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Influenza Virus RNA Structure: Unique and Common Features

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The influenza A virus genome consists of eight negative-sense RNA segments. Here we review the currently available data on structure-function relationships in influenza virus RNAs. Various ideas and hypotheses about the roles of influenza virus RNA folding in the virus replication are also discussed in relation to other viruses.

Keywords influenza virus, RNA structure, negative-sense RNA virus, segmented RNA genome

INTRODUCTION

Influenza A virus is an important epidemic infectious agent in animals and humans [1–3]. It is a negative-sense segmented RNA virus of the family *Orthomyxoviridae*, and its genome consists of eight segments. These segments can be exchanged via reassortment upon infection of a single host by two strains. Gene segments 4 and 6 encode the main viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), respectively, and various combinations of these genes define different antigenic subtypes, such as H1N1, H3N2, etc. Wild waterfowl are thought to be the main reservoir of influenza A virus diversity, with

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16 known HA subtypes and 9 NA subtypes. Only three HA subtypes (H1, H2, and H3) and two NA subtypes (N1 and N2) have circulated in the human population in the last century, during pandemics (caused by reassortant viruses) and subsequent annual seasonal epidemics.

The molecular basis of influenza virus evolution and host adaptation is usually sought in differences between protein sequences from various strains, and various important determinants of host range and virulence have been identified [4–7]. On the other hand, relatively little is known about influenza virus RNA folding and its influence on influenza virus replication. In contrast, for many other viruses with RNA genomes, many data on RNA structure-function relationships have accumulated and building of structural models has considerably contributed to the understanding of virus replication cycles [8–10].

Here we summarize the available data on influenza virus RNA folding. We also discuss the various ideas and hypotheses about influenza virus RNA structure and its role in virus replication. In particular, we try to identify the analogies and differences between influenza viruses and other viruses. Apparently, such a comparison should not be restricted to the most closely related viruses, such as segmented negative-sense RNA viruses, but has to include the knowledge on RNA structures from other virus orders.

GLOBAL FOLDING OF INFLUENZA VIRUS GENOME SEGMENTS

In comparison with positive-strand RNA viruses, negative-strand RNA viruses, influenza virus included, seem to have less structured RNA on a global genome scale. The genomes of many positive-strand RNA viruses contain sequences that may be folded with free energies significantly lower than randomized sequences retaining the same dinucleotide and codon frequencies [11]. Such a pattern, coined genome-scale ordered RNA structure (GORS), is much more pronounced in positive-sense RNA viruses than in negative-sense RNA genomes.

This difference may be due to differences in replication mechanisms. The genomic RNA of negative-sense RNA viruses can serve as a template for viral polymerase only in a ribonucleoprotein (RNP) complex with the viral nucleoprotein, NP [12, 13]. In such a complex, the RNA secondary structure is completely or partially melted [13–15]. Thus, a potential for the formation of highly stable structures would probably present a barrier for efficient assembly of RNP particles.

However, in some influenza A virus genome segments, in particular, PB1, PB2, PA, and NP, a global pattern of RNA thermodynamic stability has been identified [16]. Furthermore, this pattern turned out to be host-specific, as the segments in human strains exhibited a slight trend

of lowering their stability (as judged from the increase in the lowest free energy of folding) as compared to those from avian strains. One of the explanations for this is adaptation of the virus to the host cell temperature, which is higher in birds than in humans [16]. On the other hand, the lowest free energy values of full-length segments are unlikely to represent the native structures, as such large RNA molecules usually fold into metastable conformations with folding free energies much higher than the global energy minima. Furthermore, the trend of the folding-energy increase in human influenza A strains actually follows the changes in their nucleotide and dinucleotide composition biases (decrease in C, G, and CpG contents), which may be attributed to reasons other than RNA folding [17, 18].

Irrespective of the GORS-like patterns, an RNA molecule can form local secondary structures. In fact, it is known that the low value of free energy is not the main determinant of a functional RNA structure [19]. Furthermore, even though influenza virus RNA is assumed to be mostly melted in mature RNP particles, local structures can still remain and they can also fold at those steps of the virus replication cycle when NP is not (yet) associated with certain RNA regions.

A ROLE OF RNA STRUCTURE IN INFLUENZA VIRUS RNA TRANSCRIPTION AND REPLICATION

The 5′- and 3′-termini of the influenza virus genome segments contain highly conserved sequences that are complementary to each other, and a role of interactions between them in virus replication has been suggested more than 30 years ago [20, 21]. Later, detailed studies of RNA structure in these regions, carried out by different groups, confirm the functional importance of the pairing between the termini of influenza virus RNA segments. This structure, so far, remains the only one in influenza virus RNA for which structure-function relationships are known in detail (reviewed in [22]). Remarkably, its functioning turned out to have a dynamic character, with different structures formed at various steps.

