNE–1% NP40 and five times with RIPA buffer, the bound proteins were eluted in Laemmli sample buffer and separated by electrophoresis in a 7–20% SDS–polyacrylamide gel. (A) The proteins separated by electrophoresis were transferred to a filter and exposed for autoradiography. (B) The gel was processed for Western blotting and developed with rabbit anti-NS1 serum (NS1), anti-polymerase subunit sera (PB1, PB2, PA), anti-NP serum (NP) or anti-M1 serum plus anti-NS1 sera (M1 + NS1) by enhanced chemiluminescence. (C) In some experiments, the immunoprecipitates, after washing with TNE–1% NP40 buffer, were treated with RNase A (60 µg/ml) for 60 min at 4 °C (+ RNase) or left untreated (Ctrl), further washed with RIPA buffer and processed for immunoblot as indicated above. M and F refer to mock-infected or influenza virus-infected cells, respectively.

presented in Fig. 1(B). The precipitation of NS1 protein was specific, since no NS1 protein could be detected by precipitation with a control serum (Fig. 1B; NS1). The Western blot analysis using sera specific for the polymerase subunits revealed that all three proteins, PB1, PB2 and PA, were co-immunoprecipitated specifically by using anti-NS1 serum (Fig. 1B; PB1, PB2, PA). Likewise, NP was also co-immunoprecipitated (Fig. 1B; NP). However, no association of M1 protein could be detected by Western blot using an M1-specific serum (Fig. 1B; M1 + NS1). For quantification of these results, small portions of the total cell extracts used were analysed in parallel to the immunoprecipitates (Fig. 1A; Total Extracts) in order to estimate the proportion of each protein that was present in the precipitates. In this way, it could be calculated that about 22% of the total NS1 protein present in the extract was immunoprecipitated, whereas 3±5, 3 and 1±5% of the PB1, PB2 and PA proteins were found in the precipitates, respectively. The proportion of NP in the immunoprecipitates was 5%. These results indicated that about 13% of the polymerase complex (average of the results obtained for PB1, PB2 and PA proteins, corrected for the efficiency of NS1 immunoprecipitation) and about 25% of the NP present in the extract are associated with NS1 protein. It should be emphasized that these are rough estimates that refer to the proteins present in the soluble extracts and not to total infected cell protein.

Since NS1 protein has been shown to interact with several types of RNA (Park & Katze, 1995; Hatada, 1997; and our unpublished results; Qiu & Krug, 1994; Qiu et al., 1995), it could be argued that co-immunoprecipitation of the polymerase complex with NS1 might be due to unspecific binding of both to RNAs present in the extract. To address this question, experiments similar to those presented in Fig. 1(B)
were carried out. After washing, the immunoprecipitates were treated with an excess of RNase A (60 µg/ml; 60 min at 4 °C), further washed with RIPA buffer and analysed as indicated above for the presence of NS1 and PB2. The results are presented in Fig. 1(C) and indicate that approximately the same amount of PB2 protein was co-immunoprecipitated with NS1, irrespective of treatment with RNase (Fig. 1C; Ctrl and + RNase). Thus, we conclude that the association of the polymerase complex and NS1 is not the result of co-aggregation to an unspecific RNA molecule. However, this experiment cannot rule out the presence in the complex of a specific RNA that might be protected from RNase attack.

The results presented so far do not rule out the possibility that the anti-NS1 sera used could cross-react with any of the components of the RNP which would account for the co-immunoprecipitation of the transcription–replication complexes. To eliminate such a possibility, we reconstituted the active RNPs in vivo by transfection of cDNAs encoding the polymerase subunits and the NP as described and further transfected with vNS2 RNA (C + RNA) (Mena et al., 1994; Perales & Ortín, 1997). Alternatively, transfection was carried out with empty pGEM vector (VT7). The co-transfection of NS1-encoding plasmid is indicated at the bottom of the figure (+ NS1). About 20 h p.i., extracts were prepared and the samples processed for immunoprecipitation and Western blot. Parallel samples were developed for NS1 protein (NS1) and PB2 subunit (PB2) as described in the legend to Fig. 1.

