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Location on the evolutionary tree of influenza H3 haemagglutinin genes of Japanese strains isolated during 1985–6 season

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SUMMARY

The nucleotide sequences of the haemagglutinin (HA) genes of influenza A (H3N2) isolates from the 1985–6 season in Japan along with those of several viruses isolated between 1982–5 from other countries were analyzed to determine the origin of the 1985–6 Japanese strains. The HA genes of these viruses consisted of 1762 nucleotides and had a three-nucleotide deletion downstream from the stop codon when compared to the sequences of earlier Hong Kong H3N2 viruses. An evolutionary tree of the HA genes of these viruses was drawn using the A/Bangkok/1/79 sequence as the starting point. Eight strains isolated from Asian and Pacific regions including Japan in the 1985–6 season (one in May) had the HA genes located closely on the evolutionary tree but away from those of the isolates in North America and Europe during the 1984–5 season, and a common ancestry for these viruses was suggested.

INTRODUCTION

Influenza viruses circulate constantly among human populations around the world, evading neutralizing antibodies by changing antigenic phenotypes. These antigenic changes (antigenic drift) are mainly due to amino acid substitutions in the antigenic determinants of the major surface antigen, the haemagglutinin (HA) molecule. The three-dimensional structure of the Hong Kong (H3N2) HA molecule has been determined and four to five antigenic sites on the HA molecule are proposed (Wilson, Skehel & Wiley, 1981; Wiley, Wilson & Skehel, 1981; Underwood, 1982, 1984; Daniels *et al.* 1983). The molecular basis of the antigenic changes of the HA molecule during the evolution of influenza A H1N1 and H3N2 viruses has been analyzed by determining the nucleotide sequences of the HA genes of field strains (Both *et al.* 1983; Raymond *et al*, 1983, 1986). Both *et al.* (1983) showed the sequential amino acid changes at the key antigenic sites of the HA molecule of the H3N2 subtype influenza viruses since their appearance in 1968–80 and suggested that the possibilities for further antigenic drift among

H3N2 viruses might be limited. However, in 1982 H3N2 subtype viruses drifted from A/Bangkok/1/79 (BKl/79)-like to A/Philippines/2/82 (Phil/82)-like viruses and caused major epidemics of influenza in the 1982–3 season. Subsequently further antigenic drift occurred causing outbreaks during the 1984–5 season in many countries (not in Japan) and during the 1985–6 season including Japan (WHO, 1984, 1986, 1987). In Japan, the chronological sequence of virus isolation of each subtype (or type) reported to NIH, Japan by prefectural and municipal public health laboratories throughout the country showed that Phil/82-like H3N2 viruses were introduced into the country during the 1982–3 season and caused outbreaks. The viruses causing the majority of outbreaks during the next two seasons, i.e. 1983–4 and 1984–5, were type A (H1N1) and type B influenza viruses, respectively. H3N2 subtype viruses again started to circulate in September 1985, reached a peak in December and disappeared rapidly in January 1986. The 1985–6 epidemic began about 2 months earlier than usual. During this period, three groups of antigenically different H3N2 viruses were cocirculating.

In the present study, we analyzed the nucleotide sequences of the HA genes of H3N2 isolates from the 1985–6 season in Japan along with the HA sequences of several viruses isolated between 1982–5 from other countries in order to determine the origin of the 1985–6 Japanese strains and also to analyze the evolutionary pathways of the H3 HA genes after the BKl/79 strain. These viruses were also compared by amino-acid sequencing of the HA molecule and HI reactivity using post-infection ferret sera.

MATERIALS AND METHODS

Viruses and purification of viral RNAs

The strains and passage history of the influenza A viruses used in the present study are shown in Table 1. The viruses were obtained from the stocks in the National Institute of Health, Japan and Centers for Disease Control, Atlanta, GA, USA (kindly provided by Dr Alan P. Kendal). The viruses were grown in MDCK cells at 37 °C and purified by 30–60% discontinuous sucrose gradient centrifugation in a Beckman SW28 rotor. Viral RNAs were extracted by the procedure described by Palese & Schulman (1976).

