Binding of influenza A virus NS1 protein to dsRNA in vitro

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The non-structural protein NS1 of influenza A virus exhibits two modes of RNA-binding activity. One is sequence-specific binding to minus-sense virus RNA with either a 5'- or 3'-terminal common sequence as reported previously. The other was identified as binding to dsRNA and this activity did not show sequence specificity. The affinity of binding to dsRNA was much higher than that to ssRNA. A short miniature virion RNA forming a panhandle structure by pairing between the 5'- and 3'-terminal common sequences bound NS1 with higher affinity and stability than did a dsRNA of similar sequence and length.

Non-structural protein 1 (NS1) of influenza virus accumulates in the nucleus and nucleolus of infected cells. It is also associated with polysomes, but is not incorporated into virions (for a review see Lamb, 1989). The protein is encoded by RNA segment 8 together with another non-structural protein, NS2 (Inglis et al., 1980; Lamb & Lai, 1980). The function(s) of NS1 remains to be determined despite many studies including analysis of temperature-sensitive mutants of the protein (Lamb, 1989; Hatada et al., 1990; Alonso-Caplen et al., 1992), having been performed.

Recently, we have demonstrated that NS1 purified from Escherichia coli cells carrying a cloned NS1 cDNA exhibits specific binding activity with minus-sense but not plus-sense virus (v) RNA. NS1 binds preferentially to the regions of the RNA containing either 5'- or 3'-terminal common genomic sequences (Hatada et al., 1992). In this communication, we identify another NS1 RNA-binding activity, binding to dsRNA, and compare it to the binding activity with ssRNA, including a miniature vRNA.

As reported previously (Hatada et al., 1992), the RNA-binding activity of NS1 was studied using an RNA mobility shift assay and a ribonuclease protection assay. NS1 was purified from E. coli cells, in which the synthesis of the protein from a cDNA copy of A/PR/8/34 (H1N1) virus segment 8 inserted in the expression vector pKK233-2 had been induced using IPTG. RNAs (32P-labelled or unlabelled) were transcribed in vitro from linearized plasmids containing virus cDNAs and other DNAs under the control of bacteriophage SP6, T7 or T3 promoters.

In Fig. 1(a), [32P]RNA (0-6 pmol) containing 59 bases from the 3'-terminal sequence of genome segment 8 [NS(−)(832–890)] exhibits an upward shift on an RNA mobility shift assay in the presence of excess NS1 (38 pmol) (lane 1), indicating the formation of an NS1-binding complex resistant to RNase (lane 2), in agreement with previous results (Hatada et al., 1992). When the [32P]RNA was annealed to unlabelled cRNA [NS(+)(832–890)] (1 pmol), the hybrid RNA thus formed exhibited a strong NS1-binding activity (lane 3) which was RNase-resistant (lane 4). The hybrid RNA between NS(−)(832–890) and NS(+)(832–890) carried non-complementary oligonucleotide tails originating from the vector sequences which were removed by pretreating with RNase T2 to obtain the dsRNA used in the binding experiment for lane 5. The samples in lanes 3 to 5 revealed the same level of strong NS1 binding to the hybrid RNA; no free dsRNA was detected (lanes 4 and 5). The binding to dsRNA appeared to be much stronger than the binding to ssRNA, which was specific for virus minus-sense RNA carrying either 5'- or 3'-terminal common sequences (compare lanes 3 to 5 with lane 1). In Fig. 1(b), M(−)(739–891) ssRNA, with a deletion of both common terminal sequences, exhibited no binding to NS1, in agreement with previous results (Hatada et al., 1992) (lanes 1 and 2). In contrast, all [32P]RNA was shifted to the NS1 binding complex when converted to the dsRNA form (lanes 3 and 4).

