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Impact of Mutations in Nucleoprotein on Replication of Influenza Virus A/Hong Kong/1/68/162/35 Reassortants at Different Temperatures[1]

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Abstract—The nucleoprotein (NP) of influenza virus is a multifunctional RNA binding protein. The role of NP in the adaptation of influenza viruses to a host has been experimentally proved. Ambiguous data are available on the role of nucleoprotein in the attenuation of influenza A viruses, which is characterized by ability to replicate at low temperature (26°C) and inability to replicate at high temperature (39°C). Influenza virus donor strain A/Hong Kong/1/68/162/35 (H3N2), adapted to growth at low temperature, differs from the wild type virus by 14 amino acid mutations in the internal and non-structural proteins. Two mutations occurred in the NP: Gly102Arg and Glu292Gly. We have obtained viruses with point reverse-mutations in these positions and compared their replication at different temperatures by measuring infectious activity in chicken embryos. It has been shown that reverse mutation Gly292Glu in the NP reduced virus ability to replicate at low temperature, the introduction of the second reverse mutation Arg102Gly completely abolished virus cold adaptation.

Keywords: influenza virus, nucleoprotein, reverse-mutation, cold adapted phenotype, temperature sensitive phenotype, reassortant

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INTRODUCTION

Influenza and other acute respiratory infections occupy the leading position in the damage caused to public health. Annual epidemics are characterized by high morbidity and increased mortality, especially among people from high risk groups (with chronic somatic and infectious diseases). Severe complications often accompanying influenza lead to serious economic losses. Hence comes the need for development of the means for protection, prevention and control of influenza infection, that is impossible without a thorough study of the pathogenesis and replication mechanisms of influenza virus.

The influenza virus nucleoprotein (NP)—multifunctional RNA-binding protein. NP is involved in the processes of viral genome transcription, replication and packaging into virions. Monomer of NP can bind to other NP monomers forming NP oligomers, can interact with other viral proteins and also with different cellular factors: α-importin, F-actin, nuclear export receptor CRM1 [1]. Experimental data shows the involvement of NP in the adaptation of influenza virus to the host organism [2] and its role in the formation of the attenuated (*att*) phenotype of influenza B viruses that are used in the production of live influenza vaccines [3]. Influenza viruses with a*tt* phenotype replicate at low temperature (26°C) and do not replicate at high temperature (39°C), which corresponds to cold-adaptated (*ca*) and temperature sensitive (*ts*) phenotype [4]. During the process of attenuation influenza viruses acquire mutations in all genes of internal and non-structural proteins, which differs them from the wild type precursor viruses. Gene mutations in the polymerase complex play the major role in attenuation. For example, *ts* phenotype of attenuated donor strains A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 is determined assured by mutations in the genes encoding the polymerase complex proteins: PB2, PB1, PA [5, 6]. It should be noted that the effect of individual mutations on the emer-

Abbreviations: att, attenuated (phenotype); ca, cold adapted (phenotype); NP, nucleoprotein; RCT_{37}, RCT_{26}, reproductive capacity at different temperatures; ts, temperature sensitive (phenotype); CE, embryonated chicken eggs; EID_{50}, 50% embryo infectious dose.

[1] The article was translated by the authors.

[2] The authors contributed equally.

