Binding of the influenza virus NS1 protein to model genome RNAs

Eriko Hatada,¹ Sakura Saito,² Nobuyuki Okishio¹ and Ryuji Fukuda¹

1 Department of Biochemistry, Kanazawa University School of Medicine, Kanazawa, Ishikawa 920, Japan
2 Laboratory of Cytokines, Department of Viral Disease and Vaccine Control, National Institute of Health of Japan, Musashimurayama, Tokyo 190-12, Japan

We have previously shown that the influenza virus NS1 protein exhibits stable binding to a model mini vRNA which is stronger than its binding to dsRNA. In this study, we confirmed that the binding depended on a higher-order structure of the model RNA, probably the panhandle structure formed by pairing between the 5′- and 3′-terminal common sequences. The formation of the structure was enhanced by the NS1 protein itself. We propose that an A bulge in a stretch of double helix results in a binding site with greater affinity for the NS1 protein.

Various RNA-binding activities of influenza virus NS1 protein have been reported (Skorko et al., 1991; Hatada et al., 1992; Hatada & Fukuda, 1992; Qiu & Krug, 1994; Qiu et al., 1995; Park & Katze, 1995), and correlated with regulation operating at various post-transcriptional steps (Qiu & Krug, 1994; Qian et al., 1994; Qiu et al., 1995; Lu et al., 1995), although no NS1 mutants harbouring the predicted defects have been reported. We previously demonstrated that the NS1 protein exhibited two modes of RNA-binding activity (Hatada & Fukuda, 1992). One, a strong binding to dsRNA, which has no base sequence specificity and does not depend on temperature; the other, a base-sequence-preferential binding to virion RNA (vRNA). vRNA fragments containing either the 5′- or 3′-terminal common sequence exhibit temperature-dependent NS1-binding activity which is much weaker than that of dsRNA. In contrast, a model mini vRNA, NS(−)(1–38, 859–890)ssRNA (segment 8 vRNA with nucleotides 39–858 deleted), carrying both 5′- and 3′-terminal common sequences of vRNA, which is expected to form a panhandle structure (Robertson, 1979), binds NS1 with greater affinity and stability than dsRNA of a similar nucleotide sequence and length (Hatada & Fukuda, 1992). This finding suggested that the panhandle structure of the mini vRNA, bearing unpaired bases and bulges, endows the RNA with a greater NS1-binding ability.

For the binding experiments in Fig. 1, base substitutions were introduced in the mini vRNA, and the NS1-binding activities of these mutant RNAs were examined in the presence or absence of a 10-fold molar excess of dsRNA competitor, NS−(1–174)dsRNA. Deletion of a single A, either A10 or A11 (%AA10/11), from the mini vRNA considerably reduced binding resistant to dsRNA competition (Fig. 1b, lanes 3 and 4). On substitution of U for C at position 1 in this A10/11-deletion mutant (thus giving complete complementarity to the panhandle) (%AA10/11C63U), the binding activity of the resulting double-mutant vRNA was also greatly decreased (lanes 7 and 8). In contrast, reintersion of A into this double-mutant RNA (resulting in mini vRNA C63U) increased the binding activity to more than that of the original RNA (compare lanes 5 and 6 with 1 and 2). It was thus inferred that A10/11 was important for the strong NS1-binding ability of the mini vRNA. In contrast to the mini vRNA, the mini cRNA [NS(+)1–28, 865–890]ssRNA] exhibited weak NS1-binding ability, and scarcely gave any NS1-binding complex resistant to dsRNA competition (lanes 9 and 10, respectively). Baudin et al. (1994) reported a detailed analysis of the secondary structure of a model vRNA, the panhandle domain of which is almost identical to ours. Based on their panhandle model, we predicted the possible conformation for our model vRNAs and mutant RNAs shown in Fig. 1a (the internal sequences of our RNAs didn’t give significant complementarity, and have been omitted from the figure). The panhandle domain consists of a helix containing an inner loop and a bulge. C9 and C65 interact together by non-canonical hydrogen bonds, and C9A10 and G62C63 form an inner loop. A4 forms a single-base bulge, which may be buried inside the helix. Deletion of A10/11 presumably led to base pairings between A10/11 and U61, and between C9 and G62, resulting in the formation of a helix without the bulge at A4, which now paired with U67. The mutant AA10/11 RNA formed a helix containing 14 bp and an inner loop (A8 and C65), while the ΔA10/11C63U RNA formed a double helix (15 bp) without loops and bulges. The NS1-binding activities of these RNAs were very sensitive to competition with dsRNA. The reintroduction of A10/11...
Fig. 1. NS1 binding by model mini vRNA mutants. (a) Sequences and presumed secondary structures of the panhandle domains of the mini vRNAs and a mini cRNA. The A10/11 deletion is shown by (y) and the mutated base is underlined. The secondary structure of the wild-type mini vRNA was predicted from Baudin et al. (1994), while those of the mutant vRNAs are based on speculation (see text), and that of the mini cRNA was predicted by a computer method (Zuker & Stiegler, 1981). The bars and dots between bases indicate Watson–Crick bonds and non-canonical hydrogen bonds, respectively. These RNAs, 32P-labelled or not, were prepared by transcribing in vitro the linearized plasmid DNAs, which were constructed as described by Hatada & Fukuda (1992), and mutagenized according to Kunkel et al. (1987). The transcripts were purified by PAGE. (b) RNA mobility shift assay to detect the NS1-binding ability of the mini vRNAs. Binding buffer containing 0±5 µg of the recombinant NS1 protein (19 pmol), which was expressed in E. coli and purified as described (Hatada et al., 1992), was preincubated in the absence (odd-numbered lanes) or in the presence (even-numbered lanes) of 1 pmol unlabelled competitor dsRNA, NS(–) (1–174)dsRNA, for 30 min at 30°C, then mixed with 0±1 pmol 32P-labelled RNAs – wild mini vRNA (lanes 1 and 2), ∆A10/11 (lanes 3 and 4), C63U (lanes 5 and 6), ∆A10/11C63U (lanes 7 and 8), mini cRNA (lanes 9 and 10), NS(–) (1–35)dsRNA (lanes 11 and 12) – and further incubated for 30 min at 30°C. They were then subjected to non-denaturing 4% PAGE. The bands marked with an asterisk were specific to the model vRNAs incubated with NS1 and were very sensitive to RNase digestion, in contrast with the binding complexes which were RNase-resistant. They are usually not as prominent as shown here. We did not consider them in this paper, as their nature has not been defined. At the bottom of the figure is shown the density of the binding complex, measured by a BAS 1000 bio-imaging analyser (Fuji), corrected for the content of U, the labelled nucleotide, and expressed as a percentage of the value of the complex in lane 1. For additional details of the methods, see Hatada et al. (1992) and Hatada & Fukuda (1992).