All segments of the influenza A virus can form a stem-loop structure with two stems separated by a bulge, the so-called “panhandle” (Fig. 1A). On the basis of electron microscopy and nuclease S1 mapping, the panhandle was suggested to determine the circular viral RNA (vRNA) conformation [23]. Similar structures can be folded in other segmented negative-strand RNA viruses, such as members of the *Orthomyxoviridae*, *Bunyaviridae*, and *Arenaviridae* families and Tenuiviruses [22, 24–26].

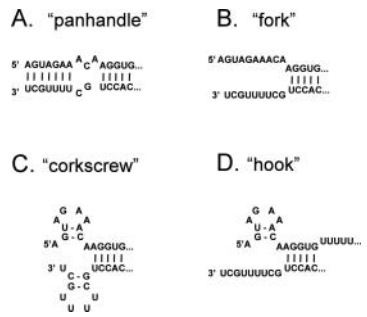

FIGURE 1 Variations on the panhandle structure at the ends of influenza virus vRNAs.

Mutagenesis studies pointed to different functional roles of the panhandle base pairs, showing that the interactions between the panhandle ends were not necessary for initiation of transcription [27, 28]. This led to the proposal of the "RNA-fork" model, in which the panhandle is only partially double-stranded (Fig. 1B). Mutations in this part of the panhandle abolished promoter activity, which could be restored by complementary double mutations. In contrast, the effect of mutations at positions closer to the RNA 5′-end could not be compensated by substitutions at the 3′-end [27, 28].

The latter effect can be explained by the "corkscrew model" (Fig. 1C), with two small hairpins at the vRNA ends [29, 30]. The corkscrew conformation turned out to be required for tight polymerase binding and the endonuclease activity of the polymerase complex [29–31].

Furthermore, the small hairpin at the vRNA 5′ end in the "5′-hook model" (Fig. 1D) turned out to be necessary for polyadenylation of mRNA [32]. The polyadenylation of influenza A virus mRNA occurs by reiterative copying of a track of five to seven uridine residues [33, 34], adjacent to the panhandle stem (Fig. 1D). The 5′-hook hairpin seems to be important for stable binding of the polymerase complex to the 5′ end of vRNA during mRNA synthesis that serves as a physical barrier resulting in reiterative copying of the U-stretch [27, 32].

The panhandle and the corkscrew structures are conserved in vRNAs of *Orthomyxoviridae* and other groups of segmented negative-sense RNA viruses, such as *Bunyaviridae* and Tenuiviruses [24, 26, 35]. However, the corkscrew structure does not seem to be universally required in all viruses of these groups. For instance, in Thogoto virus no evidence for the hairpin at the 3′ arm of the corkscrew structure was obtained [35].

It is likely that at different steps of the virus replication cycle the vRNA panhandles undergo conformational transitions between structures required for various functions. The switch between open and closed forms of the influenza RNP is guided by the antagonistic effects of NP and polymerase: NP melts RNA structure, while polymerase complex anneals it [15]. An RNA chaperone role, similar to that of the influenza virus NP protein, was suggested for hantavirus nucleocapsid protein [36]: the protein recognizes the terminal panhandle, but then unwinds it in the complex. Interestingly, the extent of pairing between the ends of the defective-interfering RNAs from vesicular stomatitis virus (VSV) has been directly shown to modulate the balance between transcription and replication; more stable panhandles enhanced replication, but suppressed transcription [37].

Such a switch is not unique for influenza and other negative-strand RNA viruses. Conformational transitions at both or one of the RNA termini were shown to switch between various RNA functions in both animal and plant positive-strand RNA viruses (e.g., [38–41]).

A ROLE OF RNA STRUCTURE IN INFLUENZA VIRUS RNA ENCAPSIDATION

The eight vRNA segments of influenza A virus are separately encapsidated as RNP complexes, each containing one copy of three polymerase subunits (PB2, PB1, and PA) and multiple NP molecules, with an estimated stoichiometry of one NP monomer per 24 nucleotides [12, 13, 42]. At least one copy of each segment has to be packaged into a virion, and the currently accepted model assumes that this is mediated by specific packaging signals in vRNA rather than via a random process (reviewed in [43]).

Exact location and structure of these signals are still enigmatic, but multiple mutagenesis studies by different groups tracked down their location at the vRNA termini, in both untranslated regions (UTRs) and the proximal parts of the coding regions [43]. The terminal panhandles seem to be an important structural determinant of packaging [23, 44]. Obviously, however, the panhandles, which are conserved in all segments, are not sufficient for segment-specific packaging.

Specificity can be realized by interactions between the segments. In support of this model, a characteristic "7 + 1" configuration of 7 segments surrounding a central one is seen by transmission electron microscopy [45]. Furthermore, the signals of all segments are necessary and sufficient for efficient packaging, as evidenced by reverse genetics experiments manipulating various combinations of signals and packaged genes [46–48]. The existence of intersegment interactions was also

concluded from the observation that mutation of the proposed packaging signals of one segment affected the packaging of other non-mutated segments [49–52].

