Specific binding of influenza A virus NS1 protein to the virus minus-sense RNA in vitro

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The non-structural protein NS1, encoded by genome segment 8 of influenza A virus, was expressed in Escherichia coli from cloned cDNA and purified. The NS1 protein had a specific RNA-binding activity, binding to influenza A virus minus-sense but not plus-sense RNA synthesized in vitro from cloned DNA using phage RNA polymerase. NS1 bound preferentially to the regions of RNA containing either 5'- or 3'-terminal common sequences of the genomic RNA. Binding was inhibited by virion RNA, but not by single-stranded minus-sense cDNA and oligo DNAs having the common sequences. In addition, binding was also inhibited by 28S rRNA but not 18S rRNA prepared from MDCK cells.

Introduction

The genome of influenza A virus is composed of eight ssRNA segments of negative polarity (reviewed in Lamb & Choppin, 1983; McCauley & Mahy, 1983). These RNAs (vRNAs) have 13- and 12-nucleotide sequences at the 5' and 3' termini, respectively, that are common to all segments of type A influenza viruses. The common terminal sequences show partial inverted complementarity, and are postulated to have roles as recognition signals for viral transcription, replication or encapsidation (Hsu et al., 1987). Three types of virus-specific RNAs for each segment, mRNA, cRNA (the template for vRNA synthesis) and vRNA, are synthesized in the nuclei of infected cells (Herz et al., 1981). The mRNA has a 5' capped primer oligonucleotide and a 3' poly(A) tail, and is an incomplete transcript of vRNA in that it lacks a copy of the last 17 to 22 nucleotides at the 5' end, whereas the other type of transcript, cRNA, is a full-length copy of vRNA (Krug et al., 1979; Hay et al., 1977).

RNA segment 8, the smallest segment, encodes two overlapping non-structural polypeptides, NS1 and NS2 (Inglis et al., 1979; Lamb & Choppin, 1979), which are found in infected cells, but not in the virion. NS1 is encoded by a collinear mRNA transcript, whereas NS2 is encoded by an interrupted mRNA (Inglis et al., 1980; Lamb & Lai, 1980). NS1 is found in the nucleus and nucleolus of infected cells, and is also associated with polysomes (Dimmock, 1969; Lazarowitz et al., 1971; Compans, 1973; Krug & Etkind, 1973). Recently, it has been shown that NS1 contains two independent signals for nuclear localization (Greenspan et al., 1988). In some strains of type A influenza virus NS1 forms electron-dense paracrystalline inclusion bodies late in infection (Morrongiello & Dales, 1977; Shaw & Compans, 1978; Petri et al., 1982), which contain a mixture of cellular RNA species including 28S ribosomal RNA (Yoshida et al., 1981). However, relatively little is known about the function of NS1 during the virus growth cycle. Studies of several temperature sensitive (ts) mutants of NS1 have suggested that the protein is involved in the synthesis of vRNA or the shutoff of host cell protein synthesis (Wolstenholme et al., 1980; Koennecke et al., 1981). Previously we have reported that synthesis of the late proteins and NS1 by two independent ts mutants, each having a single amino acid substitution in NS1 (Hatada et al., 1990), decreases markedly at a temperature of 40 °C (compared with that at the control temperature of 34 °C). At 40 °C, however, the levels of individual mRNAs, including those for late proteins, are almost the same as those at 34 °C (permissive temperature) and attain wild-type levels later in infection when the synthesis of the late proteins and NS1 decreases greatly. This observation suggests that NS1 is involved in some post-transcriptional process in the synthesis of the late proteins and NS1.

To examine the possible role of NS1 in translational control, we carried out experiments to determine whether NS1 binds to viral mRNAs in vitro. We found that NS1 forms RNase-resistant complex(es) with virus minus-sense but not plus-sense RNAs, possibly by interacting with one of the common terminal sequences.