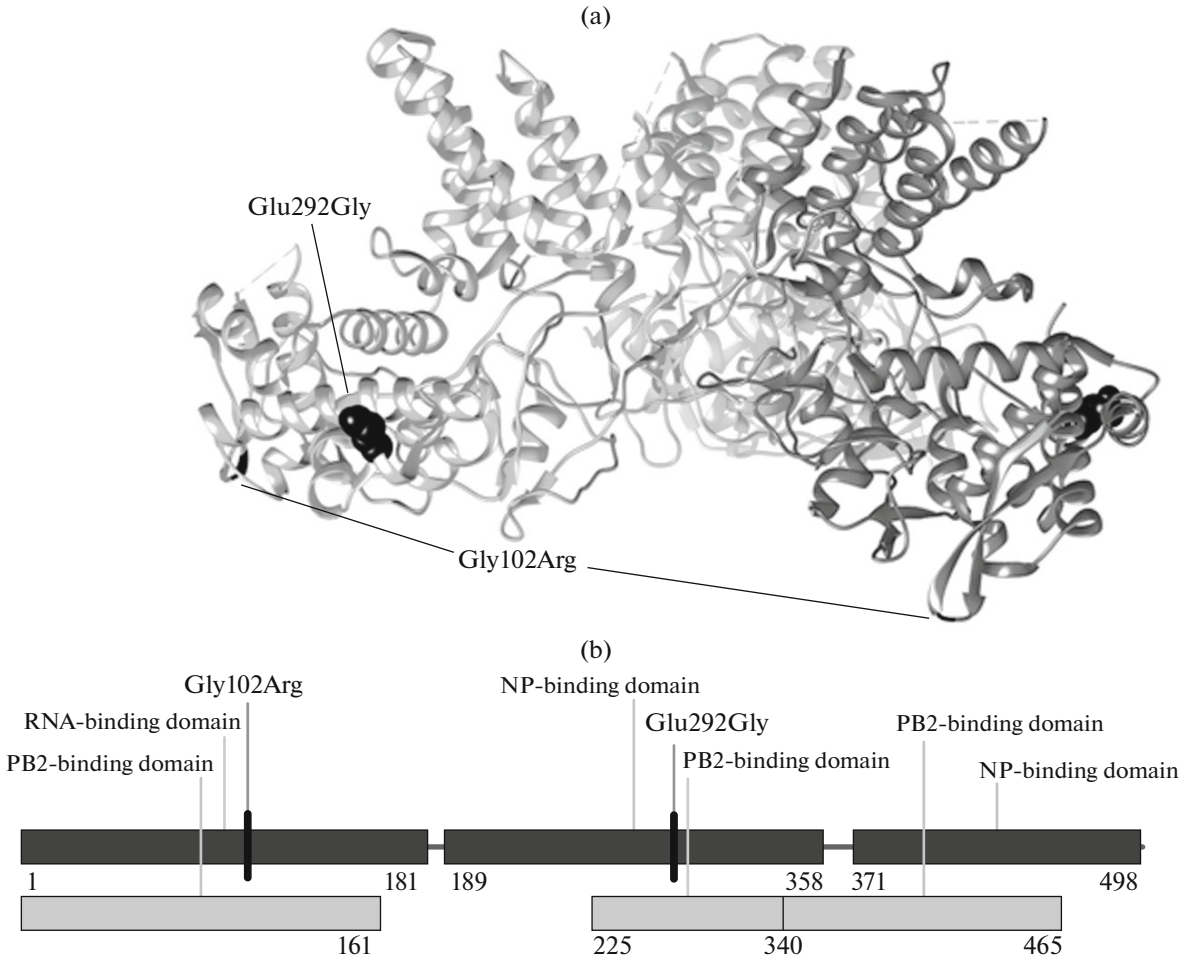

Fig. 1. The structure of the influenza A virus nucleoprotein (a) influenza A virus NP trimer. Mutated amino acid positions are indicated. The image was built in UCSF Chimera [8] (PDB Acc. No. 2IQH). (b) The scheme of functional domain location in the influenza A virus NP.

gence of *ca* phenotype experimentally hasn't been studied, and there are contradictory data about the role of NP mutations in the formation of *att* phenotype of influenza A viruses.

Developed in the Research Institute of Influenza cold adapted (ca-phenotype) donor strain A/Hong Kong/1/68/162/35 (H3N2) differs from the wild type precursor by 14 amino acid substitutions in internal and non-structural proteins [7]. Furthermore two mutations appeared in NP. The first mutation Gly102Arg located in the potential RNA-binding site occurred during virus adaptation to the embryonated chicken eggs (CE) at the permissive temperature. The second mutation Glu292Gly located in the oligomerization domain appeared during the process of cold adaptation (Figs. 1a, 1b).

The aim of this study was the experimental assessment of the Glu292Gly and Gly102Arg NP mutations impact on the replication of reassortant viruses containing NP of influenza A/Hong Kong/1/68/162/35 donor strain at low and high temperature.