A network of specific interactions between various segments of the RNA genome could either be mediated by yet-unidentified protein factors or realized via direct RNA–RNA interactions [43, 49, 53]. In both scenarios, a role of RNA structure can be suspected, analogous to other virus families (e.g., *Retroviridae*). However, besides the terminal panhandle region, functional structures formed by the influenza RNA packaging signals remain unknown.

In principle, the high-order structure of RNA in packaged RNP particles, formed by each segment, is strongly influenced by NP binding. The shape of an RNP is mostly dictated by the structure of NP, as particles obtained without RNA are structurally indistinguishable from RNA-containing complexes [54]. However, RNA can still form some structures in RNP complexes, for instance, relatively short secondary structures that may be involved in intersegmental interactions [43]. RNA structure probing indicated periodic patterns of double-stranded RNA regions in RNP complexes [55]. Interestingly, in probing experiments with a "mini-vRNA" construct binding to NP [14], a small hairpin retained its structure upon protein binding. Although the authors attribute this to deficiencies in complex formation, it is also possible that some of the hairpin base pairs remain intact in the complex.

Intriguingly, experiments with selective incorporation of randomized NS segment packaging sequences suggest that their functional high-order structure might also be formed by sequences very different from the wild type [56]. However, attempts to identify functional secondary structures in the packaging signals of NS and other segments by predictions of low free energy conformations did not lead to reasonable models [50, 53, 56, 57]. Apparently, a straightforward search for (sub)optimal orthodox secondary structures in naked vRNAs without additional experimental or phylogenetic data is not sufficient to identify functional vRNA motifs and interactions in RNP complexes.

A ROLE OF RNA STRUCTURE IN INFLUENZA VIRUS RNA TRANSLATION

Infection of cells by the influenza A virus results in a shutoff of host protein synthesis and selective translation of the virus mRNAs [58, 59]. The molecular mechanism of such a discrimination involves both virus and host factors.

Some of the determinants of preferential translation of influenza virus mRNAs have been suggested to be located in their 5′-UTRs

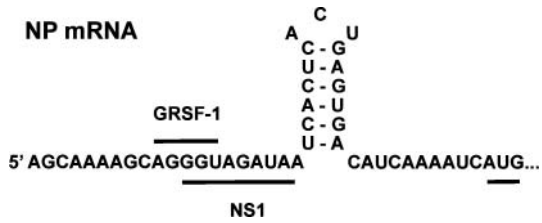

FIGURE 2 The hairpin at the 5′ end of NP mRNA of influenza A viruses, located between the proposed protein binding sites and the start codon [61, 63, 64]. The start codon is underlined. The putative sequence motifs AGGGU and GGUAGAUA, recognized by host factor GRSF-1 and viral NS1 protein, respectively, are indicated.

[60–63]. The sequence GGUAGAUA, present in NP and M segments, and AGGGU, present in NP and NS segments, have been shown to have stimulatory effects on translation due to the binding of the virus NS1 protein and the host GRSF-1 protein, respectively [61, 64].

One nucleotide downstream of the GGUAGAUA motif in the NP segment, a stable hairpin is predicted (Fig. 2), which may be involved in translational regulation [61, 63]. However, deletion of this hairpin did not have a significant effect on GRSF-1 binding, so its role is presently unclear [63, 64]. It has also been noted that the influenza A virus 5′-UTRs seem to have little stable secondary structure, which may be important for ensuring a single-stranded character of potential protein binding sites [63, 64].

Recently a correlation between locations of mRNA secondary structure domains and encoded protein domains was described for HIV [65]. Such a correlation could be explained by translational pausing caused by a stable RNA structure, which may ensure an efficient correct folding of important protein domains. Such an effect has also been proposed for the RNA bacteriophage MS2 [66], and could apply to many other viruses. This mechanism is one of the possible rationales for the presence of conserved local structures in influenza mRNAs as well.

A ROLE OF RNA STRUCTURE IN THE EXPRESSION OF INFLUENZA VIRUS OVERLAPPING READING FRAMES

Two segments of the influenza A virus genome (segments 7 and 8) contain introns. The ratio of spliced and unspliced mRNAs, encoding proteins with overlapping reading frames, M1/M2 and NS1/NS2, respectively, is tightly regulated [58, 67]. Homologous proteins in influenza B and C viruses are also encoded by overlapping open reading

frames (ORFs), but influenza B virus exploits a termination-reinitiation strategy in its segment 7 [67].

