AVIAN AND HUMAN INFLUENZA A VIRUS STRAINS POSSESS DIFFERENT INTRACELLULAR NUCLEOPROTEIN OLIGOMERIZATION EFFICIENCY

E.N. PROKUDINA*, N.P. SEMENOVA, I.A. RUDNEVA, V.M. CHUMAKOV, S.S. YAMNIKOVA

D.I. Ivanovsky Institute of Virology, Gamaleya St.16, Moscow 123098, Russia

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Summary. – We have previously shown (Prokudina-Kantorovich EN and Semenova NP, *Virology* **223**, 51–56, 1996) that the nucleoprotein (NP) of influenza A virus forms in infected cells oligomers which in the presence of SDS and 2-mercaptoethanol (ME) as reducing agent are stable at room temprature (RT) and dissociate at 100°C. Here we report that the efficiency of intracellular NP oligomerization depends on the host origin of influenza A virus strain. Thus, in the cells infected with avian influenza A virus strains the viral NP was almost completely oligomerized and only traces of monomeric NP were detected by polyacrylamide gel electrophoresis (PAGE) in unboiled samples. However, in the cells infected with human influenza A virus strains, besides oligomeric NP also a significant amount of non-oligomerized monomeric NP was detected in unboiled samples. In purified virions of avian and human strains the same difference in NP monomers/oligomers ratio was detected as in the infected cells. A reassortant having all internal protein genes from a human strain and the glycoprotein genes from an avian strain revealed the same intracellular pattern of NP monomers/ oligomers ratio as its parental human virus. These findings suggest that the type of NP oligomerization is controlled by the NP gene. The possible connection between the accumulation of protease-sensitive monomeric NP in cells infected with a human influenza strain and the parallel accumulation of cleaved NP in these cells is discussed.

Key words: influenza A virus; human strains; avian strains; reassortant; nucleoprotein; glycoproteins; nucleoprotein oligomerization

Introduction

In our previous studies (Prokudina-Kantorovich and Semenova, 1996) we have observed that in the cells infected with influenza A virus (*in vivo)* NP forms boiling-sensitive oligomers. Earlier Ruigrok and Baudin (1995) have described an *in vitro* formation of influenza A virus NP oligomers and pointed out that the oligomerized NP must contain at least two independent binding sites. Recently Elton *et al.* (1999) have studied the *in vitro* NP oligomerization and identified positive and negative sequence elements. The authors have shown that NP contains two discrete regions capable of NP to NP binding as well as a C-terminal sequence that inhibits this self-association.

Comparison of the *in vitro* and *in vivo* influenza A virus NP oligomerization has revealed some differences. Whereas the optimal temperature for the *in vitro* NP oligomerization was 4°C (Ruigrok and Baudin, 1995), the *in vivo* NP oligomerization proceeded only at 37°C and was absolutely absent at 4°C (Semenova *et al.*, 1998). Whereas the *in vitro* NP oligomerization took place only at high NP concentration (Ruigrok and Baudin, 1995), the monomeric NP molecules synthesized in infected cells were immediately oligomerized (Prokudina-Kantorovich and Semenova, 1996; Semenova

*E-mail: vpr@zebratelecom.ru; fax: +7095-1902867.

Abbreviations: HA = hemagglutinin; MAb = monoclonal antibody; ME = 2-mercapto-ethanol; MOI = multiplicity of infection; NP = nucleoprotein; PA = polymerase acid protein; PAGE = polyacrylamide gel electrophoresis; p.i. = post infection; RT = room temperature

et al., 1998). Thus, it seems that the *in vivo* NP oligomerization is not a physical NP self-association but probably a complicated biochemical process.

It is known that some differences exist between influenza A virus strains of human and avian host origin. First of all, their NP gene structure is different (Buckler-White and Murphy, 1986; Gammelin *et al.*, 1989; Scholtissek *et al.*, 1985). In the cells infected with human but not with avian strains the cleaved NP is accumulated at late stages of infection (Zhirnov and Bukrinskaya, 1981). In contrast to a human strain NP, an avian strain NP does not possess a proteolytic site for caspases (Zhirnov *et al.*, 1999). Some differences between human and avian strains in the level of proteolysis associated with viral polymerase acid protein (PA) have also been described (Naffakh *et al.*, 2000). It is known that the host origin of influenza A virus strains is connected with their NP structure (Gorman *et al.*, 1991). There are some differences in NPs of avian and human influenza virus strain groups (Buckler-White and Murphy, 1986) and that is why we aimed this study at comparison of the *in vivo* NP oligomerization efficiency of these groups.