Methods

Construction of a recombinant plasmid for expression of the NS1 protein. The Escherichia coli expression vector pKK233-2 (Amann & Brosius, 1985) has the tac promoter and the lacZ ribosome-binding site followed
by an ATG initiation codon which is contained within a unique NcoI site. A cDNA copy of genome segment 8 (containing the NS1 gene) of influenza virus A/PR/8/34 (H1N1) was cloned in the plasmid pSP64 (a gift from Dr. K. Nagata). The recombinant plasmid has two BamHI sites, one between the first and the second codons of the NS1 gene, and the other in the vector sequences downstream from the NS1 gene. An NS1 cDNA fragment was excised from the recombinant DNA using BamHI and the recessed 3′ termini were filled with the Klenow fragment of DNA polymerase I. The fragment was then inserted by blunt-end ligation into the filled NcoI ends of pKK233-2, resulting in the correct NS1 reading frame without any extra amino acid sequences.

Preparation of 32P-labelled virus RNAs. Template DNAs linearized as mentioned above were transcribed in an in vitro system using T7 or SP6 phage RNA polymerase, and [α-32P]UTP as described previously (Hatada et al., 1989).

RNA binding assay. To study the RNA-binding activity of NS1, we modified the procedures published previously for an RNA mobility shift assay and a ribonuclease protection assay (Garner et al., 1981; Zapp & Green, 1989; Heaphy et al., 1990; Marciniak et al., 1990). About 1 μg of NS1 (38 pmol) and 100 to 600 fmol RNA labelled with [α-32P]UTP (3 x 10^4 to 5 x 10^4 c.p.m./pmol) (see above) were incubated in 15 μl binding buffer containing 6.6 mM-Tris-Cl pH 7-6, 33 mM-KCl, 1 mM-EDTA and 0.1 mM-DTT for 30 min at 30°C. The incubation was continued for 10 to 30 min in the presence or absence of either 0.75 μg of RNase A or 1-5 units of RNase T2. After addition of gel loading buffer (5%, glycerol, 1 mM-EDTA, 0.05% xylene cyanol and 0.05% bromphenol blue; final concentrations), the mixtures were applied to a non-denaturing 4% polyacrylamide gel (acrylamide:bis-acrylamide, 60:1) in 0.5 x TBE buffer (1 x TBE contains 90 mM-Tris-base pH 8.0 and 2.5 mM-EDTA). Electrophoresis was carried out at a constant voltage of 2.5 V/cm in 0.5 x TBE buffer for 14 h at 4°C. The gels were dried and exposed to Fuji RX X-ray film for autoradiography. For competition experiments NS1 was preincubated with competitor RNA for 30 min at 30°C prior to addition of the 32P-labeled RNA.

Immunoprecipitation assay. Antibody against NS1 was raised in a rabbit, from the serum of which the IgG fraction was precipitated using 33% ammonium sulphate. The collected pellets were dissolved and dialysed against PBS, and then applied to a DE52 cellulose column. The flow-through fraction of the column was used as the IgG fraction. Complexes between NS1 and 32P-labeled RNAs in the binding buffer (15 μl) were diluted with PBS up to 50 μl, and incubated with the anti-NS1 IgG fraction (0.5 to 5 μl) or a non-specific IgG fraction (5 μl) in the presence of 2 μl of 30% Protein A-Sepharose for 1 h at room temperature. After washing several times in 100 μl of buffer containing 50 mM-Tris-Cl pH 7.5, 150 mM-NaCl and 5 mM-EDTA, the beads were resuspended in 10 μl of 80% formamide, 10 mM-Tris-Cl pH 7.5, 1 mM-EDTA and 1% SDS, and heated to 95°C for 3 min to release the bound RNA. The beads were then removed by centrifugation and the supernatant containing the released RNA was applied to a 6% denaturing polyacrylamide gel containing 7% urea in 0.5 x TBE buffer. Bands were visualized by autoradiography as described above.

Detection of the NS1 protein in the shifted RNA bands. The radioactive bands on the dried gel corresponding to the shifted RNA bands (complex) were excised and swollen in 200 μl of H2O for 3 h at 37°C. They were then supplemented with 50 μl of gel loading buffer containing 250 mM-Tris-Cl pH 6.8, 20 mM-EDTA, 20% glycerol and 0.05% bromphenol blue, heated for 2 min at 100°C, and subjected to 12.5% SDS–PAGE (Laemmli, 1970). Gels were silver-stained (Oakley et al., 1980) to visualize proteins.