Nucleotide sequencing of the HA genes

Nucleotide sequences of the HA genes were determined from total virion RNAs using the dideoxy chain termination method (Sanger, Nicklen & Coulson, 1977) essentially as described previously (Nakajima *et al.* 1986). Briefly, 3 μg of total viral RNA was mixed with 1 μg of a specific synthetic primer and incubated for 30 min at 42 °C before the reaction was started. One-quarter of this primed template was extended with AMV reverse transcriptase (2 U/μg RNA, Life Sciences) in the presence of 50 mM Tris/HCl, pH 8·0, 10 mM DTT, 5 mM $MgCl_2$, 50 mM KCl, 50 μM each of dCTP, dGTP, dTTP, 1 μM dATP, 5 μCi of [α-^{32}P]dATP (3000 Ci/mmol, New England Nuclear), and one of the following dideoxynucleotides: ddGTP (1·5 μM), ddTTP (6 μM), ddCTP (3 μM), or ddATP (0·6 μM). The reaction mixture was incubated for 30 min at 37 °C and then an excess (0·1 mM) of dNTPs was added. This was incubated for 30 min at 37 °C and the reaction

Table 1. *Description of the H3N2 strains used in the present study*

Strain name	Abbreviation	Month of specimen collection	Place of isolation	Passage history*
A/Bangkok/1/79	BK1/79	Sept. 79	Thailand	E15CK2
A/Philippines/2/82	Phil/82	Mid-summer 82	Philippines	E1OCK5
A/Oita/3/83	OI/83	Jan. 83	Japan	E5CK2
A/New Jersey/4/85	NJ/85	Jan. 85	U.S.A.	E3CK2
A/Michigan/1/85	MI/85	Jan. 85	U.S.A	MK2E1CK2
A/Stockholm/4/85	SH/85	Jan. 85	Sweden	CKXMK1E1CK2
A/Yamagata/96/85	YG/85	May 85	Japan	E4CK2
A/Bangkok/2467/85	BK15/85	July 85	Thailand	E3CK2
A/Bangkok/2746/85	BK25/85	July 85	Thailand	E3CK2
A/Chaing Mai/4/85	CM/85	Aug. 85	Thailand	E3CK2
A/Tonga/23/85	TG/85	Aug. 85	Tonga	CK2E1CK2
A/Yokohama/C-5/85	YH/85	Oct. 85	Japan	CK2E1CK2
A/Gumma/346/85	GM/85	Nov. 85	Japan	CK1E1CK2
A/Yamanashi/497/85	YN/85	Nov. 85	Japan	E3CK2
A/Fukuoka/C-29/85	FO/85	Nov. 85	Japan	E6CK2

* E, eggs; CK, MDCK cells; MK, primary monkey kidney cells; X, passage number unknown. The passage numbers are indicated.

was terminated by quickly freezing in dry ice/ethanol. The reaction products were resolved by electrophoresis through 8% polyacrylamide-7 M urea slab gels in TBE buffer (100 mM Tris-borate, pH 8·3: 1 mM EDTA). The gels were transferred to filter papers (Whatman 3 MM; Whatman Ltd., England), covered with Saran Wrap, and subjected to autoradiography (X-ray film, Cronex 4; Dupont).

Oligonucleotide primers

The 12 oligonucleotides synthesized by an automatic oligonucleotide synthesizer (Model 381A, Applied Biosystems) corresponded to the nucleotide positions, 11–25, 149–163, 277–289, 424–438, 578–590, 741–755, 883–897, 1050–1064, 1197–1211, 1354–1367, 1482–1496, and 1622–1636, numbered according to the positive strand sequence of the A/Aichi/2/68 (H3N2) HA gene (Verhoeyen *et al.* 1980). For sequences that could not be read unambiguously with the use of the above 12 primers, 5 additional primers corresponding to the nucleotide positions, 419–433, 449–461, 565–577, 714–728, and 1035–1049, were employed.

HI tests

Haemagglutination–inhibition (HI) tests were performed using ferret post-infection sera treated with receptor-destroying enzyme. Ferret sera were kindly provided by Dr Kuniaki Nerome, NIH, Japan.