Materials and Methods

Viruses. Human influenza A virus strains A/WSN/33 (H1N1), A/Singapore 1/57 (H2N2), A/Aichi/2/ 68 (H3N2), A/Udorn/72 (H3N2), A/USSSR/90/77 (H1N1), A/FM/1/47 (H1N1), A/PR/8/34 (H1N1) and avian influenza A virus strains A/Duck/ Ukraine/63 (H3N8), A/Chicken Germany/49 (H10N7), A/Duck/Pennsylvania/10218/84 (H5N2), A/Duck/Czechoslovakia/56 (H4N6), A/FPV/Weybridge (H7N1), A/Duck/Ho-Chi-Minh/014/78 (H5N3), A/Pintail/Primorie/695/76 (H2N2), A/Tern/South Africa (H5N3) were used.

All these virus strains have been maintained at this Institute and propagated in the embryonated chicken eggs.

Cells. The continuous MDCK cell line derived from canine kidneys was used.

Assay of intracellular boiling-sensitive NP oligomers. The cells were infected with different strains of influenza A virus at a multiplicity of infection (MOI) of about 10 PFU/cell and incubated for 18 hrs at 37°C. From 4 to 18 hrs post infection (p.i.) the cells were labeled with [^{35}S]-methionine (50 μCi/ml) and suspended in the Laemmli sample buffer containing 2% SDS and 5% ME (Laemmli, 1970). Each sample was divided into two equal portions, one of which was boiled (100°C) for 3 mins before PAGE. and the other was not boiled but kept at RT. Portions (50 μl) of cell lysates were subjected to PAGE (Laemmli, 1970). This assay enabled to determine NP oligomers which in denaturing (SDS) and reducing (ME) conditions are stable at RT and dissociate at 100°C.

Virus purification. The virus-containing culture medium collected 24 hrs p.i. was clarified by low-speed centrifugation and centrifuged in SW41 rotor (Beckman L50 ultracentrifuge) at 24,000 rpm for 1 hr. Then the pellets were resuspended in STE buffer (0.1 mol/l NaCl, 0.01 mol/l Tris-HCl pH 7.4, and 0.001 mol/l EDTA) and layered on a 15–30% sucrose gradient in STE and centrifuged at 24,000 rpm for 1 hr. The virus-containing fractions were collected, pooled and pelleted at 24,000 rpm for 1 hr. The pellets resuspended in STE buffer were analyzed by radioimmunosorption.

Radioimmunosorption. The virus purificates were incubated with pooled anti-NP monoclonal antibodies (MAbs) kindly provided by Dr. R.G.Webster, St. Jude Children' Research

Hospital, Memphis, TEN, USA, and Dr. L. Stitz, Justus Liebig Universitat, Giessen, Germany. The immune complexes were adsorbed on Protein A Sepharose, dissolved in the Laemmli buffer and divided into two portions subjected to the different thermal treatment and PAGE as indicated above.

Results

The type of NP oligomerization in cells infected with avian and human influenza A virus strains

The cells were infected with eight avian and seven human influenza A virus strains each. From 4 to 18 hrs p.i. the cells were labeled with [^{35}S]-methionine. At 18 hrs p.i. the cells were harvested and suspended in the Laemmli sample buffer. Each sample was divided into two equal portions, one of which was boiled for 3 mins before PAGE and the other was subjected straight to PAGE. Fig. 1 demonstrates a different radioactivity of labeled proteins at 18 hrs p.i. in cells infected with different avian strains. However, it is evident that in unboiled samples of the cells infected with all eight avian strains each (the odd lanes) the amount of monomeric 56 K NP was small or invisible. At the same time the material in high molecular mass zone (bands 1 and 11) or at the start of the gel was visible in the unboiled samples. It was shown earlier that the high-molecular mass material in unboiled samples is NP oligomers which dissociates into NP monomers after boiling (Prokudina-Kantorovich and Semenova, 1996).

With all avian strains tested the monomeric 56 K NP appeared only after sample boiling (the even lanes) as result of dissociation of NP oligomers located normally at the gel start and at the bands 1 and 2. These data suggest that in the course of infection with avian strains the most NP molecules are oligomerized.

