ABSTRACT

Influenza A virus (IAV) causes annual epidemics, often with more than 500 thousand casualties due to complications during infection. Effective therapy against influenza hasn't been developed so far. One of the trends of research suggests segmented RNA influenza genome as a therapeutic target. The antisense therapy (antisense oligonucleotides, ribozymes, DNAzymes, RNAi) is the main interest of scientists. This therapy is aimed at conservative sequences of vRNA and mRNA. However, little is known about secondary structure of vRNA and its regulatory role during viral life cycle. Simultaneously, there are some assumptions indicating that the secondary structure of mRNA and vRNA play important role in virus propagation.

The aim of this study was to determine the secondary structure of segment 7 vRNA (vRNA7) of influenza A virus (strain A/VietNam/1203/2004 (H5N1)). The next step was to indicate conservative structural motifs for influenza A, as these motifs could be involved in interactions between the segments. Also, the conservative and thermodynamically stable motifs may be recognized by a protein factor or other vRNAs during viral packing.

Conducted studies led to determination of secondary structure of the entire segment 7 vRNA (1027 nucleotides) of influenza virus. Experimental data from chemical mapping (SHAPE method, DMS modification) were introduced to *RNAstructure 5.3* software to generate the structure. As a result, six domains were determined within the model. Also, numerous structural motifs were pointed out, for instance the *panhandle* motif, described earlier in the literature.

The bioinformatic analysis of 15946 unique sequences of vRNA7 from NCBI database revealed that the proposed vRNA7 secondary structure could be conserved among another IAV strains. The estimated secondary structure conservation among strains is 91,4%.

Furthermore, microarray mapping was used to verify secondary structure model of vRNA7 and to indicate accessible sites for potential oligonucleotides. The analysis showed probes binding to sites (the middle nucleotide of complementary region): 674, 692, 699, 700, 941, 953, 954. Moreover, selective hydrolysis with ribonuclease H revealed high accessibility of other vRNA7 sites in domain II vRNA7 (98, 99, 220, 313, 314), domain III (345, 346, 347, 365, 414, 415, 417, 428, 433, 437), domain IV (464, 580), domain V (684, 685, 689, 702, 732, 791, 816, 819) and domain VI (926, 927, 943, 953, 955).

Intersegment interactions are suggested to play important role during incorporating the eight vRNA segments into progeny virions. BLAST software was used to indicate sequences

potentially responsible for vRNA7 interaction with other vRNAs. The complementary sequence between vRNA7 and other vRNAs were determined. Some of the complementary regions of different vRNAs overlapped. The sequence in region 186-223 was complemented to four other segments: vRNA3, vRNA4, vRNA5, vRNA6. In region 161-177, there was sequence complementary to three other segments: vRNA3, vRNA5, vRNA8. Twelve regions showed sequences complementary to two other segments and next twelve regions indicated sequences complementary to one segment.

The accessible sites in vRNA7 were revealed by isoenergetic microarray mapping, selective hydrolysis with ribonuclease H and chemical mapping. Moreover, the probability of existence of the determined structural motifs among another strains and the location of complementary sequences to other vRNAs suggest that these regions are important in intersegment interactions. The obtained results led to designing of 21 antisense oligonucleotides (ASO). The antiviral effect of antisense oligonucleotides was studied in MDCK cell lines infected with A/California/04/2009 (H1N1) virus strain. Four ASO (28A, 313A, pair 313A/298A) reduced virus propagation to 50%. Six antisense oligonucleotides inhibit virus life cycle to below 20%, classified as a significant antiviral factor. The pair of oligonucleotides, namely 346A and 360A (binding region 341-369) showed the highest inhibition of virus propagation. The two ASO together reduced virus multiplication to 7%. Oligomer 428A (binding region 423-433) inhibited viral life cycle to 19%. ASO 686A and 701A (binding region 678-706) were studied together and reduced viral propagation to 19% as well as ASO 940A (binding region 936-946). Interestingly, ASO 940A sequence was universal for both H5N1 and H1N1 subtypes. Moreover, gapmer G686A (binding region 678-695) inhibited viral life cycle to 17%.

Several structural motifs of vRNA7 secondary structure model were selected to determine their folding and accessible binding sites in vRNA7: A7 (domain III, region 320 - 458), F7 (domain V, region 668 – 756), R7 (domain VI, region 858 - 962). All three motifs contain target sites for ASO and probably play important role during viral life cycle. Isolated fragments were studied using: chemical mapping (SHAPE, DMS modification), microarray mapping, lead ion cleavage. The resulting secondary structure models of A7, F7, R7 are in agreement with the secondary structure of corresponding motifs in full length vRNA7. The results for the A7 and F7 allowed also to determine alternative folding in domain III and V of vRNA7.

Information about vRNA/vRNA interactions of segment 7 vRNA made it possible to indicate putative sites important for viral packing. It was observed, that vRNA7 forms

complexes with five other vRNAs: vRNA1, vRNA3, vRNA4, vRNA5, vRNA8. The ratio of the vRNA molecules involved in complex formation (in %) was: 23, 18, 25, 34 and 50 for segments 1, 3, 4, 8, and 5, respectively.

In order to understand the mode of action of the selected ASO, which inhibition were observed in MDCK cell line, the *in vitro* studies of vRNA/vRNA interactions were performed with these ASO. It was shown that oligonucleotide 360AV inhibited formation of vRNA7/vRNA8 complex and oligonucleotide 346AV limited interaction between vRNA7 and vRNA5. In addition, the pair of oligonucleotides 360AV/346AV promoted formation of vRNA7/vRNA4 complex. Oligonucleotide 428AV inhibited interaction between: vRNA7 and vRNA3, vRNA7 and vRNA4, vRNA7 and vRNA8. Additionally, oligonucleotide 940AV inhibited formation of vRNA1/vRNA7 complex but stabilized interaction between vRNA4 and vRNA7. It was also noticed, that binding of oligonucleotides 360AV and 940AV to vRNA7 disrupts vRNAsecondary structure and creates a new region of RNA accessible for other vRNAs. The latter result suggests that the packing of the viral genome is a multi-step process. The pair of nucleotides 686A/701A didn’t affect intersegment interactions significantly. This means that the antiviral effect of these ASO is associated with other regulatory function of vRNA.