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Evolution of the NS Genes of the Influenza A Viruses. I. The Genetic Relatedness of the NS Genes of Animal Influenza Viruses

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Abstract

We compared the nucleotide sequences of the NS genes of 13 animal influenza viruses belonging to human, swine, avian, and equine viruses for the study of the genetic relatedness of the NS genes in animal influenza viruses. The NS genes of three virus strains A/chicken/Brescia/02, A/equine/Prague/56, and A/equine/Miami/63 were newly sequenced. The base sequence homologies between the NS genes of avian, human, swine, and the A/equine/Miami/63 viruses were 87.8% or higher. On the other hand, the base sequence of the NS gene of the A/equine/Prague/56 virus differed widely from those of other viruses analyzed in the present study. We constructed a model of the genetic tree of the NS genes of avian and equine influenza viruses by a modified Farris method (1). For comparison of the NS genes between human and avian viruses, we estimated the speed of the nucleotide substitutions of the avian influenza NS genes. It was roughly constant, even though the substitutions did not occur sequentially. The nucleotide substitution rate of the NS genes of avian influenza viruses was one-third to one-fourth that of human influenza viruses. We deduced the time of separation between the NS genes of human and avian influenza viruses during evolution.

Introduction

Influenza A viruses have been isolated from animals of various species including humans. The genetic aspect of the host specificity of influenza A viruses has been analyzed by sequencing each virus genome (2–4). For NS genes of human influenza A viruses, it has been shown that the number of nucleotide substitutions increases linearly with time and the rate of nucleotide sequence variation has been determined to be 1.73 nucleotide substitutions/NS gene/year (5). In our previous paper (3), we analyzed the nucleotide sequences of the NS genes in avian influenza viruses and confirmed the sequence homology of human and avian influenza viruses. However, the number of nucleotide substitutions in the NS genes of avian influenza viruses did not change sequentially with time and were arranged in multiple evolutionary lineages. We did not estimate the substitution rate of the NS genes of avian influenza viruses or the time of possible separation between the NS genes of human and avian influenza viruses.

We wanted to know the genetic relatedness among various animal influenza viruses. We newly sequenced NS genes of two different types of equine influenza viruses and the oldest isolate of avian virus. In the present study, we analyzed the genetic relationships of the NS genes of human, swine, equine 1, equine 2, and avian influenza A viruses. We also estimated the time of possible separation between the NS genes of human and avian influenza A viruses.

Materials and Methods

Viruses, cloning, and sequencing

Animal influenza A viruses, A/chicken/Brescia/02 (H7N1) (Ck/Bre/02), A/equine/Prague/56 (H7N7) (Eq1/Pra/56), A/equine/Wisconsin/1/69 (H7N7) (Eq1/Wis/69), A/equine/Miami/63 (H3N8) (Eq2/Mia/63), and A/equine/Wisconsin/2/69 (H3N8) (Eq2/Wis/69) were grown in embryonated chicken eggs. The Ck/Bre/02 virus had been maintained at the National Institute of Animal Health in Ibaraki, Japan. The Eq1/Wis/69 and Eq2/Wis/69 viruses were kindly provided by Dr. B. C. Easterday. The cDNA cloning and sequencing methods of the NS genes were described previously (3).

DNA-RNA hybridization

^{32}P-labeled, single-stranded complementary DNA (cDNA) of the NS genes of the Eq1/Pra/56 and Eq2/Mia/63 viruses were synthesized from viral RNA (vRNA) by using an NS-specific primer (3). Each of the ^{32}P-labeled cDNA fragments was isolated by 2.7% polyacrylamide gel electrophoresis (6). About 2000 cpm of the cDNA was hybridized for 24 hr at 65°C with 1–3 μg of the vRNA in a reaction

mixture (200 μl) containing 10 mM Tris-HCl, pH7.5; 0.2M NaCl; 2 mM EDTA; 0.05% $NaDodSO_4$; and 10 μg of tRNA. After incubation, 2 ml of a solution consisting of 2 units of S1 nuclease (Sankyo); 0.1 M NaCl; 0.2 mM zinc sulfate; 33 mM sodium acetate, pH4.5; and 30 μg of denatured calf thymus DNA was added. After incubation for 90 min at 45°C, the TCA-insoluble radioactivity was determined.