Fig. 2 demonstrates the pattern of NP oligomerization in cells infected with human strains. In contrast to avian strains, in the unboiled samples of cells infected with human strains (the odd lanes) the amount of monomeric non-oligomerized 56 K NP was rather abundant. After boiling (the even lanes), which led to dissociation of NP oligomers (bands 1 and 11), the amount of monomeric 56 K NP increased. Thus it seems that in the course of infection with human strains a portion of NP does not form oligomers and remains in monomeric form.

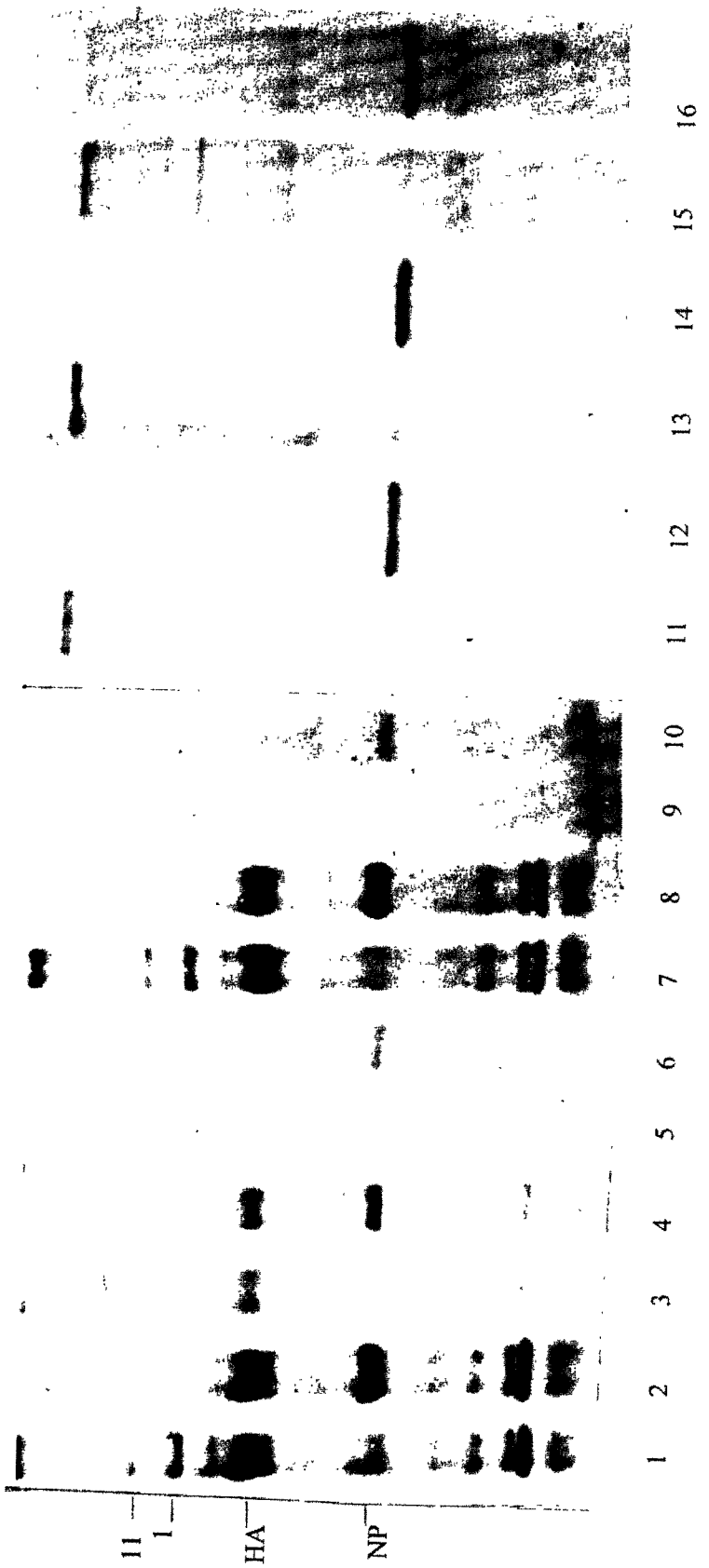

Fig. 1
NP oligomerization efficiency in MDCK cells infected with avian influenza A virus strains
