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Genetic relationship between the HA genes of type A influenza viruses isolated in off-seasons and later epidemic seasons

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SUMMARY

From January 1985 to March 1989, off-season viruses of H1N1 and H3N2 subtypes of influenza A viruses were isolated on five occasions in Japan. The HA gene sequences of the influenza A(H1N1) and A(H3N2) viruses isolated in Japan from 1985–9 were analysed and the phylogenetic tree for each subtype virus was constructed to determine any genetic relationship between viruses isolated in off-seasons and the epidemic viruses of the following influenza seasons. In one instance with H1N1 viruses in 1986 and in two instances with H3N2 viruses in 1985 and 1987, the spring isolates were genetically close to some of the winter isolates and were considered to be the parental viruses of the following influenza seasons. However, even in these cases, influenza viruses of the same subtype with different lineages co-circulated in Japan.

INTRODUCTION

In 1977, a pandemic caused by influenza A(H1N1) viruses represented by the A/USSR/90/77(H1N1)(USSR/77) strain occurred. After the previous antigenic shift in 1968 from influenza virus A (H2N2) to A(H3N2), the H2N2 influenza viruses disappeared completely. However after 1977 the hitherto prevalent H3N2 influenza viruses did not disappear. As a consequence, recent influenza epidemics were caused by influenza A(H1N1) or A(H3N2), or influenza B virus. The main epidemic viruses were not necessarily the same in Japan, the USA, or the UK [1]. Recently, the influenza surveillance system in Japan has been improved considerably. For instance, a simple method for the detection of respiratory viruses using cell culture has been developed [2]. As a result, influenza viruses are being isolated in Japan throughout the year. For at least the last four influenza seasons (1985–9) in Japan, the influenza virus type or subtype isolated during spring to summer became the dominant epidemic type or subtype in the following influenza season. This phenomenon, which has been observed elsewhere, has been

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called the 'herald wave', and was expected to forecast the virus most likely to be prevalent during the next influenza season [3, 4].

Oligonucleotide fingerprinting methods have been used to determine whether the viruses isolated during the spring season were genetically related to the viruses isolated later in the epidemic season, but the results were inconclusive [5]. On the other hand, nucleotide sequence analysis showed that the haemagglutinin (HA) gene in human influenza A(H1N1) and A(H3N2) viruses evolves essentially in one direction [6–9]. Thus, the analysis of the RNA sequences of the HA genes seemed to give a clearcut answer to the question whether the viruses isolated in off-seasons are genetically related to the following epidemic viruses.

We have analysed the HA gene sequences of the influenza A(H1N1) and A(H3N2) viruses isolated in Japan and Southeast Asia during the period of 1985–9, and the results obtained support the herald virus hypothesis in three instances.

MATERIALS AND METHODS

Viruses and purification of viral RNA

The strains and passage history of the influenza A(H1N1) and A(H3N2) viruses used in the present study are shown in Table 1. The viruses were isolated by us or obtained from the National Institute of Health, Japan, or municipal and prefectural public health laboratories. The viruses were grown in MDCK cells at 37 °C and purified by centrifugation through 30–60% discontinuous sucrose gradient centrifugation in a Beckman SW28 rotor. Viral RNA was extracted by the procedure described by Palese and Schulman [10].

Nucleotide sequencing of the HA genes

Nucleotide sequences of the HA gene were determined from virion RNA using the dideoxy chain termination method [11] as described previously [8].

Oligonucleotide primers

Eleven and seven synthetic oligonucleotides were used as the primers for H1 and H3 genes, respectively. For the H1 gene, the oligonucleotides corresponding to the nucleotide positions, 5–15, 161–171, 310–321, 527–540, 598–610, 802–814, 1025–1039, 1173–1187, 1338–1352, 1446–1458, and 1584–1596, numbered according to the positive strand sequence of the HA gene of A/PR/8/34 [12] were employed. The first four primers were the same as those used by Stevens and colleagues [13]. For the HA1 region of the H3 gene, seven primers which were employed in the previous study were used [8].

RESULTS

Influenza surveillance in Japan during 1985–9

Figure 1 shows the chronological sequence of isolation of influenza viruses by municipal and prefectural public health laboratories throughout Japan during 1985–9 [14]. An antigenic variant of influenza A(H1N1) virus, A/Yamagata/120/86, was isolated in April, 1986. H1N1 viruses antigenically similar to this strain were the major epidemic viruses in the 1986–7 and 1988–9 influenza seasons. On the other hand, H3N2 viruses were the major epidemic viruses in the 1985–6