Results and Discussion

Genetic relatedness of the NS genes among animal influenza viruses

The nucleotide sequences of the NS genes of Ck/Bre/02, Eq1/Pra/56, and Eq2/Mia/63 viruses are shown in Fig. 1. The nucleotide sequences of the NS genes of ten animal influenza viruses—Ck/Bre/02, A/chick/Japan/24 (Ck/Jap/24) (H7N7), A/fowl plague/Rostock/34 (FPV/34) (H7N1), A/duck/England/56 (Dk/Eng/56) (H11N6), A/tern/South Africa/61) (Tern/SA/61) (H5N3), A/duck/Ukraine/63 (Dk/Ukr/63) (H3N8), A/mynah/Haneda-Thai/76 (My/H-T/76) (H3N1), Eq2/Mia/63, A/swine/Iowa/30 (Sw/Ia/30) (H1N1), and Eq1/Pra/56—were compared with each other and the results are shown in Fig. 2. Three human influenza viruses—A/PR/8/34 (PR/8/34) (H1N1), A/Fort Warren/1/50 (FW/50) (H1N1), and A/Udorn/72 (Udorn/72) (H3N2)—and one avian influenza virus—A/duck/Alberta/76 (Dk/Alb/76) (H12N5)—are also included in Fig. 2 for comparison. Since we used a synthetic primer, described in Materials and Methods, for the cDNA cloning of the NS genes, the sequences of the first 34 bases could not be determined and were excluded from comparison. The comparison of the NS gene of the Sw/Ia/30 virus with those of other viruses was done only for the nucleotide sequence corresponding to the NS1 polypeptide. The percentage of base differences in the NS genes in pairwise comparisons of avian (except Dk/Alb/76), human, swine, and Eq2/Mia/63 viruses were within the range of 4.0–12.2%, that is, the base sequence homologies of the NS genes of these viruses were fairly high. On the other hand, the base sequences of the NS genes of the Dk/Alb/76 and Eq1/Pra/56 viruses differed more widely from those of other viruses analyzed in the present study. Epidemiology and genetic findings suggested that the Sw/Ia/30 virus had been derived from a 1918 human virus (11). A common ancestry of the human and most avian influenza viruses has been suggested (2, 3). Attention was focused then on the difference between the NS genes of Eq1/Pra/56 and Eq2/Mia/63 in genetic relatedness to those of avian influenza viruses. The genetic homology of the NS genes in equine influenza viruses was studied for two viruses, each of serotype 1 (Eq1) and serotype 2 (Eq2). Table 1 shows that the NS gene of the Eq1/Pra/56 virus was close to that of the Eq1/Wis/69 virus, but not to that of the Eq2/Mia/63, nor to that of the Eq2/Wis/69 virus; while the NS gene of the Eq2/Mia/63 virus was close to that of the Eq2/Wis/69 virus, but not to that of the Eq1/Pra/56, nor to that of the Eq1/Wis/69 virus. These results suggested that the NS genes of the Eq1 and

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Ck/Bre/02  ATG GAT TCC AAC ACT GTG TCA AGC TTT CAG TTA GAC TGC TTT CTT TGG CAT
Eq2/Mia/63 --- --- --- --- --- --- --- --- --- --- G-- --- --G --- --- --- ---
Eq1/Pra/56 --- --- --- --- --A --- --- --T --- --- G-- --T --G --- --- --- --C

Ck/Bre/02  GTC TGC AAG CGA TTT GCA GAC CAA GAA ATG GGT GAT GCC CCA TTC CTT GAC
Eq2/Mia/63 --- C-- --A --- --- --- --- --- --- C-- --- --- --- --- --- --- ---
Eq1/Pra/56 A-T C-- --A --- --- --- --- --- --- A-- A-- --- --- --- --G --T --- ---

Ck/Bre/02  CGA CTT CGC CGA GAT CAA AAG TCC CTG GGA GGA AGA GGC AGC ACT CTT GGT
Eq2/Mia/63 --- --- --- --- --C --G --- --- --A A-- --- --- --- --- --- --- ---
Eq1/Pra/56 --- --- --- A-- --- --- --- --- T-A AA- --- --G --- --- --- --- ---

Ck/Bre/02  CTG GAC ATC GAG ACA GCT ACT CGT GCT GGA AAG CAG ATA GTG GAG CGG ATT
Eq2/Mia/63 --- --- --- --A --- --C --- --- --A --- --- --- --- --- --- --- ---
Eq1/Pra/56 --A --- --T -G- -GC T-A --A -TA --A --G -G- --- --- --A A-- --- ---