RESULTS

Nucleotide sequence changes of the HA genes from BK1/79 sequence of the H3N2 strains isolated between 1982–5

Figure 1 shows the nucleotide sequence changes in the H3 HA genes of 14 human influenza strains from that of BK1/79 (Both & Sleigh, 1981) isolated

between 1982–5 from different regions of the world, along with the changed amino-acids. Since the synthetic primers described in Materials and Methods were used for the sequence determination, the first 29 bases could not be determined. The nucleotide bases of the HA gene of BK1/79 strain which had not been shown by Both & Sleigh (1981) were either taken from the results by Both *et al.* (1983) or newly determined by us. All viruses including BK1/79 consisted of 1762 nucleotides with a three-base deletion downstream from the stop codon (1687–1689) when compared to the sequences of the A/Aichi/2/68 (Aichi/68), A/NT/60/68/29C (NT/68), and A/Victoria/3/75 (Vic/75) viruses (Verhoeyen *et al.* 1980; Both & Sleigh, 1980). Nucleotide changes occurred at least at 101 positions in the 14 HA genes when compared to the HA sequence of BK1/79. These changes were classified either as sporadic changes, occurring in only one or at most a few strains, or mainstream changes inherited by most subsequent strains as described by Raymond *et al.* (1986). Thirty-one mainstream, 67 sporadic, and 3 intermediate (i.e. the one which did not fit the above mentioned criteria) changes occurred. These nucleotide changes resulted in deduced amino-acid changes at 45 residues, 5 of them located in the leader peptide, 32 in the HA1, and 8 in the HA2 polypeptides. Among these, 10 amino-acid changes resulted from mainstream nucleotide changes. The nucleotide changes were scattered all over the HA gene, whereas the amino-acid changes were rather clustered between residues 124 and 261 in HA1. At residues 138 and 248 in the HA1 polypeptide, two different amino-acid changes were observed.

Evolutionary tree of the HA gene based on the nucleotide changes between different virus isolates

An evolutionary tree for different virus isolates can be drawn based on the number of nucleotide differences separating them (Both *et al.* 1983). An evolutionary tree for the HA genes of the 1982–5 H3N2 strains from BK1/79 based on nucleotide changes is shown in Fig. 2. Between the HA genes of BK1/79 and Phil/82, Phil/82 and OI/83, OI/83 and SH/85, and SH/85 and TG/85 viruses, there were 14, 10, 1 and 6 mainstream changes, respectively. These viruses represent the epidemic strains either of the 1979–80, 1982–3, 1984–5, or 1985–6 influenza seasons in the Northern Hemisphere. Each of the pairs Phil/82 and CM/85, NJ/85 and MI/85, and YN/85 and FO/85 belongs on the same branch. Five Japanese strains, YG/85, YH/85, GM/85, YN/85, and FO/85, are located closely on the evolutionary tree, together with the other three 1985 isolates (BK15/85, BK25/85, TG/85). Two American strains (MI/85, NJ/85) and a European strain (SH/85), each of which represents epidemic strains of the 1984–5 season, are located closely to OI/83. SH/85 had the largest number of sidestream mutations.

Antigenic analysis of the H3N2 isolates

The results of the HI tests of four H3N2 isolates from the 1985–6 season in Japan and ten viruses isolated between 1982–5 from other countries are shown in Table 2. BK1/79 was used as the reference strain. Phil/82 and all other 13 strains

