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# STUDIES ON SOME GENETIC PROPERTIES OF ANTIGENIC RECOMBINANTS OF INFLUENZA VIRUSES

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*Summary.* — Two groups of antigenic recombinants Hav4N1 were obtained by recombination of human influenza virus H0N1 with two avian influenza viruses isolated from ducks in 1956 and terns in 1978 and possessing the same surface antigen Hav4 Nav1. Recombinants obtained by crossing A/PR/8/34 and A/duck/ČSSR/56 viruses showed a lower ability to reproduce at optimal and lowered temperatures and differed in the thermosensitivity of haemagglutinin and neuraminidase. An analysis of virus-specific proteins of the recombinants revealed different combinations of genes coding for internal (Pl, NP) and nonstructural (NS1) proteins. Recombinants obtained by crossing A/PR/8/ /34 and A/tern/Frunze/334/78 viruses possessed a thermostable haemagglutinin; they produced plaques of a size characteristic of avian influenza but, as distinct from the latter, they were practically not eluted from fowl erythrocytes. Polypeptide analysis of these recombinants showed that the genes coding for NP, M and NS1 proteins were inherited from the A/tern/Frunze/334/78 strain.

*Key words*: *influenza virus*; *recombinants*; *genetic markers*; *electrophoresis*; *polypeptides*

## *Introduction*

The possibility of experimental obtaining of antigenic recombinants in mixed infections with human and animal influenza viruses has been repeatedly demonstrated (Tumova and Pereira, 1965; Kilbourne, 1968; Webster, 1970; Webster *et al.*, 1971; Podchernyaeva *et al.*, 1972; etc.).

The aim of the present work was to compare some genetic markers and the polypeptide composition of animal influenza viruses possessing the antigenic structure Hav4Nav1 and of antigenically similar recombinants (Hav4N1) obtained by crossing animal influenza viruses with human influenza virus.

## *Materials and Methods*

*Viruses.* Reference strains of human influenza virus A/PR/8/34 (H0N1) and avian influenza virus A/duck/ČSSR/56 (Hav4Nav1) were used along with the strain A/tern/Frunze/334/78 (Hav4Nav1) isolated in 1978 from a tern in the Kirghizian S.S.R.

*Sera.* To select the recombinants, we used a hyperimmune rabbit serum to the H0Navl recombinant, obtained by Dr. Webster by crossing influenza virus strains A/NWS (H0N1) and A/duck/ /England/56 (Hav3Nav1); the serum was kindly provided by Dr. Webster. The parent strains and recombinants were identified using antisera to strains A/PR/8/34, A/duck/ČSSR/56 and recombinants with an antigenic structure of H0N2 and Hav6N1 that had been obtained by Podchernyaeva and Blinova (1980) on crossing the strains A/PR/8/34 and A/rein deer/Chukotka/76 (Hav6N2).

*Methods.* Recombination experiments were carried out in chick embryos with two live influenza viruses ($10^7$ $EID_{50}$/0.2 ml per embryo). The infected embryos were incubated at 36°C for 18 hr, after which three passages were carried out in the presence of immune serum, followed by cloning by the limiting dilution method at 36° C. Genetic markers ($rct_{28, 36, 40}$; $T_{56}$; $T_N$; $E_{rc}$; Pl) were investigated as described (Podchernyaeva *et al.*, 1972).

In the neuraminidase inhibition (NI) reaction, 0.1 ml volumes of 10-fold serum dilutions were added to 0.1 ml of the working dose of antigen. Ovomucoid was used as substrate. The residual neuraminidase activity was determined according to Aminoff (1961). Sera used in the haemagglutination inhibition (HI) test were treated with receptor destroing enzyme (RDE). For immunodiffusion and polypeptide analysis, the viruses were concentrated and purified in 10–40% sucrose linear density gradients in Tris-HCl buffer at pH 7.4. The immunodiffusion reaction in 1% agarose was carried out according to Schild and Pereira (1969).

The polypeptide composition of the viruses was investigated by electrophoresis in 10% polyacrylamide gels (PAG) according to Laemmli (1970). Electrophoresis was carried out under reducing conditions for 18 hr at 8 mA. Protein zones in the gels were evaluated after staining of the gels with 0.5% Cooomassie brilliant blue solution.

To investigate the synthesis of virus-specific proteins, chick embryo cell (CEC) cultures were inoculated with virus. At 4 hr after inoculation, $^{35}S$-methionine and $^{3}H$-leucine were added to the cultures in a dose of 0.37 MBq per 2.5 $cm^2$ cell surface area and the cultures incubated further for 2 hr. Proteins were analysed by electrophoresis in 17% PAG. After electrophoresis, the gels were dried and examined by autoradiography (Russel and Skehel, 1972).