In the regions close to the 3′ splice sites in NS mRNA of influenza A and B viruses, a remarkably conserved folding has been predicted [68, 69]. In both virus types, a stable pseudoknot structure can be formed, comprising the 3′ splice site in one of its loops (Fig. 3). The pseudoknot conformation is supported by nucleotide covariations and by structure probing and NMR spectroscopy of oligonucleotides comprising the region around the 3′ splice site [68]. To the best of our knowledge, apart from the terminal panhandle structures, this is the only structure shown to be conserved in both influenza A and B virus genomes.

Interestingly, in influenza A virus NS mRNA a dynamic equilibrium between this pseudoknot and an alternative hairpin conformation (Fig. 3) can be suggested [68]. Furthermore, the equilibrium turned out to be very sensitive for point mutations in some strains. For instance, a G563-C substitution, observed in recent H5N1 viruses, favored a shift to the hairpin conformation, while the pseudoknot became less stable (Fig. 3B). The C563 mutation seems to be unique for H5N1 viruses, and it is possible to trace its first occurrence in China in 2001 [68]. It is stably inherited in the dominant H5N1 lineages spreading over the world [70]. C563 has been present in all H5N1 viruses isolated from humans since 2003 [68].

Potential functions of the postulated pseudoknot and hairpin structures (Fig. 3) remain to be elucidated. Their location near the 3′ splice site suggests that they are involved in splicing regulation. Splicing of NS mRNA in vitro is known to be strongly suppressed, and this block may result from an inactive RNA conformation, presumably including a structurally inaccessible 3′ splice site [71, 72].

The suppression of the influenza A NS mRNA splicing has been shown to be dependent on inhibitory regions, located both in the intron (positions 153–465) and in the 3′ exon (positions 775–860) [72]. It has been suggested that this may be attributed to an inhibition of proper NS1 mRNA folding required for splicing. While in the chimeric constructs, the intronic inhibitory region could inhibit the splicing of other mRNAs as well, the exonic sequence was inhibitory only in the NS1 mRNA. The structural context of these inhibitory elements has not been investigated, but it is possible that they can somehow interfere with the alternative foldings near the 3′ splice site shown in Figure 3.

The influenza A virus NS1 protein itself is an inhibitor of splicing [73, 74]; on the contrary, influenza C virus NS1 upregulates splicing [75]. Interestingly, in the influenza C virus NS1 mRNA we could not find any structure similar to the pseudoknot suggested for the A and

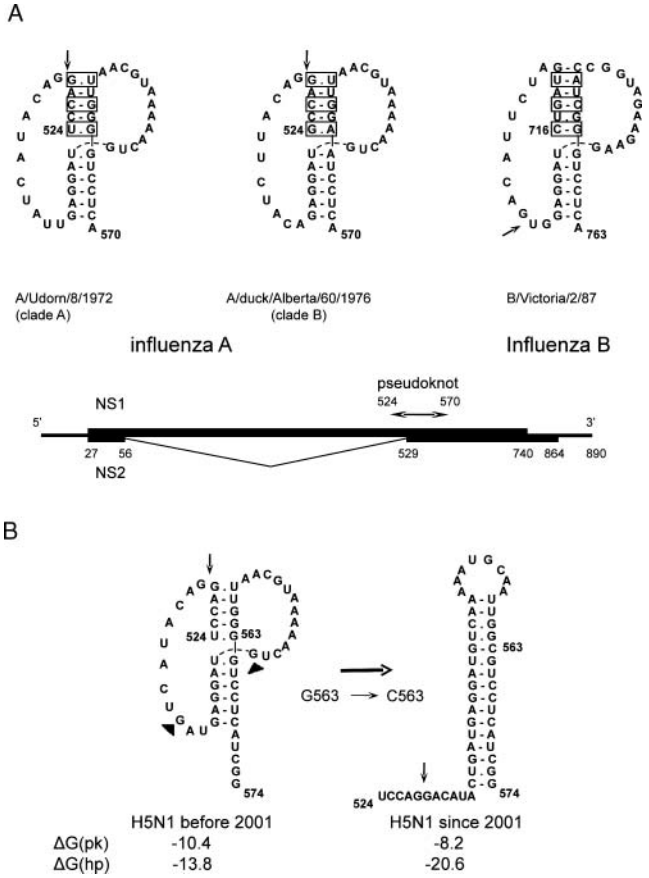

FIGURE 3 (A) The pseudoknot structure conserved in NS segments of influenza A and B viruses [68, 69]. The 3′ splice sites are indicated by small arrows. The nucleotide covariations supporting the pseudoknot are boxed. The location of the pseudoknot in relation to the coding regions of influenza A virus NS1 and NS2 mRNAs is shown schematically. (B) The pseudoknot/hairpin conformational transition caused by a unique C563 substitution in the H5N1 influenza A viruses, observed since 2001. The G-residues demonstrating the most considerable changes in RNase T1-susceptibility [68] upon the transition are shown by triangles. The indicated estimates of free energies (in kcal/mol) of the pseudoknot ΔG(pk) and the hairpin ΔG(hp) for the typical H5N1 sequences before and after 2001 are calculated [68] using the program kinefold (http://kinefold.curie.fr).