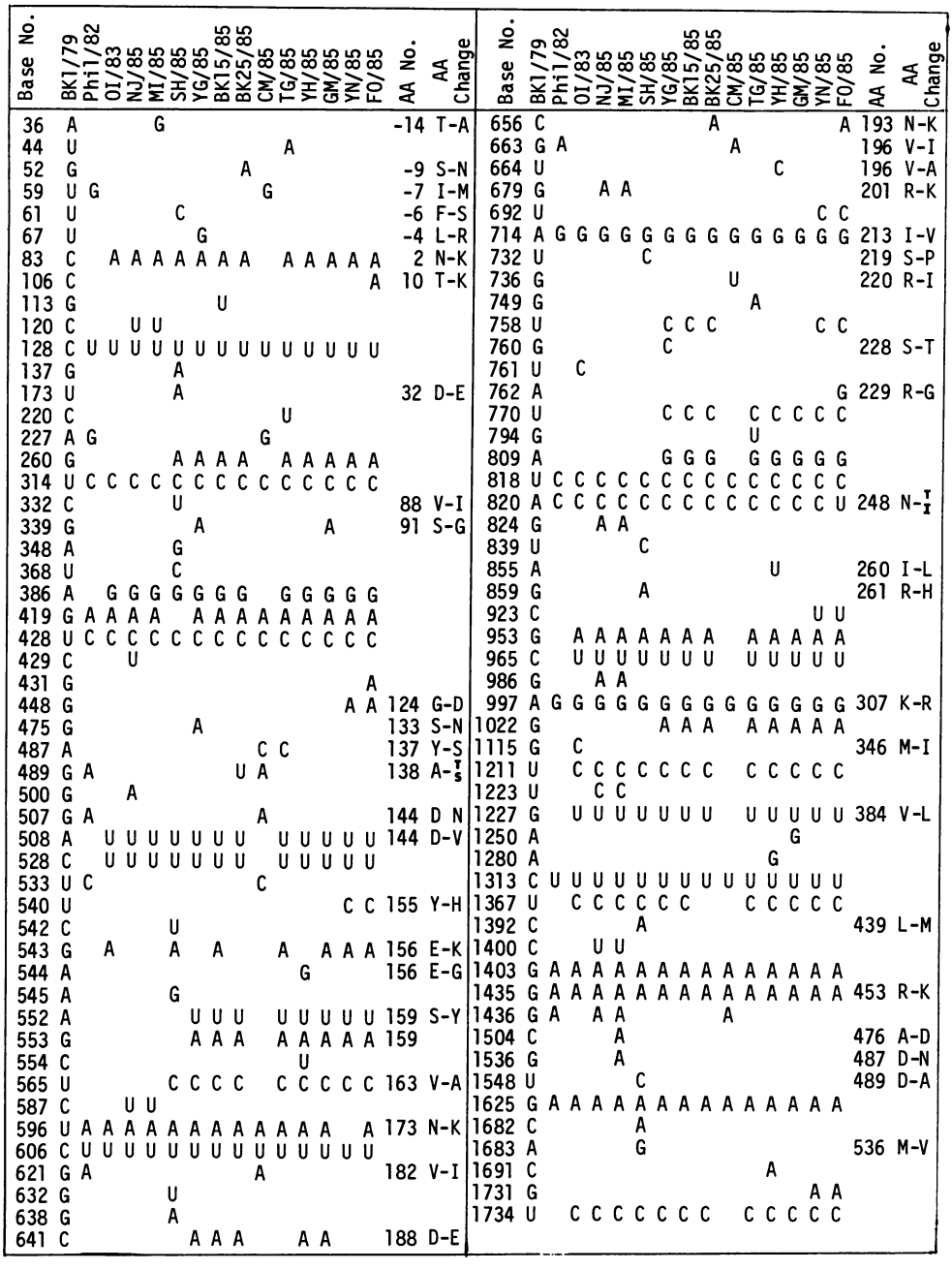

Fig. 1. Nucleotide changes in the HA genes of 14 H3N2 strains from 1982–5. Only positions which drifted from the HA gene of BK1/79 are shown. The position of the amino-acid changes are also shown in the last column.

had the HI titres of $\leqslant 80$ against BK1/79 ferret sera and are drifted from BK1/79 strain. From the HI pattern, 14 viruses could be tentatively divided into 5 groups. Only the viruses which have HI titres within fourfold lower of that obtained with the homologous virus are considered to be inhibited by the ferret