Ck/Bre/02  CTG GAG GAA GAA TTT GAT GAG GCA CTT AAA ATG ACC ATT GCC TCT GCC ACT
Eq1/Mia/63 --- --A --G --G -CA --- --- --- --- --- --- --- --- --- --- -TT C--
Eq1/Pra/56 --A A-A --- --- -C- --- AGT -A- -CA --- GG- --T --- A-- --A -TA C-C

Ck/Bre/02  GCT TTC AGC TAC CTA ACT GAC ATG ACT CTT GAG GAG ATG TCA AGG GAC TGG
Eq2/Mia/63 --- -CA C-- --- --- --- --- --- --- --- --T --- --- --- --A --- ---
Eq1/Pra/56 A-- -CA TAT --T G-- --- --- --- --- --- --A --A --- --- --- --- ---

Ck/Bre/02  TTC ATG CTC ATG CCC AAA CAG AAA GTG GCA GGC TCC CTT TGC ATC AGA ATG
Eq2/Mia/63 --- --- --- --- --- --G --- --- --A --- --- --- --C --T --A --- ---
Eq1/Pra/56 --- --- --T --A --- --C --A --- AGA -T- --A --A --C --- --- --- ---

Ck/Bre/02  GAC CAG GCA ATC ATG GAC AAA AAC ATC ACA CTG AAA GCA AAC TTC AGT ATA
Eq2/Mia/63 --- --- --- --- --- --T --G --- --- -T- --A --- --- --- --T --- G-G
Eq1/Pra/56 --T --A --C --A --- --T --G G-A C-- --- --- -C- --- --- --T --- G-G

Ck/Bre/02  ATT TTT GAT TGG CTG GAG ACT CTA ATA CTA CTA AGG GCT TTC ACC GAG GAG
Eq2/Mia/63 --- --C --C C-- --- --- --A --- --- --- --T --- --- --- --- --A --A
Eq1/Pra/56 G-C --- A-C AAA --- --- --- --- -C- --T T-- C-A --A --- --A -GT --T

Ck/Bre/02  GGA GCA ATT GTT GGC GAA ATC TCA CCA TTG CCT TCT CTT CCA GGA CAT ACT
Eq2/Mia/63 --- --- G-C --- --- --- --T --- --- --- --- --- --- --- --- --- ---
Eq1/Pra/56 --- --- --- A-- --A --- -CT -T- --- A-A --- --- --- --- --- --- ---

Ck/Bre/02  GAT GAG GAT GTC AAA AAT GCA ATT GGG GTC CTC ATC GGA GGA CTT AAA TGG
Eq2/Mia/63 A-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- G-- ---
Eq1/Pra/56 A-- --A --- C-- --- --- --- --- -A- A-- --- --- --- --- --- G-- ---

Ck/Bre/02  AAT GAT AAC ACA GTT CGA GTC TCT GAA ACT CTA CAG AGA TTC GCT TGG AGA
Eq2/Mia/63 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Eq1/Pra/56 --- A-- --- --- --- --- A-- --- --G -T- --- --- --- --- A-- --- ---

Ck/Bre/02  AGC AGT AAT GAG GAT AGG AGA CCT CCA CTC CCT ACA AAG CAG AAA CGG AAA
Eq2/Mia/63 --- --- C-- --- A-- G-- --- --- T-- T-- --- --- --- --- --- --A ---
Eq1/Pra/56 -A- --- --- --- A-- G-- G-- TT- TT- --- T-- --- --A --A --- -AA ---

Ck/Bre/02  CTG GCA AAA ATG GCT GAG TCA GAA GTT TGA AGA AAT AAG ATG GTT GAT TGA
Eq2/Mia/63 A-- --G --- -CA AT- --- --- --- --- --- --- --- --- G-- --- --- ---
Eq1/Pra/56 A-- -AG GG- -CA A-- -G- --- --- --- --- --- --- --- --- -C- --- ---

Ck/Bre/02  AGA AGT GAG ACA TAA ATT GAA GAT AAC AGA AAA TAG TTT CGA ACA AAT AAC
Eq2/Mia/63 --- --- -C- --- --G -C- --- A-- T-- --- --- --- --- T-- --- --- ---
Eq1/Pra/56 --- -A- A-- G-- --- -C- A-- A-A T-- --- G-- C-- --- T-- --- --- ---

Ck/Bre/02  ATT TAT GCA AGC CTT ACA ACT ATT GCT TGA AGT GGA ACA AGA CAT AAG AAC
Eq2/Mia/63 --- --- --- --- --- --- --- --- --- --- --- --- --- G-- --- --- ---
Eq1/Pra/56 --- --G --- --- A-- --- --- --- --- --- --- --- G-- --- G-- --- G--

Ck/Bre/02  TTT CTC GTT TCA GCT TAT TTA ATG ATA AAA AAC ACC CTT GTT TCT ACT
Eq2/Mia/63 --- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
Eq1/Pra/56 --- --- --- C-- --- --- --- --- --- --- --- --- --- --- --- ---
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Fig. 1. The nucleotide sequences of the NS genes of Ck/Bre/02, Eq1/Pra/56, and Eq2/Mia/63 viruses.