B types. The influenza A virus NS1 protein does not require its RNA-binding activity for splicing inhibition, but it is required for specific blocking of the nucleo-cytoplasmic transport of unspliced NS1 mRNA [76]. The mechanism of this specificity and the potential (structural) signals in NS1 mRNA required for nuclear retention are not known.

Coupled translation of M1 and BM2 proteins from segment 7 mRNA of influenza B virus occurs via a termination-reinitiation mechanism [77]. The M1 stop codon and the BM2 start codon overlap at the pentanucleotide UAAUG, the site of ribosome "stop-start." It has recently been suggested that the stop-start is regulated by the BM2 mRNA secondary structures able to affect the pairing between complementary sequences in 18S rRNA and mRNA [78]. Structure probing and folding predictions have revealed a potential for alternative coexisting secondary structures in BM2 mRNA with various accessibilities of the complementarity region (Fig. 4). Mutagenesis experiments identified the region required for efficient reinitiation, and both probing and structure predictions indicated that this region can adopt multiple metastable structures that could regulate BM2 expression [78, 79]. Regulation of translation reinitiation by RNA folding seems to be a widespread mechanism, and it has recently been proposed for caliciviruses [80, 81].

A ROLE OF RNA STRUCTURE IN INFLUENZA VIRUS EVOLUTION

A potential for the folding of functional RNA structures in the influenza virus genome implies specific constraints in its evolution [82, 83]. As some structures can be folded within protein coding regions, requirements for protein-coding capacity of viral mRNA pose an obvious constraint on RNA structure evolution, and vice versa. As predicted for RNA viruses in general [83], multiple constraints affecting the same genome can seriously limit the sequence space areas available for influenza virus adaptation.

This can explain, for instance, a remarkable conservation of codons in the influenza A virus genome, in particular, of those located in the regions important for packaging [84]. On the other hand, some regions in the influenza virus genome can fold into alternative structures that may be functional [68, 78]. Furthermore, a potential for the formation of alternative structures may also lead to conformational shifts in specific virus strains, as observed in the H5N1 lineage (Fig. 3B). In such shifts, novel nucleotide substitutions may be stably inherited if they become important in the new structural context. Thus, the evolution of influenza virus RNA structure may be punctuated [68], similar to