EXPERIMENTAL DESIGN

Plasmids

Gene segments of cold-adapted virus A/Hong Kong/1/68/162/35 (HK) were synthesized and cloned into the pAL-TA vector by Evrogen company (Russia). After that genes were subcloned into bidirectional plasmid pHW2006 designed for assembly of viruses by reverse genetics method [9]. Subcloning was performed by restriction of available pHW2006-based plasmid, ligation, cloning and transformation of *Escherichia coli DH5α*. Plasmids with point mutations in the NP were obtained by site-directed mutagenesis using special designed primers and QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, USA). Plasmids encoding genes of surface proteins of influenza viruses A/Texas/04/2009 (Tex) (subtype H1N1)) and A/Astana/06/2005 (Ast) (subtype H5N1), genetically close to highly pathogenic virus A/Kurgan/05/2005 (H5N1) isolated on the territory of Russia [10]), and internal proteins of influenza virus

A/Puerto Rico/8/34 (PR8) were designed and kindly provided by the Laboratory of Molecular Virology and Genetic Engineering of the Research Institute of Influenza.

Viruses

The assembly of reassortant viruses was made by reverse genetics method. This method is based on the transfection of sensitive Vero cells by cassette of 8 bidirectional plasmids. Inside the cells plasmids provide synthesis of virion RNA and mRNA in forward and reverse direction respectively, the latter provide translation of viral proteins. Eventually complete viral particles assemble in cells [3]. The culture fluid containing viral particles was then used to infect CE. Further viruses were grown and titrated in the CE. As a results the following recombinant influenza A viruses with reverse mutations in NP were obtained: H5N1 subtype with one reverse-mutation A/HK/Astana/6:2/Gly292Glu/2015 (Ast/HK/NPmut) and with two reverse-mutations A/HK/Astana/6:2/Gly292Glu,Arg102Gly/2015 (Ast/HK/NPmut2), and H1N1 subtype with one reverse-mutation A/HK/Texas/6:2/Gly292Glu/2014 (Tex/HK/NPmut). Corresponding viruses without reverse-mutations: A/HK/Astana/6:2/2009 (Ast/HK) and A/HK/Texas/6:2/90/2014 (Tex/HK) were obtained by classical genetic reassortment as described previously [11]. The control virus A/PR8/Texas/6:2/2009 (Tex/PR8) (H1N1 subtype), possessing non-temperature sensitive (*non-ts*) and non-cold adapted (*non-ca*) phenotype was obtained by reverse genetics.

Sequencing

The amplification of *NP* gene fragment was done by reverse transcription PCR (RT-PCR) using specially designed primers (Syntol, Russia) and reagents and enzymes for reverse transcription and PCR (Federal Budget Institution of Science Central Research Institute for Epidemiology, Russia). Sequencing of PCR products and plasmids were carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and capillary sequencer ABI GA3130 (Applied Biosystems, USA). Sequences were assembled and analyzed using Vector NTI software (Invitrogen, USA) on the basis of theoretical reference sequences.

Analysis of Virus Temperature Phenotype

Infectious activity, *ts* and *ca* phenotypes of viruses were estimated by parallel titration in 10-day old CE at permissive (34°C), low (26°C) and high non-permissive (39°C) temperatures. CE were infected by 10-fold serial dilutions of virus-containing allantoic fluid in phosphate buffer. Infected embryos were incubated for 2 days at 34 or 39°C or 7 days at 26°C. The virus presence in the allantoic fluid of infected CE were controlled in hemagglutination assay [12]. The 50% embryo infectious dose (EID_{50}) was calculated by the method of Reed-Muench [13]. The infection titer was expressed in $\log EID_{50}/0.2$ mL. Analysis of the temperature phenotype was carried out by RCT marker (reproductive capacity at different temperatures) calculated by the difference in infectious titer at permissive (optimal) and the specified temperatures [14]. The virus was considered as temperature sensitive if RCT_{39} marker was not less than $5 \times \log EID_{50}/0.2$ mL (Formula 1). The virus was considered as cold-adapted if the difference in reproduction at optimal and low temperatures, RCT_{26} (Formula 2) was less than $3 \times \log EID_{50}/0.2$ mL.

$$RCT_{39} = \log EID_{50}/0.2\ \text{mL (at 34°C)} - \log EID_{50}/0.2\ \text{mL (at 39°C)}, \quad (1)$$

$$RCT_{26} = \log EID_{50}/0.2\ \text{mL (at 34°C)} - \log EID_{50}/0.2\ \text{mL (at 26°C)}. \quad (2)$$

RESULTS

To study the impact of mutations in the NP protein on the replication of influenza virus A/Hong Kong/1/68/162/35 reassortants at various temperatures the plasmids coding for NP with reverse mutations Gly292Glu, Arg102Gly or their combination were constructed. The obtained mutant NP genes and other 7 gene segments were subcloned in bidirectional vector pHW2006.