For the experimental procedure see Materials and Methods. Unboiled (odd lanes) and boiled samples (even lanes). Bands 1 and 11 represent NP oligomers. For the abbreviations see their list on the front page of the article. Lanes 1 and 2: A/Duck/ Ukraine/63 (H3N8), lanes 3 and 4: A/Chicken Germany/49(H10N7), lanes 5 and 6: A/Duck/Pennsylvania/10218/84 (H5N2), lanes 7 and 8: A/Duck/ Czechoslovakia/56 (H4N6), lanes 9 and 10: A/FPV/Weybridge (H7N1), lanes 11 and 12 A/Duck/Ho-Chi-Minh /014/78 (H5N3), lanes 13 and 14: A/Pintail/Primorie/695/76 (H2N2), lanes 15 and 16: A/Tern/South Africa (H5N3).

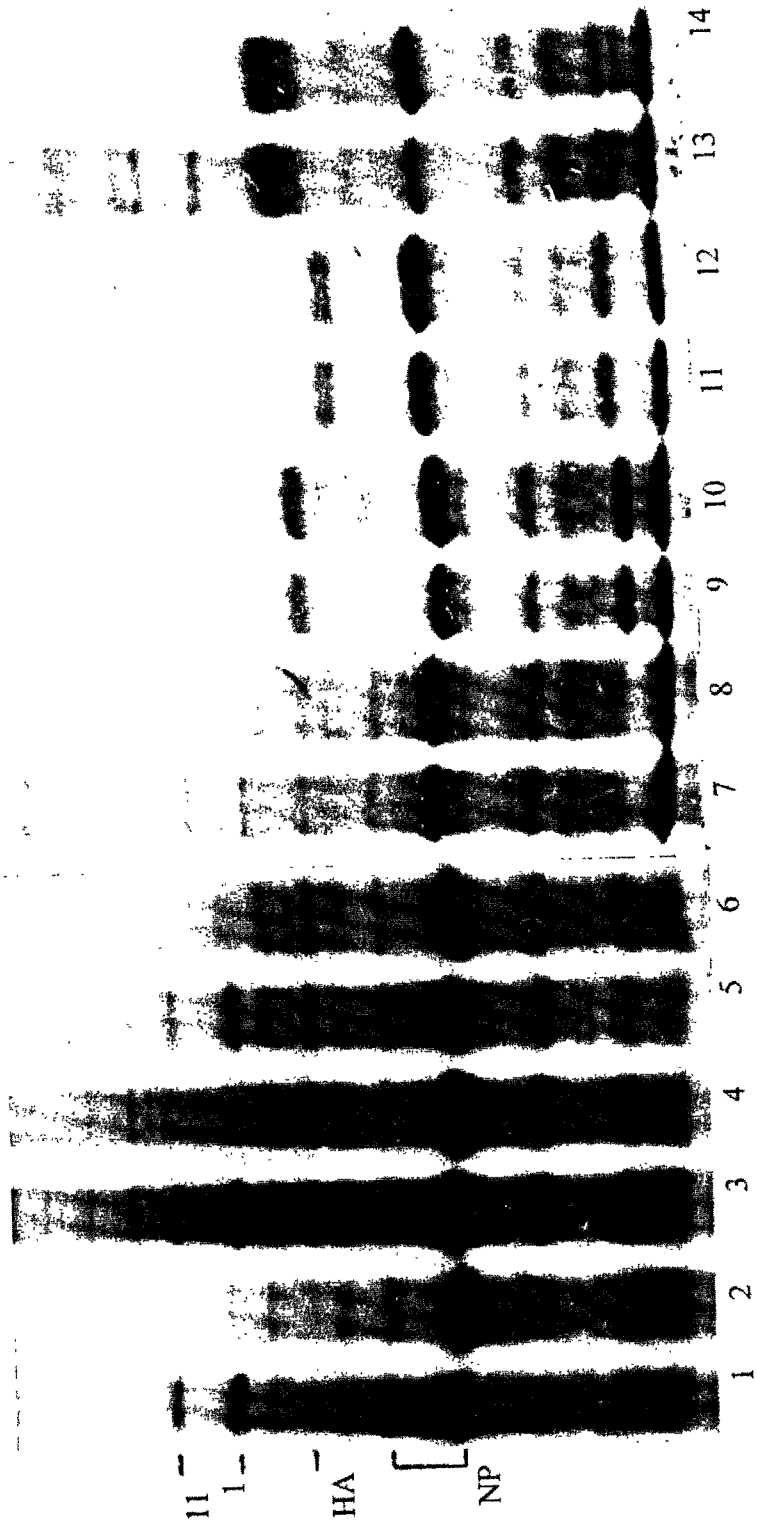

Fig. 2

NP oligomerization efficiency in MDCK cells infected with human influenza A virus strains