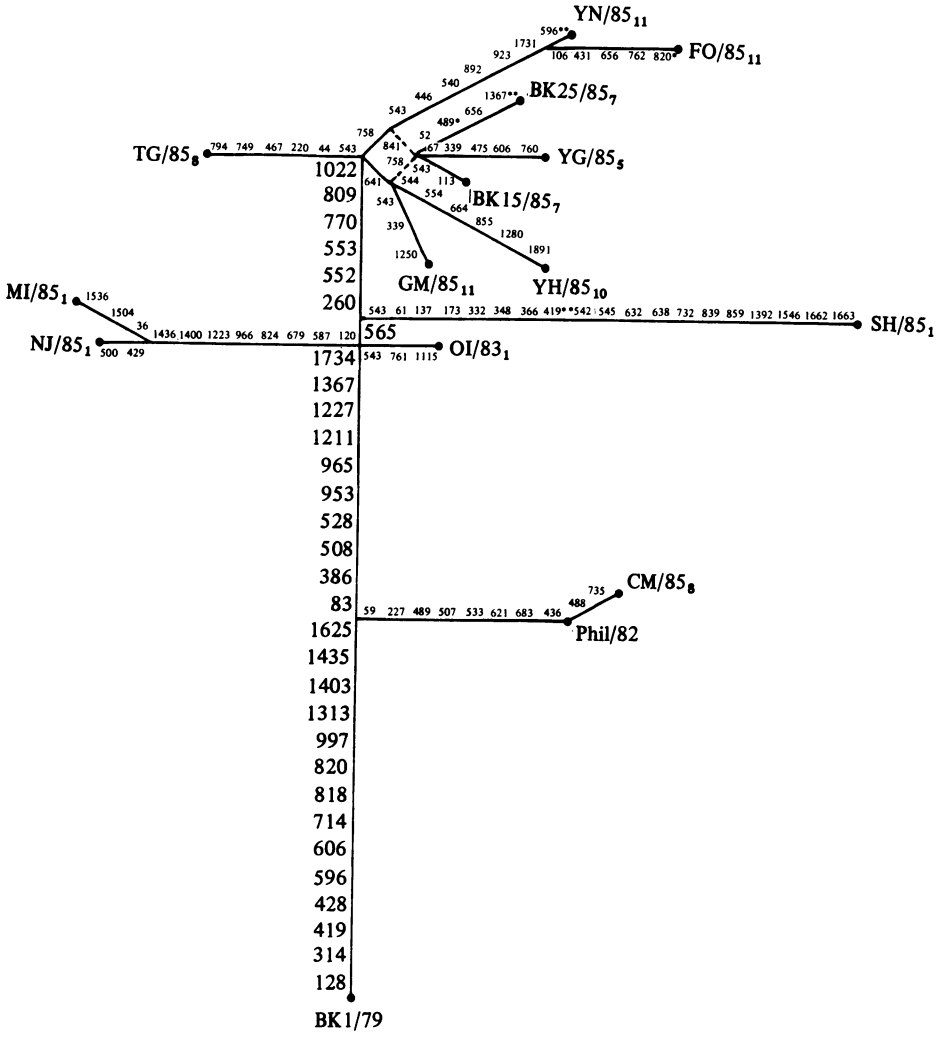

Fig. 2. The evolutionary tree for the HA genes of the 1982–5 H3N2 strains from BK1/79 based on the nucleotide changes. *denotes the nucleotides which changed differently from the preceding ones. **denotes the nucleotides which returned to those of the BK1/79 strain. Since the evolutionary pathway of YG/85, BK15/85, and BK25/85 could not be determined from the analysis, a dashed line was used in the figure. The suffix after the strain shows the month of isolation.

sera. Group 1 viruses (Phil/82, NJ/85, MI/85, YG/85, CM/85) were well inhibited with Phil/82 and YG/85 antisera; group 2 viruses (OI/83, SH/85, TG/85, YH/85) were inhibited with anti-Phil/82, anti-YG/85, and anti-YN/85; group 3 viruses (BK15/85, GM/85, YN/85) were inhibited with anti-YG/85 and anti-YN/85; group 4 virus (BK25/85) was inhibited with anti-YG/85 and anti-FO/85; group 5 virus (FO/85) was inhibited with anti-YG/85, anti-YN/85, and anti-FO/85. Anti-YG/85 ferret sera had the broadest HI spectrum against H3N2 viruses examined in this study, while anti-FO/85 ferret sera inhibited only BK25/85 and

Table 2. *Haemagglutination inhibition reactions of H3N2 influenza viruses isolated from 1979–85*

Group	Virus strain	Ferret sera* BK1/79	Phil/82	YG/85	YN/85	FO/85
	BK1/79	1280†	160	160	40	<40
1	Phil/82	40	320	200	40	<30
	NJ/85	40	480	480	120	60
	MI/85	40	400	480	120	120
	YG/85	<40	240	640	160	120
	CM/85	80	480	480	40	<30
2	OI/83	40	320	480	320	60
	SH/85	<40	120	320	320	40
	TG/85	<40	100	400	400	100
	YH/85	<40	240	640	480	120
3	BK15/85	<40	60	240	480	60
	GM/85	<40	60	320	640	80
	YN/85	<40	60	240	960	80
4	BK25/85	<40	60	180	180	480
5	FO/85	<40	80	320	320	1280

* All ferret sera were treated with receptor-destroying enzyme to inactivate non-specific inhibitors. Eact titre is the mean of duplicate tests. The viruses which have HI titers within 4-fold of that obtained with the homologous virus are in the same group.