## *Results*

In two series of experiments on recombination between human influenza virus H0N1 and 2 mutually antigenically similar avian influenza viruses (Hav4Nav1) we obtained recombinants possessing haemagglutinin Hav4 and neuraminidase N1. Upon crossing of A/PR8/34 virus with A/duck/ČSSR, /56 and A/tern/Frunze/334/78 strains we selected respectively eight ($R_{297}$ $R_{306}$, $R_{307}$, $R_{311}$, $R_{319}$, $R_{321}$, $R_{325}$, $R_{332}$) and three ($R_{17}$, $R_{18}$, $R_{19}$) recombinants Hav4N1. The antigenic properties of the parent viruses and the recombinants are listed in Table 1. The antigenic properties of the strains tested were determined based on the results of HI and NI tests.

Precipitation reactions in agarose revealed antigenic identity of haemagglutinins of strains A/tern/Frunze/334/78, A/duck/ČSSR/56 and the $R_{17}$ recombinant (Fig. 1).

The genetic markers of parent strains and the recombinants are presented in Table 2. A comparison of the properties of recipient avian influenza viruses A/duck/ČSSR/56 and A/tern/Frunze/334/78, in addition to antigenic identity, revealed differences in several genetic markers between them. Strain A/tern/ /Frunze/334/78, isolated from terns 20 years after isolation of the strain

**Table 1. Antigenic characteristics of parent strains of influenza virus and their recombinants**

| Virus strains and recombinants | HI tests | | NI tests | |
|---|---|---|---|---|
| | A/PR/8/34 | A/duck/ČSSR/56 | A/PR/8/34 | A/duck/ČSSR/56 |
| A/PR/8/34 (H0N1) | 20.000* | < 10 | 8500 | < 10 |
| A/duck/ČSSR/56 (Hav4Nav1) | < 10 | 10 240 | < 10 | 800 |
| $R_{297}$ | < 20 | 10 240 | 10 000 | < 10 |
| $R_{306}$ | < 20 | 10 240 | 8 000 | < 10 |
| $R_{307}$ | < 20 | 10 240 | 3 000 | < 10 |
| $R_{311}$ | < 20 | 10 240 | 6 000 | < 10 |
| $R_{319}$ | < 20 | 10 240 | 6 000 | < 10 |
| $R_{321}$ | < 20 | 10 240 | 3 200 | 30 |
| $R_{325}$ | < 20 | 20 480 | 6 000 | < 10 |
| $R_{332}$ | < 20 | 20 480 | 4 000 | < 10 |
| A/tern/Frunze/334/78 (Hav4Nav1) | < 10 | 2 560 | 360** | < 10 |
| $R_{15}$ | < 10 | 1 280 | 360** | < 10 |
| $R_{18}$ | < 10 | 1 280 | 360** | < 10 |
| $R_{19}$ | < 10 | 1 280 | 360** | < 10 |

* Titres expressed in dilution reciprocals.

** Neuraminidase of these viruses was identified by antiserum to Hav6N1 recombinant obtained by Podchernyaeva and Blinova on crossing strains A/PR/8/34 and A/deer/Chukotka/76.

A/duck/ČSSR/56, differed from the latter by the ability to reproduce at in-creased (40° C) temperature in chick embryos, by haemagglutinin thermostability and enhanved elution activity.

Recombinants obtained by crossing strains A/PR8/34/ and A/duck/ČSSR/ /56 differed in thermostability of haemagglutinins and neuraminidase and elution activity. Some od the recombinants ($R_{297}$, $R_{321}$, $R_{325}$, $R_{322}$) possessed a thermostable haemagglutinin, like A/PR/8/34 virus and others ($R_{306}$, $R_{307}$, $R_{311}$, $R_{319}$), by contrast, a thermolabile haemagglutinin characteristic of strain A/duck/ČSSR/56. A part of the recombinants possessed neuraminidase that completely lost its activity at 50°C, like the AjPR/8/34 strain, while in other recombinants the loss occurred at 52°C, like in the strain A/duck/ČSSR/56, and in still others (recombinants $R_{319}$ and $R_{321}$) only at 56°C. In all recombinants, with the exception of $R_{332}$, the elution activity was slow. All recombinants produced plaques in CEC cultures. The plaque size was the same as of A/PR/8/34 plaques.

Recombinants obtained upon crossing of the strains A/PR/8/34 and A/tern/Frunze/334/78 possessed several properties characteristic of the latter strain (ability to reproduce at optimal and increased temperature; typical S-marker plaques; and thermoresistant haemagglutinin) but, as distinct from this parent, they were not eluted from fowl erythrocytes for the whole period of observation (6 hr).