For the experimental procedure see Materials and Methods. Unboiled (odd lanes) and boiled samples (even lanes). Bands 1 and 11 represent NP oligomers. For the abbreviations see their list on the front page of the article. Lanes 1 and 2: A/WSN/33 (H1N1), lanes 3 and 4: A/Singapore 1/57 (H2N2), lanes 5 and 6: A/Aichi/2/ 68 (H3N2), lanes 7 and 8: A/Udorn/72 (H3N2), lanes 9 and 10: A/USSSR/90/77 (H1N1), lanes 11 and 12: A/FM/1/47 (H1N1), lanes 13 and 14: A/PR/8/34 (H1N1).

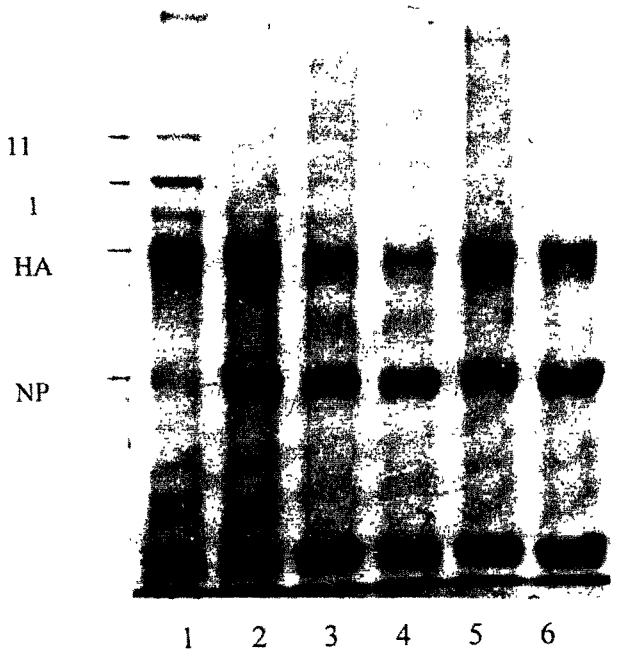

Fig. 3
NP oligomerization efficiency in MDCK cells infected with the influenza A virus avian strain Duck/Ukraine/63 (H3N8), the influenza A virus human strain USSR/90/77 (H1N1) and the reassortant R3 having the glycoprotein genes from the avian strain Duck/Ukraine/63 (H3N8) and the NP gene together with other internal protein genes from the human strain USSR/90/77 (H1N1)
For the experimental procedure see Materials and Methods. Unboiled (odd lanes) and boiled samples (even lanes). Bands 1 and 11 represent NP oligomers. For the abbreviations see their list on the front page of the article.
Lanes 1 and 2: A/Duck/Ukraine/63 (H3N8); lanes 3 and 4: A/USSR/90/77 (H1N1); lanes 5 and 6: the reassortant R2.

The role of NP gene in NP oligomerization efficiency

The difference between avian and human influenza A virus strains in NP oligomerizaion efficiency was confirmed by study of an influenza A virus reassortant. Fig. 3 presents an example of NP oligomerization efficiency in cells infected with the avian strain A/Duck/Ukraine/1/33/63 (lanes 1 and 2), the human strain A/USSR/90/77 (lanes 3 and 4) and their reassortant R3 (lanes 5 and 6) having the glycoprotein genes from the avian strain A/Duck/Ukraine/1/33/63(H3N8) and the NP gene together with other internal protein genes from the human strain A/USSR/90/77 (H1N1). This reassortant has been obtained and studied in detail earlier (Kaverin *et al.*, 1998; Rudneva *et al.*, 1993). Fig. 3 (lane 1) shows that in cells infected with A/Duck/Ukraine/1/33/ (H3N8) strain the amount of monomeric 56 K NP in the unboiled sample is small and NP is mostly represented by oligomers located in high-molecular mass bands 1 and 11. In contrast to the avian strain, in MDCK cells infected with the human strain A/USSR/90/77 (H1N1) (Fig 3, lane 3) the amount of monomeric 56 K NP in the unboiled sample is abundant and the amount of NP oligomers in bands 1 and 11 is relatively small. As to the reassortant it can be seen that its hemagglutinin (HA) band, as expected, resembles the HA band of the parental avian strain A/Duck/Ukraine/63 (H3N8) (compare HA in lanes 5 and 6 with that in lanes 1 and 2). Furthermore, in the unboiled sample of the reassortant, the presence of a large amount of monomeric non-oligomerized NP and a weak bands of NP oligomers resemble the behavior of the human parental strain A/USSR/90/77 (H1N1) (compare NP, 1 and 11 bands in lanes 5 and 3). The results obtained in this experiment suggest that the type of NP oligomerization depends on the host origin of the strain and is probably controlled by NP gene.