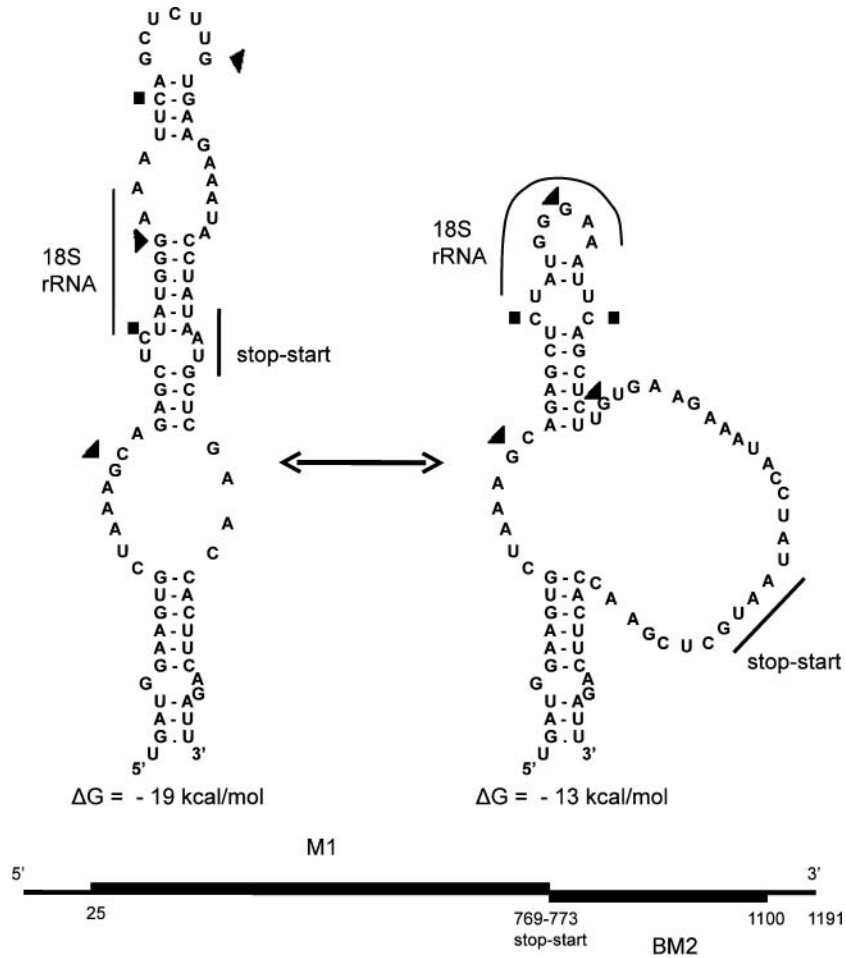

FIGURE 4 Alternative structures postulated to regulate translation reinitiation on M1/BM2 mRNA of influenza B viruses [78]. The locations of the stop-start pentanucleotide UAAUG and the sequence (UAUGGGAA) proposed to bind a complementary sequence in 18S rRNA are indicated. The locations of the M1 and BM2 coding regions in the M segment are shown schematically. Both alternative structures are in reasonable agreement with the RNA structure probing [78]. The strongest cleavage sites by the T1 (triangles) and CV1 (squares) RNases are shown here as an example. The indicated free energy estimates are calculated using the program mfold (http://frontend.bioinfo.rpi.edu/applications/mfold/).

the virus antigenic evolution [85–87]. Remarkably, such a pattern of evolution, with relatively long periods of phenotypic (e.g., structural) neutrality punctuated by rare discontinuous transitions, is also predicted from theoretical simulations of RNA adaptation [88, 89].

RNA secondary structure constraints have been suggested to act as possible additional control to eliminate mutations occurring in the regions coding for essential domains of viral proteins [90, 91]. In principle, such a function could explain the presence of high-order RNA structures in coding parts of the genome. On the other hand, in general it should be noted that in a region coding for conserved protein domains, a prediction of conserved RNA structural element may be just one of possible structures folded by a conserved nucleotide sequence. For instance, we noticed that in many conserved regions of influenza virus segments, mutually exclusive structures could be predicted (Gultyaev et al., unpublished). In the absence of experimental and/or phylogenetic (covariations) support, it is impossible to judge which of the alternative structures is actually formed in vivo.

POTENTIAL ROLES OF UNDISCOVERED INFLUENZA RNA STRUCTURES

As compared to many other single-stranded RNA viruses, the knowledge about high-order structures formed by influenza virus RNA is rather limited. In particular, this difference is striking in comparison with positive-stranded RNA viruses. In this group, reliable structural models can be developed for very large genomic regions or even complete genomes (e.g., [65, 92]), and numerous functional structures and specific motifs are identified and studied in detail (e.g., [8, 9]). In contrast, besides established structure-function relationships of the terminal panhandle structures [22], the information about the rest of the influenza virus genome is mostly limited to fragmentary folding predictions [61, 63, 68, 69, 78, 90, 91], structure probing in vitro [68, 78] and detection of global thermodynamic properties [16].

To a certain extent, this is because influenza virus does not need to exploit several mechanisms determined by highly structured RNA topologies. For instance, its mRNAs are capped and polyadenylated, thus IRES structures and translational enhancers of cap-independent translation, known to form characteristic structures in many viruses, are not likely to be encoded by the influenza virus genome. Influenza virus mRNAs do not have very long 5′-UTRs and 3′-UTRs that contain the majority of cis-acting RNA elements in positive-stranded RNA viruses [9]. Furthermore, the structure of influenza virus RNPs,

presumably with RNA uniformly distributed along the RNP [13], does not seem to allow an extensive RNA structure formation.

On the other hand, even in RNPs some high-order RNA structure can be expected. In influenza A virus RNPs, the bound RNA is accessible to treatment by RNAses [13, 14, 55]. The length of protected fragments is around 18 nt, while the average RNA fragment bound per NP monomer is 24 nt [13, 42]. Furthermore, RNPs turn out to contain unprotected vRNA regions that are sufficient for hybridization to complementary cDNA fragments, as shown by specific RNase H cleavage of bound RNPs [93]. These regions can be partially double-stranded, as evidenced by RNA structure probing of RNPs [14, 55]. Thus, some local structural motifs, presumably small hairpin loops, may be exposed at the surface of RNP particles and are able to interact with each other.

Such motifs might provide a basis for specific recognition between influenza virus genome segments during packaging [43]. Yet, no structures performing this role have been identified in influenza viruses.

In principle, the recurrent motifs that promote the formation of stable bimolecular RNA/RNA complexes are specific tetraloop binding by helical receptors and "kissing" interactions mediated by hairpin loops [94–98]. The GNRA hairpin tetraloops (where N is any base, and R is either G or A) are recognized by RNA stems (receptors) containing specific motifs [94–96]. The long-range GNRA/receptor interactions are typical for large ribozymes, where they are essential for RNA self-assembly [95, 96].

Kissing interactions are formed between hairpin loops and complementary sequences that are frequently, but not always, located in hairpin loops as well [97–99]. Viruses have been shown to exploit kissing interactions for various functions, in particular, for the interdependent packaging of genomic RNAs such as in homodimeric retroviral genomes and the bipartite genome of Red clover necrotic mosaic virus [100–105].