Table 1. *Influenza virus strains used in the present study*

Strain name	Date of specimen collection	Abbreviation*	Passage history†
H1N1 viruses			
A/Yamagata/120/86	12 Apr.	YG120/86	E8CK2
A/Tokyo/770/86	22 Apr.	TK770/86	E4CK4
A/Nagano/238/86	24 Apr.	NN238/86	CK5
A/Nagano/38/86	15 Dec.	NN38/86	CK4
A/Nagano/174/87	16 Mar.	NN174/87	CK3
A/Nagano/1396/88	2 May	NN1396/88	CK3
A/Hiroshima/C21/88	21 Dec.	HS/88	E2CK2
A/Nagano/1605/88	22 Dec.	NN1605/88	CK4
A/Nagano/1669/89	13 Jan.	NN1669/89	CK4
H3N2 viruses			
A/Sichuan/2/87	Apr.	Sichuan/87	E10CK2
A/Nagano/1046/87	17 May	NN1046/87	LLC2CK3
A/Kamata/421/87	24 Nov.	KT421/87	MK1CK4
A/Tokyo/1276/87	1 Dec.	TK1276/87	E3CK4
A/Chiba/38/88	27 Jan.	CB/88	CK4
A/Nagano/1185/88	18 Feb.	NN1185/88	CK5
A/Nagano/184/88	26 Feb.	NN184/88	CK5
A/Kobe/768/88	27 Feb.	KB/88	CK5
A/Kamata/612/88	19 July	KT612/88	MK2CK4
A/Kanagawa/107/88	25 Aug.	KG/88	CK7
A/Chiang Mai/156/88	29 Aug.	CM/88	CK4
A/Kamata/13/89	9 Jan.	KT13/89	MK1CK3
A/Aichi/1/89	28 Jan.	AI/89	CK4
A/Nagano/1749/89	29 Mar.	NN1749/89	CK4
A/Kamata/248/89	12 May	KT248/89	CK4

* Month of specimen collection is shown in text after the year by subscript number. (e.g. $YG120/86_4$).

† E = egg; CK = MDCK cells; LLC = LLCMK2 cells; MK = primary monkey kidney cells. The passage numbers are indicated.

and 1987–8 influenza seasons. Typical herald waves were observed for H3N2 viruses in the spring of 1985 and for H1N1 viruses in the spring of 1986, while the viruses belonging to the same subtypes which caused the major epidemics in the following influenza seasons were isolated sporadically during spring to summer for H1N1 viruses in 1988 and H3N2 viruses in 1987 and 1988.

Nucleotide sequence changes of the HA genes of H3N2 strains isolated between 1985–9 from that of BK1/79 sequence

Figure 2 shows the nucleotide changes in the HA gene of 15 human influenza A(H3N2) strains isolated between 1987–9 in Japan from that of the A/Bangkok/1/79(H3N2)(BK1/79) strain [6], along with the amino-acid changes. Only the nucleotide sequences of the HA1 region were determined. The first 29 bases could not be determined, because the synthetic primers described in Materials and Methods were used for the sequence determination. Nucleotide changes occurred at least at 77 positions in the 15 HA genes when compared with the HA gene of BK1/79. These changes were classified into mainstream changes inherited by most subsequent strains, strain-specific changes occurring in only one or at most a few

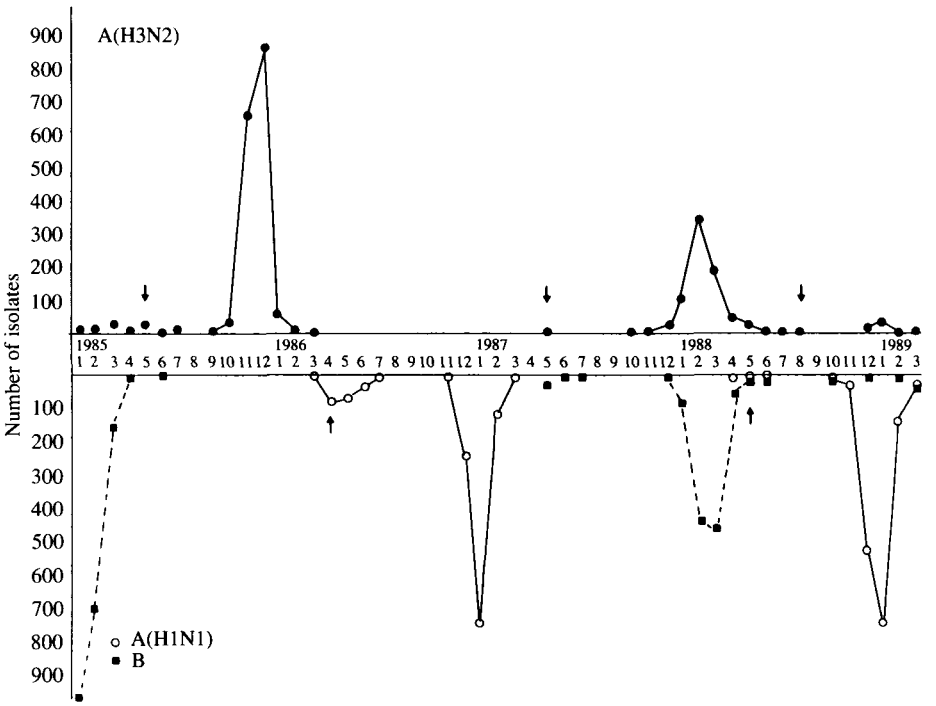

Fig. 1. Chronological sequence of isolation of influenza viruses in Japan from January 1985 to March 1989. Virus isolation was done by municipal and prefectural public health laboratories throughout the country and the number of isolated viruses was reported to the National Institute of Health in Japan [14]. Some of the off-season viruses used in the present study are shown by arrows.