Using reverse genetics method several reassortant viruses were constructed: Tex/HK/NPmut and Ast/HK/NPmut (with reverse mutation Gly292Glu), Ast/HK/NPmut2 (with a combination of Gly292Glu and Arg102Gly mutations). Reverse mutations in plasmids and viruses were confirmed by direct sequencing. Viruses without reverse mutations Ast/HK and Tex/HK that possess *ca* and *ts* phenotype were obtained by classical genetic reassortment. The control strain Tex/PR8 characterized by *non-ca* and *non-ts* phenotype was obtained by reverse genetics.

The temperature dependence of viral replication was evaluated by their ability to infect CE at optimum (34°C), low (26°C) and high (39°C) temperatures (table). All constructed reassortants except Tex/PR8 strain actually did not replicate at 39°C, i.e. had *ts* phenotype regardless of the presence of reverse-mutations in the NP.

Using the titers of infectious activity in parallel titration experiments RCT_{26} index was computed as a difference of reproduction at optimal and low temperatures. Results demonstrated on Fig. 2 show that at a low temperature reassortants with mutation 292Gly→Glu reproduced to a lesser degree than reassortants without mutations and can't be characterized with *ca* phenotype by RCT_{26} marker. The reassortant with a combination of mutations 292Gly→Glu and 102Arg→Gly replicated at low temperature to a much lesser degree

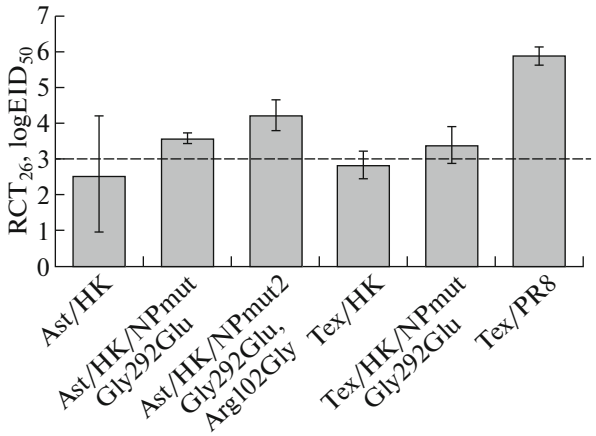

Fig. 2. RCT_{26} indexes calculated for viruses with mutations in the NP and non-mutated strains Ast/HK and Tex/HK (mean ± SD). The horizontal dotted line indicates the maximum RCT_{26} value for the cold-adapted strain.

than viruses with a single mutation and also can't be attributed to *ca* viruses.

So as single mutation Glu292Gly, so a double mutation Glu292Gly/Arg102Gly in NP protein of influenza viruse reduces the cold-adaptation of the investigated reassortants. None of the studied mutations affected development of *ts* phenotype.

THE DISCUSSION OF THE RESULTS

We have demonstrated that the introduction of a mutation Gly292Glu in the NP protein of *ca* and *ts* influenza virus A/Hong Kong/1/68/162/35 reassortants affects the level of their replication at low temperature. Reassortants with reverse mutation Gly292Glu in the NP protein Ast/HK/NPmut and Tex/HK NPmut are characterized by *non-ca* and *ts* phenotype. The mechanism of mutation's effect on reproduction at low temperature can be explained by theoretical modeling performed previously [15]. It was shown that temperature decrease causes decrease in the mobility of NP protein domains that impairs NP binding to virion RNA and may block replication. On the contrary the mobility of domains in the mutated NP (Glu292Gly) increases which may lead to increased replicative activity of mutant strain at low temperatures [15]. Thus our experimental data support the potential role of Glu292Gly mutation in the NP protein in the development of the *ca* phenotype of A/Hong Kong/1/68/162/35 virus.