Materials. Plasmids pKK233-2 and pTZ18U were purchased from Pharmacia and Bio-Rad, respectively. Protein A-Sepharose was from Sigma, proteinase K from Merck and T7 phage RNA polymerase from Takara Syuzo. The oligodeoxynucleotides were synthesized using an Applied Biosystems DNA synthesizer (Model 380 B). Sources of other materials have been described previously (Hatada et al., 1989, 1990).
Results

Specific binding of NS1 to virus minus-sense RNAs

The RNA-binding activity of NS1 was studied using a RNA mobility shift assay and a ribonuclease protection assay. Pure NS1 was prepared from E. coli cells, in which the synthesis of the protein from a cDNA copy of A/PR/8/34 (H1N1) segment 8 inserted in the expression vector pKK233-2 had been induced using IPTG (Fig. 1, lane 2 and see Methods). A mock protein preparation (Fig. 1, lane 4) was made from induced E. coli cells carrying the expression vector but not the cDNA insert. Virus plus- or minus-sense RNAs were transcribed in vitro from the virus cDNAs connected to the bacteriophage promoters of SP6, pTZ18U and pTDT7 plasmids (see Methods). These recombinant plasmid DNAs, which were linearized by restriction enzyme digestion, provided run-off transcripts of type A influenza virus [A/Udorn/72 (H3N2)] RNAs with or without the exact common 5'- or 3'-terminal sequences (Fig. 2).

32P-Labelled ssRNA transcripts with or without added NS1 were incubated for 30 min at 30 °C, and some were treated with RNase A or T2. They were then applied to a 4% non-denaturing polyacrylamide gel in TBE buffer and subjected to electrophoresis at 2-5 V/cm for 14 h at 4 °C. As shown in Fig. 3, long RNAs moved as a broad smear in this gel (lanes 5, 10, 15 and 20). In the presence of NS1 (molar NS1 : RNA ratio, about 190:1), however, the movement of a portion of the RNAs was decreased (lanes 1, 6 and 11). Upon digestion by RNase, some radioactivity in the more slowly migrating fraction remained resistant to digestion for minus-sense NS and M RNAs [NS(-) and M(-) RNAs, respectively] (lanes 2 and 12), but not that for plus-sense RNAs (lanes 7 and 17). Using a short polynucleotide consisting of the 5'-
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Fig. 3. Assay of NS1 binding to influenza virus RNAs. The binding buffer containing 1 μg of NS1 (lanes 1 to 3, 6 to 8, 11 to 13, 16 to 18 and 21 to 23) or buffer B (lanes 4, 5, 9, 10, 14, 15, 19, 20 and 24) was mixed with 32P-labelled RNA probes (see Fig. 2): 200 fmol of NS(−) (lanes 1 to 5), 200 fmol of NS(+) (lanes 6 to 10), 200 fmol of M(−) (lanes 11 to 15), 200 fmol of M(+) (lanes 16 to 20) or 600 fmol of M(−)(1-29) (lanes 21 to 24). The mixtures were incubated for 30 min at 30 °C and continued in the presence (lanes 2 to 4, 7 to 9, 12 to 14, 17 to 19, and 22 to 24) or in the absence (lanes 1, 5, 6, 10, 11, 15, 16, 20 and 21) of 0.75 μg of RNase A. Some samples were treated with 1.25% SDS (lanes 3, 8, 13, 18 and 23) for 2 min at 100 °C. The position of the NS1–RNA complex is indicated.

Fig. 4. Characterization of the RNA-binding activity of NS1. Increasing amounts of M(−)(1-29) labelled RNA (200, 400, 600 and 800 fmol; lanes 1 to 4, respectively) were added to 1 μg NS1 or increasing amounts of NS1 (0.25, 0.5, 1.0 and 2.0 μg; lanes 5 to 8, respectively) were added to 600 fmol of M(−)(1-29) labelled RNA. Using 200 fmol of labelled NS(−) RNA, the binding complex was observed with 1 μg NS1 (lane 9), but not with 0.06 A260 units of a mock protein preparation (lane 10). Prior to mixing with NS(−)(832–890) labelled RNA (600 fmol), 1 μg of NS1 was not treated (lane 11) or pretreated by heating for 2 min at 100 °C (lane 12), or digested with 0.5 mg/ml or proteinase K for 30 min at 37 °C (lane 13). Note that 32P-labelled RNA digestion was incomplete because proteinase K inactivated the RNase (lane 13).
Influenza virus NS1: RNA-binding specificity