In retroviruses, genomic RNAs are packaged as dimers formed via multiple contacts mediated by kissing hairpin structures. Compared to retroviral genomic RNAs, influenza virus vRNAs in RNPs are expected to have considerably less secondary structure, but several contacts between small structural elements, probably formed under the chaperone-like influence of NP binding, could provide both specificity and thermodynamic stability for inter-segment interactions.

Several observations may be considered as an indirect support for such a "multi-hairpin" model. It is known that even two base pairs are sufficient for efficient kissing interaction [106, 107], and the cooperative effect of multiple interactions can lead to much more stable complexes [108]. Existence of multiple interactions between influenza virus gene

segments is consistent with relatively mild effects of substitutions of certain conserved nucleotides in the packaging signals [43]. Similarly, in mutagenesis studies of retroviral dimerization, deletions of single motifs only partially affect encapsidation [101]. On the other hand, a remarkable conservation of certain wobble positions in codons putatively located within the packaging signals of influenza A virus [84] may be explained by the need to have specific loop conformations for efficient contacts. Such structural constraints in the loops involved in intermolecular or long-range intramolecular kissing interactions of viral RNAs can lead to stringent sequence requirements [100, 109–111].

In principle, one may also anticipate the existence of alternative structures and conformational switches regulating the packaging of the influenza virus genome. Folding of nascent vRNA during its synthesis, prior to the formation of the terminal panhandle and the mature RNP complex, should result in local structures that may be partially or completely disrupted upon NP binding and packaging. Such rearrangements of secondary structure regulate dimerization and packaging of retroviral genomes [102–104].

Interestingly, several studies identified potential hairpin structures in the 5′-proximal regions of some of the vRNA segments from *Bunyaviridae* genomes [112–114]. Such hairpins could be functional during packaging. The hairpins at the 5′ ends of S segments of Bunyamwera virus and Hantaan virus (Fig. 5A,B) have been suggested to be involved in recognition of transcribed nascent vRNA by N protein [112–114].

The extent of evolutionary conservation of these hairpins in related viruses has not yet been investigated. Intriguingly, very conserved hairpins can also be predicted in the 5′-proximal vRNA regions of some segments of the influenza virus genome. For instance, using various programs for RNA structure prediction [115–117], we identified a possible stable hairpin in the 5′-proximal part of segment 7 vRNA (Fig. 5C). Despite some sequence diversity in this region, the hairpin seems to be conserved in all influenza A virus strains. Furthermore, at one of the base-paired positions, a remarkable host-specific covariation can be observed; while the majority of human strains (except early viruses of 1918–1933) have a C-G pair, avian strains have mostly U-A.

A functional role of such hairpin structures remains to be elucidated. In principle, they can be specifically recognized by NP, even if they are completely or partially melted upon the assembly of mature RNP particles. Another possible role of hairpin structures could be to establish kissing interactions between the segments.

It is not surprising that a potential for the formation of conserved structures is revealed in NS segments of influenza A and B viruses

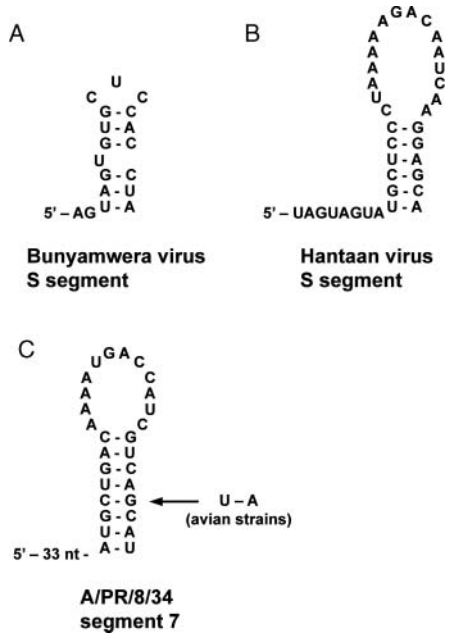

FIGURE 5 The hairpin structures predicted to fold at the 5′ ends of vRNAs from negative-sense viruses. (A) Bunyamwera virus S segment [112, 113]. (B) Hantaan virus S segment [114]. (C) Conserved hairpin in the M segment of influenza A viruses, supported by a host-specific covariation [A. P. Gultyaev, unpublished]. All structures can be predicted by various folding algorithms, including free energy minimization, such as mfold (http://frontend.bioinfo.rpi.edu/applications/mfold/).

(Fig. 3) [68] and of M segments in influenza B virus (Fig. 4) [78], which contain overlapping ORFs. Many viruses utilize mRNA (re)folding to fine-tune the balance between proteins alternatively expressed from a single gene by mechanisms, such as splicing, ribosomal frameshifting, readthrough, reinitiation, and leaky scanning (e.g., [8, 80, 118–121]).