† Homologous titres are underlined.

FO/85 viruses well. Four Japanese strains isolated in the 1985–6 season (YH/85, GM/85, YN/85, FO/85) belonged to groups 2, 3, and 5.

Amino-acid changes in the 1982–5 H3 molecule

The evolutionary relationships of the 1982–5 H3 HA molecules based on the amino-acid changes from BK1/79 are shown in Fig. 3, along with the antigenic grouping by the HI tests. Since the amino-acid change at residue 156 occurred unpredictably, we considered it to have occurred independently in each strain. The evolutionary pathway correlated well with the one based on nucleotide changes. Between BK1/79 and Phil/82, Phil/82 and OI/83, OI/83 and SH/85, and SH/85 and TG/85 HA polypeptides, there were 5, 3, 1 and 1 mainstream changes, respectively. Eight of them located in the HA1 region and two in the HA2 region. The changed amino-acid residues were located on both antigenic and non-antigenic sites in the H3 molecule. Eight 1985 strains isolated after the 1984–5 season (except CM/85) originated at the same position on the mainstream stem and contained 2–7 amino-acid changes from the divergent point. The antigenic grouping by the HI tests did not reflect the evolutionary relationships of the H3 HA molecule (Fig. 3).

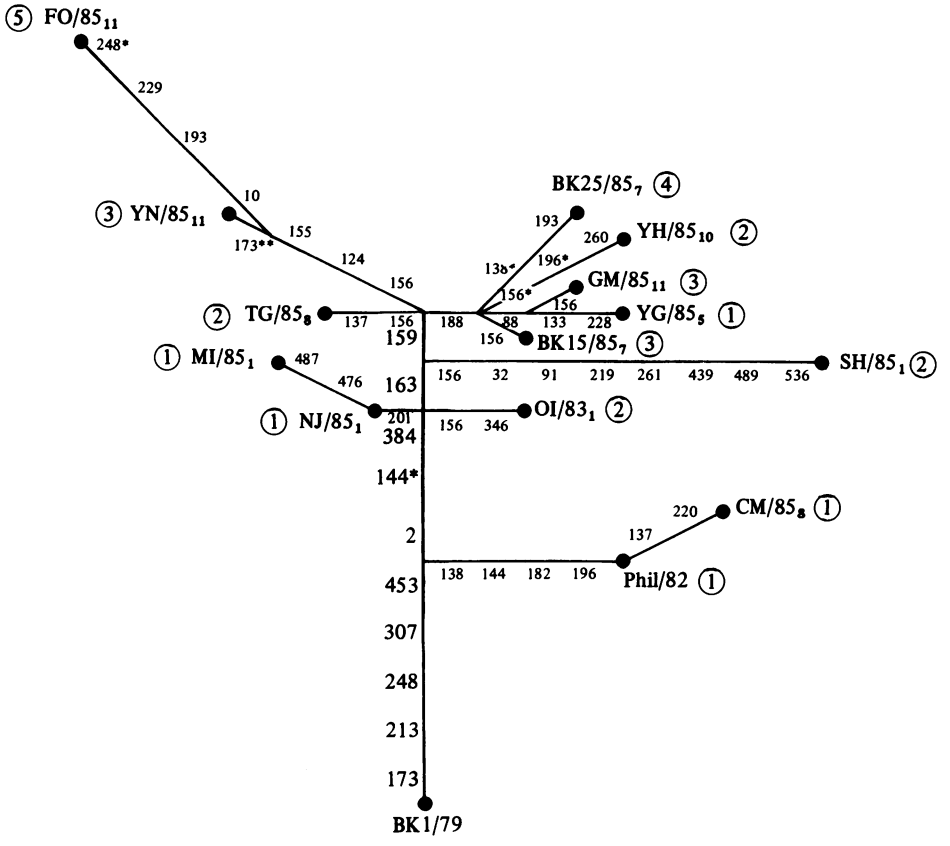

Fig. 3. The evolutionary relationships of the 1982–5 H3 molecules based on the amino-acid changes from BK1/79 H3 polypeptide. * denotes the amino acids which changed differently from the preceding ones. ** denotes the amino acids which returned to those of the BK1/79 strain. The suffix after the strain shows the month of isolation and the number in circle after the strain shows the HI grouping.