In Fig. 1(c), non-viral RNA was tested. An RNA of 28 bases and its partner of 60 bases carrying the complementary sequence were transcribed from BamHI-digested pTZ18U and EcoRI-digested pTZ19U vector DNAs, respectively. NS1 could not bind to either of these ssRNAs (lanes 1 to 3, and 4 to 6). When these RNAs were annealed and digested with RNase T2 to remove the single-stranded region, the resulting dsRNA (28 bases) (lane 7) produced a strong NS1-binding activity (lane 8). The NS1-binding complex was shifted...
Fig. 1. NS1 binding to ss- and dsRNAs. RNA binding buffer containing 1 μg NS1 (38 pmol) was incubated with 32P-labelled RNA probes as described below for 30 min at 30 °C, and subjected to 4% PAGE (acylamide : bisacylamide, 60 : 1). For details see Hatada et al. (1992). (a) 3P-labelled NS(−)(832–890) RNA probe (0-6 pmol) carrying the 3′-terminal common sequence. [32P]ssRNA (lanes 1, 2 and 6); [32P]ssRNA annealed to 1 pmol NS(+)(832–890) RNA carrying non-complementary short tails originating from the vector sequences (lanes 3 and 4); [32P]dsRNA [32P-ssRNA annealed to NS(+)(832–890) RNA and digested with RNase T2 to remove the ssRNA tails] (lanes 5 and 7). After the binding reaction, incubation was continued in the presence (lanes 2, 4 and 5) or absence (lanes 1, 3, 6 and 7) of RNase T2. Probe RNAs incubated without NS1 (lanes 6 and 7). (b) 32P-Labelled M(−)(739–891) RNA probe (0-4 pmol) with both common terminal sequences deleted. [32P]ssRNA (lanes 1, 2 and 5); [32P]dsRNA [32P-ssRNA annealed to a full-length M(+) RNA and digested with RNase T2 to remove ssRNA regions] (lanes 3, 4 and 6). After the binding reaction, incubation was continued in the presence (lanes 2 and 4) or absence (lanes 1, 3, 5 and 6) of RNase T2. Probe RNAs incubated without NS1 (lanes 5 and 6). (c) 32P-labelled non-viral RNA probes (0-6 pmol) transcribed from vector DNAs. [32P]ssRNA (28 bases, transcribed from pTZI8U DNA digested with BamHI) (lanes 1 to 3); [32P]ssRNA (60 bases, transcribed from pTZ19U DNA digested with EcoRI) (lanes 4 to 6); the following [32P]ssRNAs (0-6 pmol) were hybridized to the annealing partner of the 60 base ssRNA (0-6 pmol), and digested with RNase T2 to obtain [32P]dsRNA of 28 bp (lanes 7 and 8), [32P]dsRNA of 42 bp ([32P]ssRNA transcribed from pTZ18U DNA digested with HindIII) (lane 9), and [32P]dsRNA of 58 bp ([32P]ssRNA transcribed from pTZ18U DNA digested with HindIII) (lane 10). These probe RNAs were incubated in the presence (lanes 2, 3, 5, 6, 8, 9 and 10) or absence (lanes 1, 4 and 7) of NS1, followed by a further incubation with (lanes 3 and 6) or without (all other lanes) RNase T2. All the following figures except Fig. 5 show autoradiograms of gel shift assays. Binding complexes and free RNA probes are indicated by brackets, and closed (ssRNA) and open (dsRNA) arrowheads, respectively.

to a higher position depending on the length of the dsRNA, produced using pTZ18U DNAs digested with PstI (42 bp) and HindIII (58 bp) (lanes 9 and 10, respectively). NS1 did not bind to dsDNAs (data not presented). Thus we concluded that NS1 has a high but non-specific binding affinity for dsRNA. To characterize further NS1 binding to dsRNA and to compare it with that to ssRNA, the following experiments were performed.

In Fig. 2, an increasing amount of NS1 was incubated with a constant amount (0-1 pmol) of [32P]NS(−)(1–174) ssRNA carrying the 5′-terminal common sequence, and its [32P]dsRNA derivative. With ssRNA (Fig. 2a), an increasing amount of the binding complex was detectable when 7-6 pmol or more of NS1 was used (lane 8 of a longer exposure; data not presented). The complexes were diffuse, and unbound RNA remained even in the presence of excess NS1, indicating a weak interaction between NS1 and ssRNA. With dsRNA (b), the binding complex was detected with 0-95 pmol or more of NS1 (lane 6 of a longer exposure), and no free dsRNA was observable with >15-2 pmol NS1 (lanes 9 to 12). The binding complex moved upward in proportion to the addition of an increasing amount of NS1; the highest position was attained with a 300-fold molar excess of NS1 (lane 10), indicating that more than one molecule of NS1 bound to a dsRNA molecule. In contrast, no such upward transition of the complex was observed with ssRNA (Fig. 2a, lanes 8 to 12).