Fig. 5. Detection of NS1 in the complex (C). (a) Immunoprecipitation of M(−)(1-29)-NS1 complex with anti-NS1 antibody. Binding reactions were performed using 600 fmol of 32P-labelled M(−)(1-29) and 1 μg of NS1 (lanes 1 to 5) or 0.06 A280 units of mock protein preparation (lanes 6 and 7), and treated with an increasing amount of polyclonal anti-NS1 IgG (lanes 1 to 4, and 6; 0.5, 1, 2, 5 and 5 μl, respectively) or 5 μl of non-specific IgG (lanes 5 and 7) in the presence of Protein A-Sepharose. 32P-labelled RNA was released from the immunoprecipitates (see Methods) and subjected to electrophoresis on a 6% polyacrylamide-7 M-urea gel in 0.5 × TBE buffer, followed by autoradiography. Lane P, 50 fmol of 32P-labelled M(−)(1-29). (b) Presence of NS1 in shifted RNA bands. Lanes P, 1 and 2 show an autoradiogram of an RNA-binding assay; for the binding reaction, 1 μg of NS1 was incubated with 600 fmol of 32P-labelled M(−)(1-29) (lane 1) or 200 fmol of 32P-labelled NS(−) (lane 2); lane P, 32P-labelled M(−)(1-29) alone (150 fmol). The radioactive bands on the dried gel corresponding to complexes (as indicated in the figure) were cut out, treated as described in Methods and subjected to 12.5% SDS-polyacrylamide electrophoresis. The gel was silver-stained (lanes C, C', 3 to 6 and M). The following specimens were subjected to a second gel electrophoresis (all the gel pieces contained pieces of 3MM filter paper on which the gel had been mounted): a piece of blank gel (lanes C and C'); pieces of gel containing complexes from lanes 1 and 2 (lanes 3 and 4, respectively); a piece of gel containing free NS1 (lane 5); NS1 (lane 6); the same Mr markers as in Fig. 1 (lane M).

Fraction or a non-specific IgG fraction (Fig. 5a). In the presence of both NS1 and anti-NS1 IgG 32P-labelled RNA was recovered in the immunoprecipitates (lanes 1 to 4), but not when non-specific IgG or the mock preparation were employed (lanes 5, 6 and 7). The presence of NS1 in the RNase-resistant bands of the first gel was demonstrated directly by excising the bands, subjecting them to a second SDS-PAGE and staining with silver (Fig. 5b; RNA was not stained). As the gel pieces from the first gel also contained pieces of filter paper which could not be stripped off the gel, silver-staining bands originating from the paper were seen near the top of the gel (lanes C and C'). Excluding these bands, the only visible band comigrated with NS1 (compare lanes 3 and 4 with lane 5; see legend). These results showed that the RNase-resistant bands were NS1–RNA complexes, indicating specific binding of NS1 to viral minus-sense RNA.

Competition of RNA binding of NS1 by various RNAs

To examine the specificity of complex formation, NS1 was preincubated with an excess of various unlabelled RNAs (Fig. 6). Binding to labelled NS(−) RNA was
inhibited almost completely with a 10-fold molar excess of the homologous NS(−) RNA (lane 9). With similar quantities of M(−) RNA, binding decreased to 40% of that in the absence of competition (lane 3), but remained unchanged with M(+)-RNA (lane 2). Total virus genomic RNA (16-fold excess with regard to the number of RNA molecules) inhibited binding almost completely (lane 6). No appreciable inhibition was observed with 18S ribosomal RNA (300-fold molar excess, lane 4) or tRNA (400-fold molar excess, lane 7). In contrast, 28S ribosomal RNA (130-fold molar excess) greatly inhibited binding (lane 5), in agreement with a previous report showing that NS1 binds to 28S ribosomal RNA in vivo (Yoshida et al., 1981). No competition was observed with single-stranded viral cDNAs of either sense (data not shown).