Polypeptide analysis carried out on the parent strains A/PR/8/34 and A/tern/Frunze/334/78 in 10% PAG revealed differences in electrophoretic

**Table 2. Genetic markers of parent strains of influenza virus and their recombinants**

| Virus strains and recombinants | Antigenic formula | HA | rct | | | Pl | | $T_{56}$ | N | $T_N$ | $E_{rc}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 28° C | 36° C | 40° C | titre | size | | | | |
| A/PR/8/34 | H0N1 | 640 | 5.0 | 8.0 | 5.0 | 8.0 | 5—7 | 60 | 1.8 | 50° | 1 |
| A/duck/ČSSR/56 | Hav4Nav1 | 160 | 5.0 | 7.8 | 3.5 | 7.0 | 1—3 | 10 | 2.0 | 52° | 3 |
| $R_{295}$ | Hav4N1 | 80 | 4.5 | 7.0 | 3.5 | ; 7.0 | 5—7 | 60 | 0.5 | 50° | 6 |
| $R_{306}$ | Hav4N1 | 160 | 4.0 | 6.5 | 3.5 | 6.0 | 5—7 | 10 | 0.15 | 50° | 6 (12.5%) |
| $R_{305}$ | Hav4N1 | 80 | 4.0 | 6.5 | 4.0 | 6.0 | 5—7 | 10 | 0.1 | 52° | 6 (25%) |
| $R_{311}$ | Hav4N1 | 640 | 4.0 | 6.0 | 3.5 | 7.0 | 5—7 | 10 | 0.2 | 50° | 6 (25%) |
| $R_{319}$ | Hav4N1 | 40 | 4.5 | 6.5 | 4.0 | 6.0 | 5—7 | 10 | 0.3 | 56° | 6 |
| $R_{321}$ | Hav4N1 | 640 | 3.0 | 6.0 | 4.0 | 7.0 | 5—7 | 60 | 1.9 | 56° | 6 |
| $R_{325}$ | Hav4N1 | 40 | 4.5 | 6.0 | 4.0 | 7.0 | 5—7 | 30 | 0.75 | 50° | 6 |
| $R_{332}$ | Hav4N1 | 320 | 4.0 | 6.5 | 3.0 | 7.0 | 5—7 | 60 | 1.0 | 50° | 6 |
| A/tern/Frunze/334/78 | Hav4Nav1 | 160 | 3.0 | 8.2 | 8.2 | 7.0 | 1—5 | 360 | 0.81 | 54° | 6 (50%) |
| $R_{17}$ | Hav4N1 | 160 | 5.1 | 7.5 | 7.0 | 7.0 | 1—5 | 360 | 0.54 | 52° | 0 |
| $R_{18}$ | Hav4N1 | 160 | 4.5 | 7.5 | 8.0 | 6.0 | 1—5 | 360 | 0.52 | 52° | 0 |
| $R_{19}$ | Hav4N1 | 80 | 3.0 | 7.0 | 8.2 | 6.0 | 1—5 | 360 | 0.54 | 52° | 0 |

HA — haemagglutinin titre per 0.1 ml; rct — ability to reproduce in chick embryos at the indicated temperature (log $EID_{50}$/0.1 ml); $T_{56}$ — (absence of haemagglutinin titre after heating at 56° C for the indicated time (min); N — neuraminidase activity (in optical density units) per mg protein at a wavelength of 549 nm; $T_N$ — total inactivation of neuraminidase of the indicated temperature (°C); Erc — — elution activity from erythrocytes (complete elution after the indicated time in hr); Pl — plaque formation in CEC cultures — titre in log PFU/ml, size in mm in the presence of 2 μg/ml trypsin

mobility of haemagglutinin HA0 and its heavy chain HA1 and the greatest differences in the light (HA2) haemagglutinin chain and neuraminidase (NA). The same electrophoretic mobility of the light chain (HA2) in recombinant $R_{18}$ and strain A/tern/Frunze/334/78 proved that it originated from this parent. Fig. 3 illustrates the synthesis of virus-induced proteins in CEC cultures and control cells (ZKo) in the same parents and recombinants $R_{17}$, $R_{18}$ and $R_{19}$ under other electrophoresis conditions (17% PAG). Proteins NP, M and NS in the recombinants had the same mobility as in strain A/tern/ /Frunze/334/78. Fig. 4 presents electrophoregrams of 11 recombinants possessing the same surface antigens Hav4N1, obtained in a recombination experiment with strains A/PR/8/34 and A/duck/ČSSR/56. Comparative analysis revealed various combinations of internal (P1 and NP) proteins and the non-structural NS protein in these recombinants. No marked differences were found in the M, P2 and P3 proteins. In the strain A/duck/ČSSR/56, in addition to the nonstructural protein, we observed two additional, probably also nonstructural, proteins.