DISCUSSION

The nucleotide sequence analysis of the HA genes of recent H3N2 viruses showed that the Phil/82 and all viruses isolated thereafter which we analyzed in the present study had a three-base deletion downstream from the stop codon compared to the earlier H3N2 viruses. This suggests the single origin of recent epidemic strains. The evolutionary tree of the HA genes of 14 H3N2 viruses from the BK1/79 virus showed that the HA genes of these viruses fell into three groups. Group 1 includes Phil/82 and CM/85; group 2 includes OI/83, NJ/85, MI/85 and SH/85; group 3 includes the other eight viruses. The epidemic strains of the 1982–3 and 1984–5 seasons fell into the same group, while those strains of the 1985–6 season (except CM/85) formed a different group. Between the BK1/79 and Phil/82 viruses, isolated 3 years apart, there were at least 14 mainstream nucleotide changes in the whole HA gene with an average about five changes per year. This value is close to that of seven per year between Aichi/68 and BK1/79 HA genes. A similar value (about six) was obtained with the mainstream changes

between Phil/82 and TG/85, separated by 3 years. This suggests that the evolution of the H3 HA gene is still continuing at approximately the same rate.

CM/85 and Phil/82 viruses had almost the same HA nucleotide sequence despite a 3-year interval of isolation. The HI reaction patterns of 12 strains (including CM/85) isolated at the same period in Chiang Mai (Thailand) was the same (data not shown). Further, the oligonucleotide fingerprints of the whole RNA of CM/85 and Phil/82 viruses were not the same (data not shown). Therefore it seems to be likely that the Phil/82-like viruses had been kept for 3 years without major genetic or antigenic changes.

Previously we studied the origin of the 1978–9 H1N1 Japanese epidemic strains using the technique of T1 oligonucleotide mapping (Nakajima *et al.* 1981) and the results suggested that the viruses introduced from outside the country caused outbreaks in Japan. In the present study, we analyzed the HA gene sequences of the candidates for the origin of Japanese strains of H3N2 subtype in the 1985–6 season. The HA genes of YH/85, GM/85, YN/85, and FO/85 strains located closely on the evolutionary tree but on three different branches. The fact that the HA genes of eight H3N2 strains from the 1985–6 season isolated in the Asian and Pacific regions (including YG/85 which was isolated in the spring of the same year) originated at the same position on the evolutionary tree, suggests a common ancestry for these viruses. Two Japanese strains (YN/85 and FO/85) are derived from the same branch in spite of the difference in antigenicity, while YN/85 and GM/85 located on the different branches are antigenically similar. This shows that the closeness in antigenicity does not necessarily reflect the closeness seen on the evolutionary tree and changes in the amino acids at key points may influence the antigenicity of the viruses.

From the comparison of the amino-acid changes in the HA polypeptide of these viruses and the result of the HI tests, we deduced the amino-acid changes that were responsible for differences in the HI reactivity with ferret sera. Two strains (BK25/85, FO/85) reacted with anti-FO/85 ferret sera, and the amino acid change at residue 193 was common between these strains. Therefore, the amino-acid change at residue 193 from Gln to Lys may be responsible for the HI reactivity to anti-FO/85 sera. Similarly, the amino acid change at residue 156 from Glu to Lys/Gly was deduced to be responsible for the HI reactivity with anti-YN/85 sera, because all strains that changed this amino-acid residue (OI/83, SH/85, TG/85, BK15/85, YH/85, GM/85, YN/85, FO/85) gained HI reactivity with anti-YN/85 sera. Both amino acids 193 and 156 locate on the proposed antigenic site B of the HA molecule which comprises the external residues of an helix, and adjacent residues along the upper edge of a pocket tentativly implicated in virus receptor binding (Wilson, Skehel & Wiley, 1981). The amino acid changes that were responsible for differences in the HI reactivity with other ferret sera, however, could not be deduced from the present study.

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