Intracellular conversion of NP monomers to NP oligomers

To examine the relationship between monomeric and oligomeric NP in cells infected with avian and human

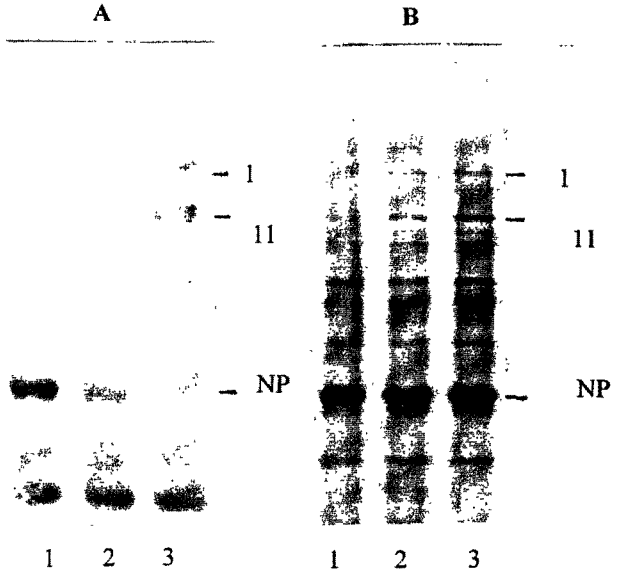

Fig. 4
Pulse-chase analysis of conversion of NP monomers into NP oligomers in cells infected with strains Duck/Ukraine/63 (H3N8) and USSR/90/77 (H1N1)
For the experimental procedure see Materials and Methods. All samples were unboiled. Bands 1 and 11 represent NP oligomers. For the abbreviations see their list on the front page of the article. 0 min (lane 1), 20 mins (lane 2) and 40 mins (lane 3).
A: avian strain A/Duck/Ukraine/63 (H3N8). B: human strain A/USSR/90/77 (H1N1).

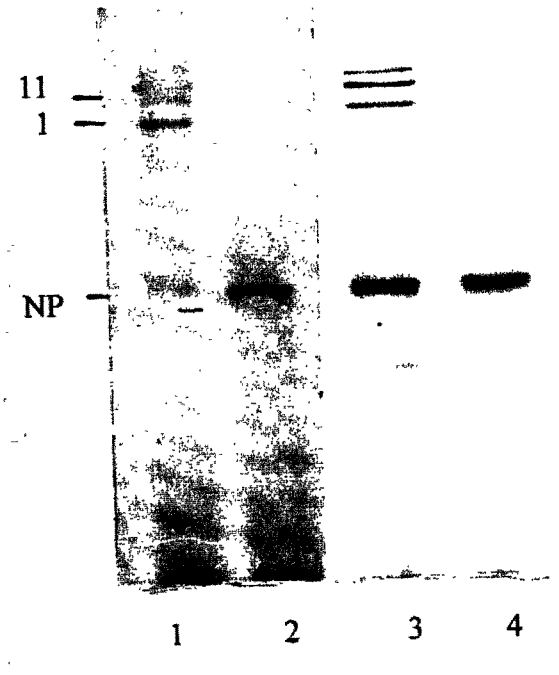

Fig. 5
Radioimmunosorption analysis of purified viral NP
For the experimental procedure see Materials and Methods. Bands 1 and 11 represent NP oligomers. For the abbreviations see their list on the front page of the article. Lanes 1 and 2: avian strain A/Duck/Ukraine/63 (H3N8); lanes 3 and 4: human strain A/USSR/90/77 (H1N1).