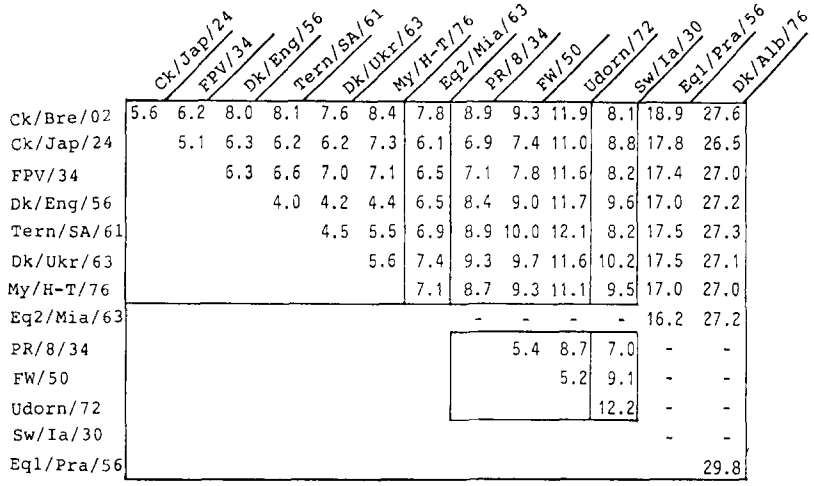

Fig. 2. Base sequence differences in the NS genes among animal influenza viruses. The percentage of the base differences in the NS gene of animal influenza viruses was calculated from the data described in the preceding paper (2,3,7–11). Besides our sequence data of the NS genes (3,11), we used the published data of the nucleotide sequences of the NS genes of the FPV/34 (7), Dk/Alb/76 (8), PR/8/34 (2), FW/50 (9), and Udorn/72 (10) viruses.

Eq2 viruses were of different origin. The Eq2/Mia/63 virus was isolated in 1963, and the subsequent spread of the virus in the equine population was reminiscent of that of a new pandemic virus in the human population (12). No evidence was obtained that horses imported from foreign countries introduced the disease into Florida (13). Although the possibility that the Eq2 subtype viruses had been circulating among horses before 1963 without causing major outbreaks could not be ruled out, it is possible that the Eq2/Mia/63 virus originated from an avian strain (14), considering that both the Eq2/Mia/63 and Dk/Ukr/63 viruses have the same serotype of hemagglutinin and neuraminidase, and similarity in the NS genes. The base differences (%) decreased with time when the NS gene of the Eq2/Mia/63 virus was compared with those of avian isolates before 1934 (Ck/Bre/02 and Ck/Jap/24 viruses), but increased with time when it was compared with those of avian isolates after 1934 (Dk/Eng/56, Tern/SA/61, and My/H-T/76 viruses). These results suggested that the NS gene of the Eq2/Mia/63 virus branched off around the time when that of the FPV/34 virus branched off from the branch of

Table 1. Base sequence homology (%) of the NS genes between the Eq1- and Eq2-type viruses

^{32}P-cDNA probe of NS gene	S1 nuclease resistance (%) after hybridization with RNAs from			
	Eq1/Pra/56	Eq1/Wis/69	Eq2/Mia/63	Eq2/Wis/69
Eq1/Pra/56	100	92	42	54
Eq2/Mia/63	50	50	100	86

The method is described in Materials and Methods.