Competition experiments were performed to compare the binding affinity of NS1 for dsRNA and ssRNA. In Fig. 3a, the NS1-binding complex of [32P]NS(−)(1–174) ssRNA was stable in the presence of a 10-fold molar excess of unlabelled NS(−)(1–174) ssRNA during 30 min incubation at 30 °C (lane 6). However, much of the complex was dissociated in the presence of a 10-fold
Fig. 2. Effect of the amount of NS1 on the formation of binding complex. [$^{32}$P]NS(−)(1–174) ssRNA (0.1 pmol) (a) and [$^{32}$P]dsRNA derivative (0.1 pmol) (b) were incubated with an increasing amount of NS1, from lanes 1 to 12, 0, 0.12, 0.24, 0.48, 0.95, 1.9, 3.8, 7.6, 15.2, 30.4, 60.8 and 121.6 pmol. The binding complex was treated with RNase A in (a). For a description of labelling see the legend to Fig. 1.

molar excess of the dsRNA derivative (lane 10). Preincubation of NS1 with these unlabelled RNAs greatly reduced complex formation (lanes, 4 and 8). In contrast, the binding affinity of NS1 for dsRNA was much stronger than that for ssRNA, and the complex was stable (Fig. 3b). Preincubation of NS1 with a 10-fold molar excess of the ssRNA did not affect NS1 binding to the dsRNA derivative (lane 4), whereas faster-moving binding complexes of broad bands and free [$^{32}$P]dsRNA were observed on preincubation with a 10-fold molar excess of dsRNA (lane 8). The dsRNA binding complex remained unchanged upon 30 min incubation at 30 °C in the presence of a 10-fold molar excess of unlabelled dsRNA, although a downward shift and a trace amount of free probe were observed (lane 10).

Fig. 4 shows the effect of temperature on the binding reaction. Only a little NS1 binding was observed with ssRNA at 0 °C and 4 °C. In contrast, almost the same level of complex was formed with dsRNA at incubation temperatures between 0 and 30 °C (and 40 °C, data not presented).

The 5'- and 3'-terminal sequences of influenza virus vRNA show partial inverted complementarity and form a hybrid in the virion and the infected cell (Hsu et al., 1987). We examined the ability of NS1 to bind to the hybrid region of vRNAs, as suggested previously (Hatada et al., 1992). To increase the likelihood of hybrid formation, a miniature vRNA of 71 bases, NS(−)(1–38, 859–890), was constructed (Fig. 5). It was expected that an incomplete hybrid would be formed between the 5'-terminal 16 and the 3'-terminal 15 bases, which were connected by an insertion of 40 bases, thus forming a panhandle structure. In Fig. 6, the NS1 binding ability of this RNA is shown in comparison with that of NS(−)(1–35) dsRNA of 35 bp. The miniature vRNA exhibited a high level of NS1 binding which was not inhibited by a 10-fold molar excess of NS(−)(1–174) ssRNA (lanes 1 to 3). The NS(−)(1–35) dsRNA also exhibited a high level of NS1 binding not inhibited by excess NS(−)(1–174) ssRNA (lanes 8 to 10). After preincubation of 38 pmol NS1 with 1 pmol of NS(−)(1–174) dsRNA, which completely inhibited NS1 binding to 0.1 pmol of NS(−)(1–35) dsRNA (lane 11), 60% of the miniature vRNA still remained as an NS1-binding complex, although the complex band broadened and moved downward (lane 4). When the miniature vRNA binding complex was incubated with a 10-fold molar excess of NS(−)(1–174) dsRNA for 30 min at 30 °C, 80% of the $^{32}$P stayed in the complex, although the complex band broadened and moved downward, indicating dissociation of some of the NS1 molecules from the complex during incubation. With the same treatment, the dsRNA binding complex disappeared, in contrast with the results shown in Fig. 3 (compare Fig. 6, lane 12 with Fig. 3b, lane 10). This contradiction might be explained as follows. (i) The competitor RNA, NS(−)(1–174) dsRNA, was about six times the length of the probe RNA, NS(−)(1–35) dsRNA, providing a 60-fold molar excess of NS1-binding sites; (ii) dsRNAs longer than 35 bp generally give a more stable complex. The same level of binding complex was formed at 0 °C as at 30 °C with the miniature vRNA as well as with the dsRNA (lanes 6 and 13).

In this report we have demonstrated that the NS1 polypeptide exhibits two modes of RNA-binding activity. One is the sequence-specific binding to ssRNA. As described previously, NS1 binds preferentially to influenza virus minus-sense RNA with either a 5'- or 3'-terminal common sequence (Hatada et al., 1992). Footprinting experiments have shown that NS1 associates with the terminal common sequences (unpublished results). The association depends on temperature, but is so weak as to have a tendency to dissociate during
Fig. 3. Competition between ss- and dsRNAs for NS1 binding. For the binding reaction, 1 μg of NS1 was incubated with 0-1 pmol of [32P]NS(-)(1-174) ssRNA (a) or 0-1 pmol of the [32P]dsRNA derivative (b). Prior to addition of the [32P]RNAs, NS1 was preincubated with the buffer alone (lane 2), or with the following unlabelled RNAs for 30 min at 30 °C (a and b): 0·1 pmol or 1 pmol of NS(-)(1-174) ssRNA (lanes 3 and 4, respectively); 0·1 pmol or 1 pmol of the dsRNA derivative (lanes 7 and 8). To investigate the stability of the complex, the binding reaction with [32P]RNAs was followed by further incubation with unlabelled RNAs for 30 min at 30 °C. These were (lanes 5 and 6) 0·1 or 1 pmol of NS(-)(1-174) ssRNA and (lanes 9 and 10) 0·1 or 1 pmol of the dsRNA derivative. In (a) the binding complex was treated with RNase A (except in lane 1) prior to gel electrophoresis. [32P]ssRNA or [32P]dsRNA (0·1 pmol each) alone was electrophoresed in lane 1 (a and b respectively). For a description of labelling see the legend to Fig. 1.