endowed the mutant RNA with greater and stronger NS1-binding ability than the dsRNA, as exhibited by the wild-type RNA and C63U. Insertion of A10/11 into the ∆A10/11 RNA results in the A bulge as described above (the original wild RNA), while insertion into the ∆A10/11C63U RNA presumably led to the formation of an A10/11 bulge (the C63U RNA). It may thus be speculated that an A bulge in a stretch of double helix forms a preferred binding site for the NS1 protein. Qiu et al. (1995) have demonstrated binding of the NS1 protein to a stem–bulge structure of human U6 snRNA, the purine-containing bulge of which directly interacts with the protein. It has been shown that irregularities in the structure of an RNA duplex, such as bulging and non-canonical base pairing, provide the basis for recognition by sequence-specific RNA-binding proteins (for a review see Draper, 1995).

The mini vRNA formed the NS1-binding complex on incubation at 0°C as well as at 30°C (Fig. 2, lanes 1, 3 and 9). After denaturation, the RNA was unable to form a binding complex at 0°C (lanes 2 and 10); neither was the denatured dsRNA (compare lane 5 with 4 or 6). When the denatured RNAs were incubated for 2 h at 55°C and then cooled to room temperature, they again formed the NS1-binding complex at 0°C (lane 11 and data not shown). These observations indicate that a higher-order structure, probably the panhandle structure of the mini vRNA, was a prerequisite for stronger NS1-binding, and such structures were not formed so long as the denatured RNA was kept at 0°C.

The experiments in Fig. 3 were undertaken to examine the possibility that NS1 enhances the formation of a higher-order structure of the mini vRNA. The binding reaction was carried...
out in a two-step incubation in a low salt buffer, which was better than the ordinary binding buffer for examining the effect of RNA structure on NS1 binding. Moreover, NS1 binding to ssRNA at 30 °C (Cₚ) was negligible in this buffer (unpublished observation). In the first incubation of experiment A (Fig. 3a, top), the denatured mini vRNA was incubated with NS1 for 0, 5, 3 or 30 min at 30 °C; the mixture was then kept at 0 °C in the second incubation for another 30 min. A higher-order RNA structure would form during the first incubation as well as during the second incubation at 0 °C. We could thus estimate the formation of the higher-order structure by measuring the amount of the NS1-binding complex, which might consist of two components: one, due to the RNA structure promoted by NS1, designated Cₚ/NS₁, the other, due to RNA structure promoted by interaction with NS1, designated Cₚ/NS₁. The amount of Cₚ/NS₁ was determined by experiment B, in which the denatured mini vRNA was incubated in the absence of NS1 in the first incubation at 30 °C. The higher-order structure formed during the incubation at 30 °C gave the binding complex, Cₚ/NS₁, on addition of NS1 in the second incubation at 0 °C. Binding to a single-stranded terminal (Cₚ) is negligible as noted above. The binding complexes in experiment A increased in amount by about 3-fold compared to those in experiment B (Fig. 3a, lanes 1–3 and 4–6). This increase might correspond to Cₚ/NS₁. It was thus apparent that the formation of a higher-order structure of the mini vRNA, which was required for stable NS1-binding, increased in the presence of NS1 protein. In contrast, no measurable binding complex was observed with the ΔA¹⁰¹/¹₁ RNA under these incubation conditions (Fig. 3a, lanes 7–12). The NS1-binding complexes shown in Fig. 3(a) as well as those bound to other mutant RNAs (autoradiogram not shown) were quantified as shown in Fig. 3(b). The same enhancing effect of NS1 was observed by using the mutant C₆₃U RNA (Fig. 3b, lanes 13–18), but not the ΔA¹⁰¹/¹₁C₆₃U RNA, nor the mini cRNA (Fig. 3b, lanes 19–24 and 25–30, respectively). It was thus inferred that NS1 enhanced the formation of a higher-order structure, probably the panhandle structure, of the model vRNA, which was required for stronger NS1-binding. Two possibilities are conceivable concerning the mechanism of the enhancing effect of NS1. One is that NS1 molecules weakly bind to each terminal sequence (Hatada et al., 1992) and may associate to form a dimer (Nemeroff et al., 1995), thus acting as a chaperone for reannealing the termini. The other is that the presence of NS1 may simply lead to displacement of the equilibrium between the denatured RNA and the folded RNA by sequestering the folded state. So far, we have no evidence to support the former possibility.