![Fig. 2. NS1 protein associates with transcription–replication complexes reconstituted in vivo. Cultures of COS-1 cells (about 5 x 10^6 cells) were infected with vaccinia virus vTF7-3 at an m.o.i. of 5–10 p.f.u. per cell, transfected with pGEM plasmids encoding the polymerase subunits and the NP as described and further transfected with vNS2 RNA (C + RNA) (Mena et al., 1994; Perales & Ortín, 1997). Alternatively, transfection was carried out with empty pGEM vector (VT7). The co-transfection of NS1-encoding plasmid is indicated at the bottom of the figure (+ NS1). About 20 h p.i., extracts were prepared and the samples processed for immunoprecipitation and Western blot. Parallel samples were developed for NS1 protein (NS1) and PB2 subunit (PB2) as described in the legend to Fig. 1.](image)

The evidence presented in Figs 1–3, as a whole, indicates that an association exists between NS1 protein and the transcription–replication complexes in influenza virus-infected cells. The simplest possibility to explain such an association would be a direct interaction between NS1 protein and any of the polymerase subunits. To test for such protein–protein interactions, we performed two-hybrid assays in yeast or in mammalian cells, as described (Chien et al., 1991; González et al., 1996; Zürcher et al., 1996). Although NS1–NS1 interaction could be easily detected, for none of the combinations between the polymerase subunits and NS1 could we obtain positive evidence for interaction (data not shown). One possible explanation for these negative results is that interaction between any of the P-proteins and NS1 only can occur when they are assembled in the polymerase complex, due to the appearance of a new interaction domain in either one of the components of the complex or in the complex itself. The fact that the polymerase binds to the panhandle region of vRNA (Tiley et al., 1994) and NS1 protein binds preferentially to vRNA, also at the panhandle region (Hatada et al., 1997), together with the negative results obtained in the two-hybrid interaction tests, suggests that the association of NS1 protein with the transcription–replication complexes might be mediated by RNA binding. However, we cannot exclude the possibility that direct protein–protein interactions play a role...
in the association. In this context, it is interesting to note that one of the temperature-sensitive mutants described for the NS1 gene could be extragenically suppressed by mutations in one of the polymerase genes (Ludwig et al., 1995; Scholissiek & Spring, 1982). In line with the proposal that vRNA binding leads to the association of NS1 protein and the viral polymerase, we could find vRNA in the immunoprecipitates (Fig. 3). In addition, we could also find mRNA associated with NS1 protein. This is not surprising, since it has been shown that NS1 protein mediates an enhanced usage of viral mRNAs by the translational apparatus (Enami et al., 1994) by increasing the rate of translation initiation (de la Luna et al., 1995). In fact, NS1 protein can be cross-linked to the 5′ untranslated sequence of NP mRNA in infected cells (Park & Katze, 1995) and binds poly(A) (Qiu & Krug, 1994).

At present, we can only speculate about the biological meaning of the NS1 interaction with the viral transcription–replication complexes. The phenotype of influenza virus temperature-sensitive mutants affected in the NS1 gene indicated alterations in late viral gene expression at either the transcriptional or post-transcriptional level (Hatada et al., 1990; Koennecke et al., 1981; Ludwig et al., 1995). Some of these phenotypes are consistent with the observed biochemical effects of NS1 protein expression in regard to nucleocytoplasmic transport or translational enhancement (de la Luna et al., 1995; Enami et al., 1994; Fortes et al., 1994). The association of NS1 protein with intracellular RNPs described here suggests that it could be involved in the induction of late transcription. In any case, the fact that NS1 protein is found in association with the viral transcription–replication complexes but is absent from the virion RNPs suggests that a specific mechanism for the displacement of NS1 protein from the intracellular RNPs has to exist for them to become encapsidated. Alternatively, there may be two independent populations of intracellular RNPs, one associated with NS1 and devoted to transcription–replication and the other one, NS1 protein-free, targeted to encapsidation.

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References


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