Requirement of terminal sequences of viral minus-sense RNA for NS1 binding

To examine the sequences required for binding of NS1 to viral RNA, various deletions were generated in M or NS RNAs as shown in Fig. 2. In Fig. 7(a), binding patterns are shown only for deletion mutations of NS(−) and M(−) RNAs (other results are summarized in Fig. 2). Full-length NS(−) RNA was the best substrate examined for NS1 binding. In the presence of either terminal sequence considerable binding activity was retained, as shown with NS(−)(189–890) and NS(−)(1–35) RNAs which had the intact common 3' and 5'-terminal sequence of genomic RNA, respectively (although the former RNA had four extra bases) (lanes 3 and 7). In contrast, the binding activity decreased appreciably with

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Fig. 7. Binding of NS1 to truncated virus RNAs. (a) NS1 binding to various truncated NS(−) and M(−) RNAs. To the binding buffer containing 1 μg NS1 (odd-numbered lanes) or without NS1 (even-numbered lanes), 32P-labelled in vitro transcripts were added: 200 fmol NS(−) (lanes 1 and 2), 400 fmol of NS(−)(189–890) (lanes 3 and 4), 400 fmol of NS(−)(189–769) (lanes 5 and 6), 400 fmol of NS(−)(1–35) (lanes 7 and 8), 400 fmol of M(−)(739–1027) (lanes 9 and 10), 400 fmol of M(−)(739–891) (lanes 11 and 12), 400 fmol of M(−)(1–29) (lanes 13 and 14). After incubation, the mixtures were digested with RNase A and subjected to gel electrophoresis. (b) NS1 binding to truncated plus- or minus-sense RNA of other virus genomes segments. These 32P-labelled RNAs were synthesized in vitro as described in Methods. The plus-sense (+) and the minus-sense (−) RNAs carry the 3'- and 5'-terminal common sequences, respectively. In lanes 1 to 6, NS1 binding to NP RNAs was examined. To the binding buffer containing 1 μg NS1 (lanes 1, 2, 4 and 5), or no NS1 (lanes 3 and 6), 400 fmol of 32P-labelled NP(−)(5') (lanes 1 to 3) or NP(+)(3') (lanes 4 to 6) was added. RNase digestion was performed in lanes 2, 3, 5 and 6, but not in lanes 1 and 4. In lanes 7 to 14, 1 μg of NS1 was incubated with 400 fmol of 32P-labelled NA(−)(5'), NA(+)(3'), HA(−)(5'), HA(+)(3'), PA(−)(5'), PA(+)(3'), PB1(−)(5') and PB1(+)(3'), respectively. RNA in lanes 7 to 14 was digested with RNase.
RNA with deletions in both terminal regions (lane 5). The binding activity of full-length M(−) RNA was moderate (Fig. 3, compare lanes 12 and 2) and increased with truncated RNAs retaining either terminal region. A 5′-terminal region as short as 29 nucleotides [M(−)(1−29)] exhibited good binding activity, as did M(−)(739−1027), which has the 3′-terminal region (lanes 13 and 9). Removal of both terminal regions eliminated binding activity (lane 11).

The binding activity of plus-sense RNAs was very weak compared to that of their minus-sense counterparts, even if they had intact common terminal sequences. Preferential binding to viral minus-sense RNAs was also observed for NP, NA, HA, PA and PB1 RNAs, as shown in Fig. 7(b). The presence of extra sequences at either end of viral RNA had little if any effect on binding activity.

The common sequences exhibiting NS1-binding activity are both common terminal sequences of viral genomic RNA. NS1 may discriminate between and bind to either of these sequences. Deoxyoligonucleotides of these common sequences neither competed with the NS1-binding activity of these RNAs nor formed complexes with NS1 (data not shown), indicating that NS1 is an RNA-binding protein.