We also investigated the influence of combination of reverse mutations 292Gly→Glu and 102Arg→Gly in the NP protein on virus replication activity at different temperatures. It was shown that the virus bearing two mutations in NP protein displayed even less infectious activity at low (26°C) temperature than viruses bearing single mutation.

It should be especially noted that investigated mutations in NP protein did not influence on viruses replication activity at high temperature (39°C).

Viruses with single reverse mutation in the 102 amino acid position in NP protein failed to be obtained. During the cold adaptation of A/Hong Kong/1/68/162/35 strain there existed two strain variants: strain with a single mutation at position 102 (Gly102Arg) and strain with a combination of substitutions at positions 102 and 292 (Gly102Arg/Glu292Gly). Strain comprising NP protein with substitution at position 292 and without mutation at position 102 did not exist in the evolution process, probably explaining why attempts to create this virus variant had failed. Perhaps the emergence of Gly at position 292 destabilizes ribonucleoprotein complex and mutation Gly102Arg eliminates the negative effect of mutation Gly292 playing the role of compensatory substitution. Of course, it is just an assumption that should be confirmed or disproved in following experiments.

We have analyzed the NP sequences of influenza A viruses that are presented in the GISAID database and found that among 21682 available human influenza A sequences only 68 (except reassortant strains) have Arg at 102 position in NP, and the same can be found for only 9 sequences among 15350 available NP sequences of the avian influenza viruses. Thus Arg in 102 position of nucleoprotein is an extremely rare event and is found in human influenza viruses in 0.33% cases and in avian strains in 0.07% cases. It is important to note that Arg at position 102 in NP protein is characteristic to the donor strain A/Puerto Rico/8/34 and its reassortants which are highly reproductive in CE viruses. In this way the mutation Gly102Arg can have an adaptive character. Confirming this hypothesis are results showing that amino acids located at 100 and 101 positions in the influenza virus NP are antigenically significant and belong to the epitope recognized by monoclonal antibodies that differentiate human and avian influenza viruses [16]. Thus amino acid residues at or close to position 102 may be involved in the adaptation of the virus to the host organism, in our case to chicken embryos.

Earlier it was reported that NP protein do not affect to the reproductive activity of reassortant influenza viruses at a low temperature [17], that contradicts to our obtained results. This discrepancy may be due to differences in methods of producing reassortants. Earlier used methods allowed to receive a reassortant containing the polymerase complex genes and NP gene from different viruses which may influence the formation of the polymerase complex. The obtained reassortants Ast/HK/NPmut (H5N1), Ast/HK/NPmut2 (H5N1) and Tex/HK/NPmut (H1N1) received all internal genes from one virus A/Hong Kong/1/68/162/35 and thus only point substitution in NP could influence their replication activity.

Replication of control strains and reassortant influenza viruses with mutations in the NP in CE at different temperatures

Viruses	Mutations in the NP	Infectious activity, $logEID_{50}$/0.2 mL*		
		26°C	34°C	39°C
Ast/HK (H5N1)	–	7.25 ± 0.71	9.25 ± 0.50	2.83 ± 0.38
Ast/HK/NPmut	Gly292Glu	4.17 ± 0.83	7.10 ± 0.45	1.25 ± 0.25
Ast/HK/NPmut2	Gly292Glu Arg102Gly	2.50 ± 0.43	6.44 ± 0.72	1.25 ± 0.00
Tex/HK (H1N1)	–	5.33 ± 0.72	8.06 ± 0.38	1.25 ± 0.35
Tex/HK/NPmut	Gly292Glu	3.42 ± 0.58	6.25 ± 0.74	1.25 ± 0.00
Tex/PR8	–	1.63 ± 0.18	7.65 ± 0.27	8.25 ± 0.00

*Results are presented as mean ± SD, calculated on the basis offrom three independent experiments.

Published data about the contribution of NP protein in the development *ts* phenotype are contradictory: there are pro [18] and contra [6]. In our research mutations in the NP had no effect on the *ts* phenotype of studied viruses. However while preparing this article for publication a work showing the role of NP protein in the formation of *ts* and *ca* phenotype of cold-adapted virus X-31 has appeared [19].

In general, the results obtained in this work extend the view of the molecular mechanisms underlying attenuation of the influenza A virus, which is important for the development of live influenza vaccines and influenza vectored vaccines against other actual viral and bacterial infections.

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