Hsu et al. (1987) have shown that a panhandle structure is formed in virus ribonucleoproteins in infected cells as well as in virions, and suggested that the structure can serve as a regulatory signal for transcription and replication, as well as for packaging RNA into virus particles. This structure in combination with a run of five to seven uridine residues serves as a transcription termination and polyadenylation signal (Luo et al., 1991; Li & Palese, 1994). It has also been suggested that both 5' and 3' ends of the viral genome are needed for activation of the virus RNA polymerase complex in vitro (Hagen et al., 1994; Cianci et al., 1995), and are involved in virus transcription initiation in vivo as well as in vitro (Fodor et al., 1995; Neumann & Hobom, 1995). The possibility of involvement of NS1 in those processes requiring the panhandle structure of vRNA is open to further studies.

![Fig. 2. Dependence of NS1-binding on the secondary structure of RNAs. Binding buffer containing 0.5 µg of NS1 was mixed with 0.1 pmol 32P-labelled RNAs which had been treated in the binding buffer as follows; incubated at 0 °C (native form, N; lanes 1, 3, 4, 6 and 9); heated for 3 min at 95 °C and then chilled at 0 °C (denatured form, D; lanes 2, 5 and 10); heated for 3 min at 95 °C, incubated for 2 h at 55 °C and then cooled down to room temperature (renatured form, lane 11). Wild mini vRNA (lanes 1–3 and 9–11), NS(1–35) dsRNA (ds, lanes 4–6). The mixtures were incubated for 30 min at 30 °C (lanes 1 and 4) or 0 °C (lanes 2, 3, 5, 6, 9, 10 and 11), and subjected to non-denaturing 4% PAGE. Lanes 7 and 8, 32P-mini vRNA alone, in the native form and the denatured form, respectively.](http://www.microbiologyresearch.org)
The NS1 protein enhanced the formation of a higher-order structure of model vRNAs to which NS1 could be stably bound. (a, top) The binding assays were performed in two-step incubations in a low-salt binding buffer (0–33 mM KCl) using 0.1 pmol of heat-denatured (3 min at 95 °C) 32P-RNAs. The incubation procedures are shown with the types of binding complex expected to be formed. In procedure A the denatured 32P-RNA was mixed with 0.5 µg NS1 and incubated for t min (0 or 30 min) at 30 °C (first incubation), and then incubated for 30 min at 0 °C (second incubation). In procedure B the denatured 32P-labelled RNA was incubated in the absence of NS1 for t min (as above) at 30 °C (first incubation), and then chilled to 0 °C, followed by incubation with 0.5 µg NS1 for 30 min at 0 °C (second incubation). For CP/PT, CP/NS1, and CPT see text. (a, bottom) The denatured 32P-RNAs used were the wild mini vRNA (lanes 1–6) and ∆A10/11 (lanes 7–12). Procedure A was applied to lanes 1–3 and 7–9, while procedure B was applied to lanes 4–6 and 10–12. The complexes were analysed by 4% non-denaturing PAGE. Asterisk as in Fig. 1(b). (b) The radioactivity of the binding complexes shown in (a) as well as those for the experiments using the denatured 32P-RNAs, C63U (lanes 13–18), ∆A10/11C63U (lanes 19–24) and the mini cRNA (lanes 25–30) (autoradiogram was not shown) was measured with a BAS 1000 bio-imaging analyser, corrected for the content of U, the labelled nucleotide, and expressed as a percentage of the value of the complex in lane 3. Procedure A was applied to lanes 13–15, 19–21 and 25–27, while procedure B was applied to lanes 16–18, 22–24 and 28–30.
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References


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