**Discussion**

In this report we have demonstrated that the NS1 polypeptide binds preferentially to minus-sense RNAs of influenza virus segments 7 and 8 having either 5′- or 3′-terminal common sequence (Fig. 3 and 7a). This preferential binding has also been observed with minus-sense RNAs of segments 2, 3, 4, 5 and 6 having the 5′-terminal sequence (Fig. 7b). These observations strongly suggest that NS1 recognizes both the 5′- and 3′-terminal common sequences of virus minus-sense RNAs. If it is assumed that the interaction is sequence-dependent, it is important to elucidate how each of the two common sequences participates in the binding, whether independently or cooperatively as a hybrid. NS1 is an RNA-binding protein because it bound neither to oligodeoxy-nucleotides of these common terminal sequences nor to ssDNA copies of virus minus-sense RNAs (data not shown). Further studies, including footprinting experiments, are now in progress to analyse the binding properties and the exact binding sequences in the viral RNAs. Thirteen and 12 nucleotides at the 5′ and 3′ termini, respectively, of influenza type A vRNAs are common to all RNA segments, except for a variation in the fourth base from the 3′ end. These common sequences show partial inverted complementarity and form a hybrid in the virion and the infected cell (Hsu et al., 1987). Thus, it is possible that NS1 interacts with the hybrid region of vRNAs which is thought to be associated with viral polymerase proteins and NP in the infected cell, and regulates either viral transcription, replication or encapsidation, for which the terminal common sequences are postulated to have roles as recognition signals. Previous studies of two ts mutants of segment 8 have suggested an involvement of the protein in the synthesis of vRNA (Wolstenholme et al., 1980). However, there is no evidence from studies of NS1 mutants that NS1 participates in viral mRNA synthesis or in vitro viral transcription.

In a previous paper we reported that the synthesis of viral late proteins and of NS1 by two independent ts mutants decreased severely without affecting the level of viral mRNA synthesis (Hatada et al., 1990). The observations suggest that NS1, most of which is localized in the nucleus at least during the first few hours of infection, is involved in some post-transcriptional process in the synthesis of the late proteins and NS1. There are several possible models for this mechanism. One possibility is that NS1 regulates the nuclear–cytoplasmic transport of mRNAs encoding these proteins, which is not the case for M mRNA (Hatada et al., 1990). Another is the gene gating hypothesis proposed by Blobel (1985). NS1 may locate vRNA (the template to be transcribed) via specific interactions with the terminal sequences, in a nuclear subcompartment favourable for translation factor assembly, processing factor and other factors necessary for post-transcriptional control. The RNA transcribed in this subcompartment would be efficiently transported to and translated in the cytoplasm.

As indicated by competition experiments (Fig. 6), NS1 interacted with 28S rRNA. This observation is in agreement with that of Yoshida et al. (1981), who reported that cytoplasmic paracrystalline inclusions are composed of NS1 in association with RNA, including 28S rRNA (in BHK cells), but not poly(A)-containing RNA.

Computer searches of rRNA sequences were performed to find those resembling the 5′- and 3′-terminal common sequences of the minus-strand. As data were not available for canine rRNAs, the sequences of human and mouse rRNA were employed, both of which gave the same results. Six matching sequences of six bases each are present in 28S rRNA and the 5′-terminal common sequence (Fig. 8, sequences a to f), and there are one seven-base and four six-base matches between 28S rRNA and the 3′-terminal common sequence. On the other hand, one seven-base (Fig. 8, sequence g) and one six-base homology (sequence b) are present in 18S rRNA and the 5′-terminal common sequence, whereas no significant homology is found between 18S rRNA and the 3′-terminal common sequence. From competition
28S rRNA (human and mouse)

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\begin{align*}
&5' \text{ consensus of minus strand} \\
&\text{AGUACAAACAAACGG} \\
&3' \text{ consensus of minus strand} \\
&\text{CCUGCUUUUCCU}
\end{align*}
\]

Six six-base homologous sequences

Fig. 8. Identification of regions of homology rRNA sequences and the common terminal sequences of the viral minus-sense RNA. For the analysis, DNASIS-DBREF50 (Ver. 7.0) (Hitachi SK software) was employed.

experiments (Fig. 6) we can deduce that sequence a, c or d (Fig. 8) of 28S rRNA is involved in the NS1 interaction. Of these, sequence d is present in the sequence of the NS plus strand and can be excluded. It is thus suggested that either sequence a or sequence c is the most probable part of the 5' terminus which is recognized by NS1. Detailed analysis of the NS1-rRNA interaction in vivo and in vitro is in progress.

When this manuscript was in preparation, Skorko et al. (1991) reported that NS1 binds non-specifically to all ssRNAs and has greater RNA-binding activity than was shown in this study. In the absence of a detailed description of their binding conditions, we cannot explain this discrepancy, although they did not perform an RNase protection assay. We clearly demonstrated that NS1 binding to minus-sense RNA was greater, even in the absence of RNase digestion (Fig. 7b, compare lanes 1 and 4).

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References


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