## *Discussion*

In the present work we obtained influenza virus recombinants possessing the same Hav4N1 antigenic formula and investigated some of their genetic and physico-chemical properties. These recombinants were selected following crossing of human influenza virus H0N1 with two different animal influenza viruses isolated in Europe in 1956 and in Asia in 1978 from two different avian species, possessing the same rather frequent combination of surface antigens (Hav4N1). A study of the genetic markers showed that strain A/tern/Frunze/ /334/78, isolated from terns in the U.S.S.R. more than 20 years after isolation in Czechoslovakia of the strain A/duck/ČSSR/56, is antigenically closely related to the latter (based on the results of HI, NI and immunodiffusion tests), but differs from it by a thermoresistant haemagglutinin, ability to reproduce at increased temperature and lowered ability to reproduce at lowered temperature.

Recombinants obtained upon crossing of strains A/PR/8/34 and A/duck/ /ČSSR/56 were characterized by a lowered ability to reproduce at lower (28°C) and optimal (36°C) temperatures. The combination of genes in these recombinants was not possibly optimal for manifestation of this marker.

An interesting phenomenon was found in investigating the monocistronic markers (haemagglutinin and neuraminidase): irrespective of that the recombinants possessed an antigenically identical haemagglutinin (Hav4), one half of them had thermolabile haemagglutinin like the A/duck/ČSSR/56 strain, while in the other half the haemagglutinin was thermoresistant like in A/PR/8/34 virus.

Similar results were obtained in studying the thermoresistance of neuraminidase: in spite of that all recombinants possessed antigenically the same neuraminidase N1 of the human A/PR/8/34 strain, in 4 recombinants the neuraminidase activity was not lost at 50° C like in this parent but at higher

temperatures (52 or 56°C), which is characteristic of the A/duck/ČSSR/56 strain possessing another type of neuraminidase. It can be concluded, therefore, that different sites in haemagglutinin and neuraminidase molecules are responsible for antigenic specificity and phenotypic manifestation of $T_{56}$ and $T_N$ markers. The mechanism of this phenomenon remains obscure. Along with a possible intragenous mutation or recombination it is not excluded that the given properties are due to the action of other proteins. In our experiments we observed no correlation between N and $E_{rc}$ markers. In studies on the ability of recombinants to be eluted from erythrocytes possessing another receptor — fucose (Tolmacheva *et al.*, 1980), the recombinant viruses proved to be incapable of being eluted from the erythrocytes under the action of neuraminidase. It is possible that in the course of genetic interaction of two viruses in the process of the formation of recombinants there occur mutantions leading to changes in the secondary structure of haemagglutinin and neuraminidase.

The mobility of HA and NA glycoproteins is known to depend on their bonds with sodium dodecyl sulphate (Russ and Poláková, 1973) and on the type of HA and NA (Bosch *et al.*, 1979). The polypeptides of viruses used in the present investigations were identified based on earlier studies of avian influenza viruses (Ivanova *et al.*, 1979). Analysis of polypeptide composition of the recombinants and their parents confirmed that the surface proteins of the recombinants belonged to a certain type revealed in serological reactions.

A comparison of virus-induced proteins in parent strains and recombinants made it possible in several instances to demonstrate that the internal proteins in the recombinants corresponded to those of one or another parent. Analysis of a great number of recombinants obtained in a single experiment (A/PR/8/ /34 × A/duck/ČSSR/56) revealed various protein combinations in the recombinants. This suggests that an as yet uncontrollable reassortment of genes occurs in recombination of influenza viruses.

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*Explanation of Figures* (Plates XXVI–XXVIII):

*Fig. 1.* Immunodiffusion reaction in agarose gel. Central well: antiserum to Hav4 haemagglutinin (A/duck/ČSSR/56). Peripheral wells: antigens A/PR/8/34 (PR8), A/tern/Frunze/334/78 (tFr), A/duck/ČSSR/56 (dCs) and recombinants $R_{17}$, $R_{18}$, $R_{19}$. The viruses were disintegrated by Triton-X-100.

*Fig. 2.* Electrophoregrams of virion proteins of strains A/tern/Frunze/334/78 (tFr 1–3), A/PR/8/34 (7–9) and recombinant $R_1$, (4–6). Electrophoresis in 10% PAG, 18 hr, 8 mA.

*Fig. 3.* Polypeptide synthesis in CEC infected with strains A/PR/8/24, A/tern/Frunze/334/78 (tFr) and their recombinants $R_{17}$, $R_{18}$ and $R_{19}$.
Proteins were labelled with $^3$H-leucine at 4 hr after inoculation. ZKo — control. Electrophoresis in 17% PAG, 20 hr, 100 V.

*Fig. 4.* Polypeptide synthesis in CEC infected with strains A/PR/8/34, A/duck/ČSSR/56 and their recombinants.
Proteins were labelled with $^{35}$S-methionine at 4 hr after inoculation. Electrophoresis in 17% PAG, 17 hr, 80 V.