Fig. 4. Effect of incubation temperature on NS1 binding to ss- and dsRNAs. Binding buffer containing 1 μg of NS1 was mixed with 0-1 pmol of the following [32P]RNAs: NS(-)(1-35) ssRNA (lanes 1 to 3) and its dsRNA derivative (lanes 4 to 6) or NS(-)(1-174) ssRNA (lanes 7 to 9), and its dsRNA derivative (lanes 10 to 12). The mixtures were incubated for 30 min and then digested with RNase A for 15 min at 30 °C (lanes 1, 4, 7 and 10), 0 °C (lanes 2, 5, 8 and 11) or 4 °C (lanes 3, 6, 9 and 12). For a description of labelling see the legend to Fig. 1.

Fig. 5. The structure of NS(-)(1-38, 859-890) RNA, the miniature vRNA. Nucleotides from the AluI site (nucleotide 36 counting from the 5' end of the NS vRNA) to the BamHI site (nucleotide 857) were removed from the influenza virus A/PR/8/34 NS gene cloned in pT3/PR8-NS (Enami & Palese, 1991). The ends of the NS gene (the 5'-terminal 35 bp and the 3'-terminal 33 bp) remaining in the plasmid were ligated using a BamHI linker. The resulting plasmid DNA was digested with BsmI and used as the template for T3 polymerase run-off transcription to obtain NS(-)(1-38, 859-890) RNA. The secondary structure of the RNA was predicted using the DNASIS program (Hitachi SK software). The total energy of this secondary structure was $-60.7$ to $-55.6$ kJ/mol, depending on the unstable secondary structure assumed by the loop region.

The gel shift assay. The other NS1-binding activity, which does not depend on temperature, is to dsRNA, and does not have sequence specificity. More than one molecule of NS1 binds per dsRNA molecule, and the binding affinity is much greater than that for ssRNA. The affinity was higher with dsRNA of longer than 35 bp. It is not known how the two modes of binding
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Fig. 6. Characterization of NS1 binding to a miniature vRNA compared with its binding to dsRNA. NS1 (1 μg) was incubated with 0.1 pmol [32P]NS(−)(1-35, 859-890) RNA (lanes 1 to 6) or [32P]NS(−)(1-35) dsRNA (lanes 8 to 13) under the following conditions. No competitor RNA (lanes 1 and 8); prior to addition of [32P]RNA, NS1 was preincubated with 1 pmol NS(−)(1-174) ssRNA (lanes 2 and 9), or its dsRNA derivative (lanes 4 and 11); after the binding reaction, the binding complex was incubated with 1 pmol of NS(−)(1-174) ssRNA (lanes 3 and 10) or its dsRNA derivative (lanes 5 and 12). All incubations were performed for 30 min at 30 °C except for those in lanes 6 and 13, in which NS1 and [32P]RNAs were incubated for 30 min at 0 °C without competitor RNA. [32P]RNAs alone were electrophoresed in lane 7 (the miniature vRNA) and lane 14 (dsRNA). For a description of labelling, see the legend to Fig. 1.

activity are interrelated in such a small molecule, except that the specific binding to ssRNA is inhibited by dsRNA, indicating close proximity of the two binding sites.

NS1 can bind to a miniature vRNA with greater affinity and stability than to a dsRNA of similar nucleotide sequence and length. Thus, it is suggested that the panhandle structure of vRNA, bearing unpaired bases and a bulge, has greater and more stable binding ability than a longer and complete hybrid of the same sequences. Several recent studies suggest that irregularities in the structure of the RNA phosphodiester backbone, such as bulging and non-canonical base pairing, provide the basis for recognition by sequence-specific RNA-binding proteins (Calnan et al., 1991; Bartel et al., 1991). In our previous report, a full-length vRNA did not exhibit such high binding affinity. Possibly the formation of other secondary structures interferes with the formation of the panhandle structure. We are investigating the functional role(s) of the NS1-binding activity during virus growth.

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References


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