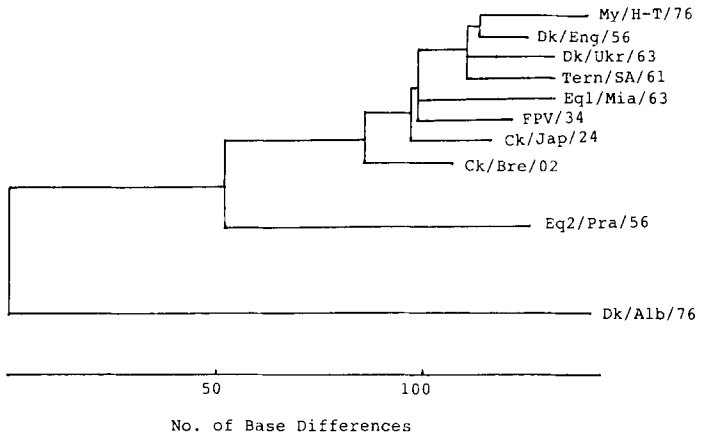

Fig. 3. The evolutionary relationships of the NS genes of avian and equine influenza viruses. The model for the evolutionary relationship was constructed according to the modified Farris method described by Tateno et al. (1). The separation point between the NS genes of the Eq1/Pra/56 and Dk/Alb/76 viruses was determined by assuming that the rate of nucleotide substitutions between them was similar. The length of each branch shows the genetic differences.

the NS evolutionary tree. Considering the above results, we constructed a model of the genetic tree of the NS genes of avian and equine influenza viruses by the modified Farris method (1). The separation point between the NS genes of the Eq1/Pra/56 and Dk/Alb/76 viruses was determined by assuming that the rate of the nucleotide substitutions between them were similar (Fig. 3).

Characteristic of the nucleotide substitutions of avian influenza viruses

In our previous paper (3) we reported that the NS genes of the avian viruses did not change sequentially with time and were arranged in separate evolutionary lineages. Nevertheless, there was a rough correlation between the extent of base differences in the NS genes and the time interval of virus isolation of avian influenza viruses (Fig. 2). Figure 4A shows the number of base differences in the NS genes of avian influenza viruses in comparison with three old strains (Ck/Bre/02, Ck/Jap/24, and FPV/34 viruses). The Dk/Alb/76 virus had an extraordinarily large number of substitutions and, therefore, was excluded. We also show the variability of the NS genes of human influenza viruses for comparison (Fig. 4B). The ordinate indicates the number of base differences in the NS genes between three virus strains taken as reference and later strains. The lines were drawn by linear regression analysis. The slope represents the nucleotide substitution rate.

For avian influenza viruses, the slopes of the lines were 0.44 nucleotide substitutions/NS gene/year when the Ck/Bre/02 virus was taken as a reference and 0.49 nucleotide substitutions/NS gene/year when either of the Ck/Jap/24 and FPV/34 viruses was taken as a reference. For human viruses, the slopes of the lines

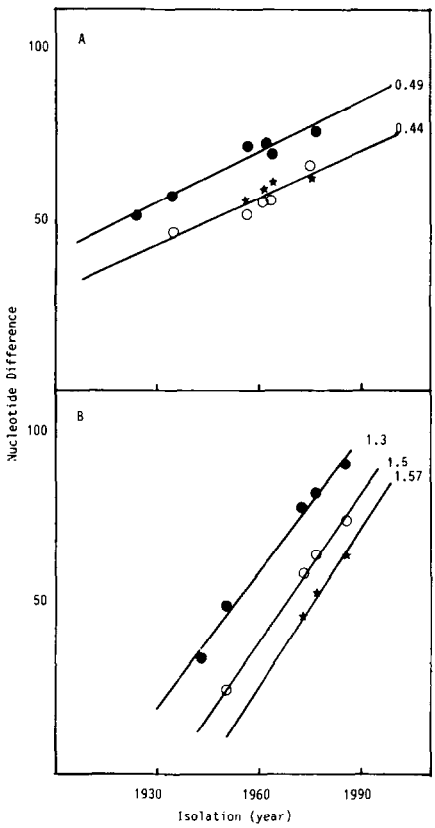

Fig. 4. Linearity with time of the number of substitutions in the NS genes of avian (A) and human (B) influenza viruses. For human viruses, besides the viruses listed in Fig. 2, we further used published sequence data of the NS genes of the A/Bellamy/42 (Bel/42), A/Alaska/77, and A/Ann Arbor/85 viruses (5). The abscissa represents the year of isolation. The ordinate indicates the number of nucleotide substitutions in the NS genes between the reference virus strain and the later strains. Reference virus strains were Ck/Bre/02 (●), Ck/Jap/24 (○), and FPV/34 (★) for avian viruses, and PR/8/34 (●), Bel/42 (○), and FW/50 (★) for human viruses. The slopes of the lines drawn by linear regression analysis are indicated.

were 1.30–1.57 nucleotide substitutions/NS gene/year by similar analysis. Our estimation of the slopes for human viruses was slightly lower than the 1.73 nucleotide substitutions/NS gene/year obtained by Buonagurio et al. (5). The respective slopes obtained from the comparisons were quite close in avian influenza viruses, even if different virus strains were used as references.