influenza A virus strains a pulse-chase experiment was carried out. The cells were infected with the strains A/Duck/Ukraine/1/33 (H3N8) and A/USSR/90/77 (H1N1) and labeled with [^{35}S] methionine (10 μCi/ml) at 6 hrs p.i. for 10 mins. Then the cells were incubated with a label-free medium (chase) and subjected to PAGE. Fig. 4 presents the results obtained with the unboiled samples. It can be seen that the pulse revealed the presence of monomeric NP for both strains (lane 1). However, the results of the chase were different for the two strains. With the avian strain the radioactivity of the 56 K monomeric NP gradually decreased and after 40 mins of chase it disappeared (lane 3). These data suggest that in the cells infected with the avian strain the synthesized monomeric NP is almost completely converted into oligomers. As to the human strain, in spite of the oligomers formation during the chase the amount of monomeric NP was rather large even after 40 mins of chase (lane 3). Thus, only a partial conversion of NP monomers into NP oligomers took place in the cells infected with the human strain.

NP oligomers and NP monomers in virions

In further experiments the NP monomers/oligomers ratio was examined in purified virions. The results of radioimmunosorbtion analysis of purified strains A/Duck/Ukraine/1/33 (H3N8) and A/USSR/90/77 (H1N1) are shown in Fig. 5. It can be seen that in the unboiled sample of the avian strain the amount of the monomeric 56 K NP was scarce (lane 1). In contrast, in the unboiled sample of the human strain the monomeric 56 K NP and NP oligomers were present in well detectable amounts (lane 3). These results show that the difference between avian and human strains in NP monomers/oligomers ratio was practically the same in infected cells as in virions. The data obtained suggest that the RNP containing monomeric NP is able to enter the virions together with the RNP containing oligomeric NP.

Discussion

In this report we showed that the intracellular NP oligomerization efficiency depends on the host origin of the influenza A virus strain of concern in general and on the NP gene in particular. Whereas the pulse-chase analysis of NP oligomers formation in cells infected with the avian strain A/Duck/Ukraine/1/33 (H3N8) demonstrated almost complete conversion of monomers into oligomers after 40 mins of chase, the same type of experiment with the human strain A/USSR/90/77 (H1N1) demonstrated only a partial conversion. Differences in the structure of avian and human strains (Buckler and Murphy, 1986) may be responsible for the difference in NP oligomerization with these strains described here. The reasons for incomplete NP oligomerization with the human strain *in vivo* are unknown. It is possible that intracellular NP molecules (synthesized *in vivo*) are heterogeneous in their NP-NP affinity, and that in the cells infected with a human strain the fraction of NP molecules with a weak binding affinity may be greater than that in the cells infected with an avian strain. Some defects (e.g. a weak binding affinity) in the portion of human strain NP molecules may be connected with special binding sites identified by Ruigrok and Baudin (1995) or with the sequence elements described by Elton *et al.* (1999). The weak NP-NP affinity of the portion of human strain NP molecules may either prevent NP-NP contacts completely or permit weak NP-NP contacts, which may dissociate even at low temperature (due to the effect of SDS during PAGE).

We have shown earlier that the successfully formed human strain NP oligomers are as stable as the avian strain NP oligomers (Semenova *et al.*, 1998). If NP monomers and oligomers are in a state of dynamic equilibrium, it may be suggested that with human influenza A virus strains this equilibrium is shifted towards NP monomers but with avian strains towards oligomers. Most probably the oligomeric form of NP is mature, while the monomeric form is immature, similarly to trans-membrane influenza A virus proteins (Doms *et al.*, 1993).

Interestingly, the fraction of monomeric NP was detected in virions in large amount in the human strain but only in traces in the avian strain. These data suggest that monomeric NP can be incorporated in RNP and the RNP with monomeric NP can be incorporated in virions. We have shown earlier that a truncated influenza A virus NP is oligomerized and can enter the RNP but such RNP cannot be incorporated in virions (Prokudina and Semenova, 1991; Prokudina *et al.*, 2001).

We have shown earlier that in contrast to oligomeric NP the monomeric NP is very sensitive to proteases (Semenova *et al.*, 1998). Due to protease sensitivity the portion of non-oligomerized monomeric NP accumulated in the cells infected with human but not with avian influenza A virus strains may be partially degraded. Thus, our observations fit the phenomenon described by Zhirnov and Bukrinskaya (1981) concerning intracellular accumulation of the degraded NP in cells infected with human but not with avian strains.

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