Thus, it is likely that functional conserved structures are present in other segments encoding more than one protein, such as M and PB1 segments of influenza A viruses. For instance, splicing of the M segment mRNA has been shown to be regulated by a purine-rich exonic enhancer that recruits splicing factor SF2/ASF [122], and such a recruitment in host pre-mRNA's may be affected by secondary structure [123]. Interestingly, in a recent genome-wide screen of human host

factors involved in influenza virus replication, multiple factors turned out to be connected with pre-mRNA splicing [124].

The PB1 segment seems to have a complicated regulation of translation of several ORFs. In addition to the primary PB1 polymerase, two other proteins (PB1-F2 and N40) are translated from downstream start codons [125, 126], and shorter polypeptides, synthesized from further downstream AUGs, have been reported as well [127]. While PB1-F2 is likely to be translated by leaky scanning past upstream AUG codons with moderate Kozak translation initiation contexts, translation of N40 and downstream polypeptides should involve additional regulation mechanisms [126].

Of course, one cannot exclude the presence of functional structures in mRNAs from other segments as well. For instance, there is growing evidence that nucleo-cytoplasmic transport and/or nuclear retention of influenza virus mRNAs are segment-specific [76, 128–131]. One of the important issues in understanding this process is to characterize the signals in viral transcripts that are recognized by the host and viral proteins involved in its regulation [130].

It has been reported that transcription of influenza virus mRNAs from recombinant plasmids in human embryo kidney cells is affected by the folded structures in the protein-coding regions [90]. This was demonstrated by a study of mutants that changed the stem-loop structure predicted in the NS gene (Fig. 6A). However, the influence of this structure on transcription by viral polymerase has not been tested.

The structure reported by Ilyinskii et al. [90] for A/WSN/33 is also repeatedly predicted as one of the most conserved elements in our simulations of NS mRNA folding for various influenza A strains [68]. In particular, a similar extended stem-loop structure may also be present in strains containing the so-called clade B NS gene (Fig. 6B). Although the top and the bottom parts of the stem-loops are formed by non-homologous base pairs, the middle parts (nucleotides 109–116/165–172) are exactly the same in both clades. The similarity of the structures from the two clades is remarkable, because their sequences differ considerably [132]. Strong conservation of the overall topology of the predicted extended stem-loop structures suggests that the shape of NS RNA folding in this region plays some yet unknown functional role.

In summary, the studies on influenza virus RNA folding suggest that we are only at the very beginning of elucidating the role of high-order RNA structures in the virus replication. More detailed knowledge of specific influenza virus RNA structures will help us to solve many puzzles in understanding the molecular mechanisms underlying the virus replication cycle.

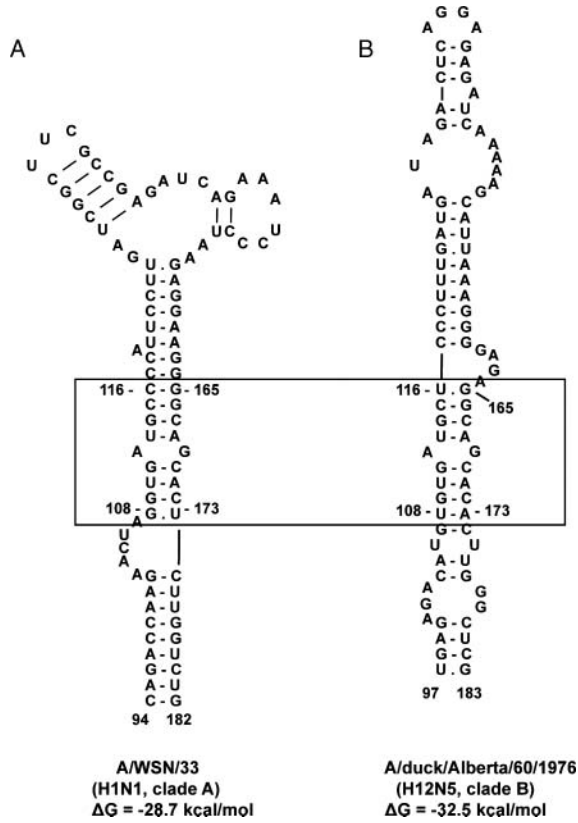

FIGURE 6 (A) A stable stem-loop structure, suggested to affect the in vitro transcription of the NS segment of clade A strain A/WSN/33 [90]. (B) Folding of a homologous structure of the NS segment mRNAs from clade B strains [A. P. Gultyaev, unpublished]. The stems formed by homologous base pairs (regions 108–116/165–173) in the two structures are boxed. Both structures can be predicted by various folding algorithms, including free energy minimization, such as mfold (http://frontend.bioinfo.rpi.edu/applications/mfold/), and supported by comparative analysis. The folding free energies are calculated using the mfold server.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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