The result indicated that the rate of nucleotide substitutions in avian NS genes was roughly constant, even though the mutations did not occur sequentially. The nucleotide substitution rate of the NS genes of avian influenza viruses was one-third to one-quarter that of human influenza viruses. Each of the oblique lines for human influenza viruses in Fig. 4 crossed the transverse axis near the year when the reference virus was isolated, suggesting the later isolated viruses were derived

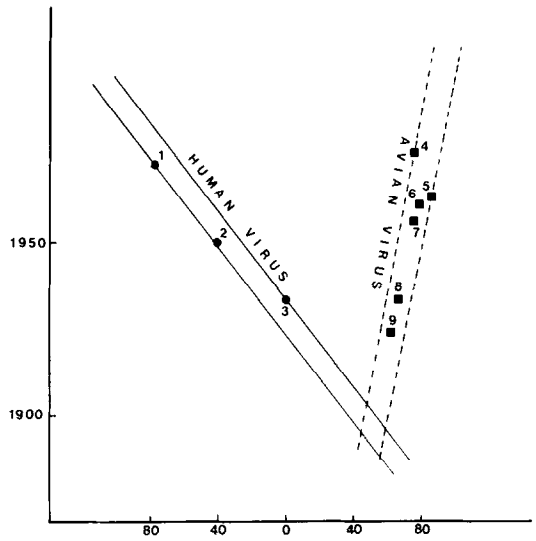

Fig. 5. Putative time of divergence between NS genes of human and avian influenza viruses. The time of divergence was estimated from the accumulation of total nucleotide substitutions of the NS genes of the human influenza viruses, Udorn/72 (1) and FW/50 (2), and the avian influenza viruses, Ck/Jap/24 (9), FPV/34 (8), Dk/Eng/56 (7), Tern/SA/61 (6), Dk/Ukr/63 (5), and My/H-T/76 (4), compared with that of the PR/8/34 virus (3). The rate of nucleotide substitutions of the NS genes of human and avian influenza viruses were estimated to be 1.53 and 0.46 nucleotide substitution/NS gene/year, respectively. The ordinate represents the year of isolation. The abscissa indicates the number of base differences of NS genes in each virus compared with that of the PR/8/34 virus.

from the reference virus or a virus close to this (1915 for PR/8/34 virus, 1934 for Bel/42 virus, and 1945 for FW/50 virus). The 19-year difference for the PR/8/34 virus may have arisen from its long time passages in eggs. On the other hand, each of the oblique lines for avian viruses did not cross the transverse axis near the year when the reference virus was isolated, suggesting that the later-isolated viruses did not derive from the reference virus or a virus close to this.

This interpretation indicates that the NS genes of these avian influenza viruses are on different branches of an evolutionary tree of the NS genes, as shown in Fig. 3. The genetic similarity of the NS genes, however, is much higher than that of the HA genes in avian influenza viruses, because the progeny viruses that arose from genetic reassortment (15,16) are mainly selected with regard to the surface antigens, HA and neuraminidase. In influenza A viruses, the HA genes have diverged and been fixed into 13 serotypes during evolution, whereas the NS genes that do not suffer selectional forces evolved independently from the HA genes.

Correlation of the NS genes in human and avian influenza A viruses

When the NS genes of the PR/8/34 and avian influenza viruses were compared, the number of base differences of the avian influenza viruses from the PR/8/34 virus

increased with time, except for the Ck/Bre/02 virus (Fig. 2). One of the possible explanations is that an avian influenza virus that was derived from the branch containing the Ck/Jap/24 virus, but not that containing the Ck/Bre/02 virus, contributed to the NS gene of the human influenza virus. The number of base differences between the NS genes of PR/8/34 and FW/50, Udorn/72, and avian influenza viruses are plotted in Fig. 5. The rates of nucleotide substitutions in the NS genes of human and avian influenza viruses were estimated to be 1.53 and 0.46 nucleotide substitutions per year, respectively (Fig. 4). Therefore, the divergence time from the hypothetical common ancestor into human and avian influenza viruses was estimated to be around 1900, although it remains to be substantiated by epidemiological evidence.

Acknowledgments

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