strains, and changes of undetermined status until the analysis of the viruses of next season is done. There were 37 mainstream, 34 strain-specific, 1 BK1/79 specific (base no. 128) and 5 undetermined changes. These nucleotide changes resulted in the changes in 28 amino acids. The nucleotide changes were scattered all over the HA1 gene, whereas most amino-acid changes were clustered between residues 124 and 213 in the HA1 polypeptide. The locations of the changed amino acids were plotted on a three-dimensional model of the HA molecule [15] (Fig. 3*a*). An evolutionary tree which shows how the HA genes of the 1985–9 H3N2 strains are derived from that of BK1/79 based on nucleotide changes is shown in Fig. 4. This was constructed by giving priority to mainstream over strain-specific changes and minimizing the chance that a strain-specific change shared by two viruses occurred independently. Five virus strains isolated in 1985 and studied by us previously [8] were included in this figure. There were 15 mainstream changes between the HA genes of A/Yamagata/96/85 ($YG96/85_5$) and $AI/89_1$. Five strains isolated in 1985 were located on two lineages separated by three mainstream changes. $NN1046/87_5$ was the first H3N2 isolate after the 1985–6 influenza season. Five strains ($NN1046/87_5$, $KT421/87_{11}$, $NN1185/88_2$, $KT612/88_7$, $CM/88_8$) were located close to each other on three branches derived from the same point in the stem, three mainstream changes away from $YN/85_{11}$. Four strains (Sichuan/87_4, $TK1276/87_{12}$, $CB/88_1$, $KG/88_8$) were also closely located on different branches derived from the same point in the stem one mainstream change

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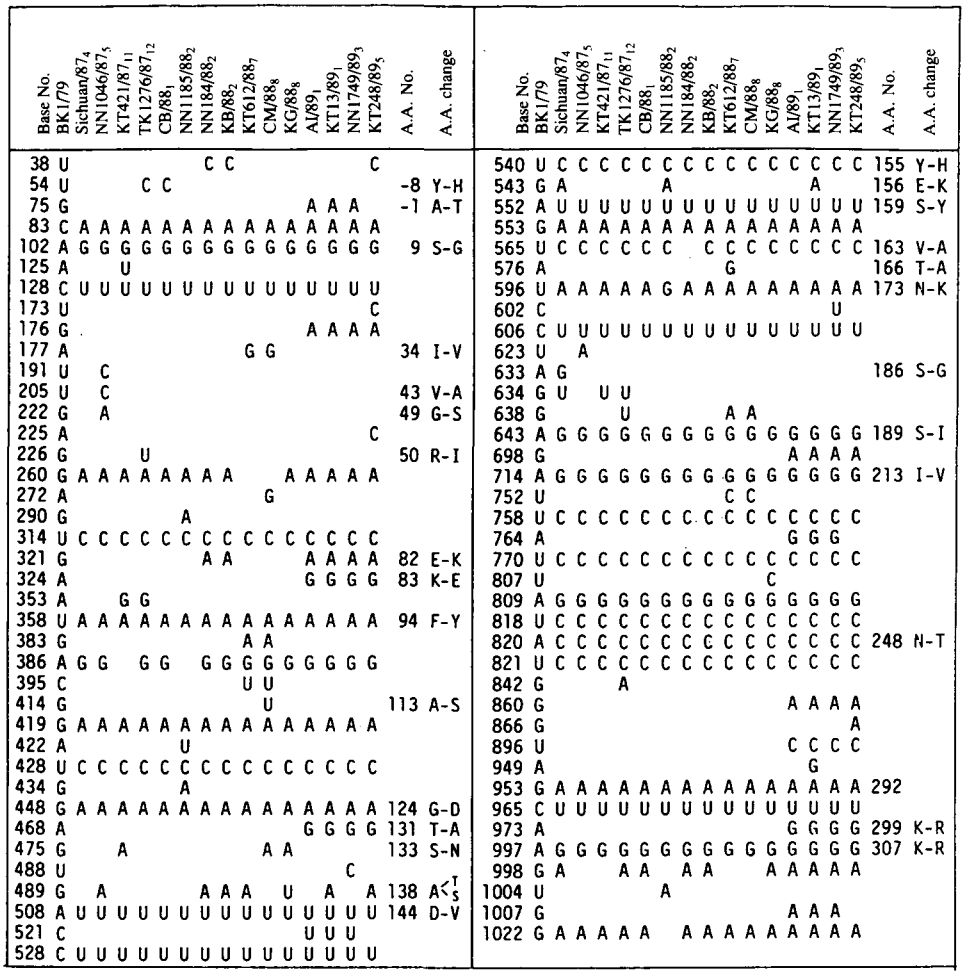

Fig. 2. Nucleotide changes in the HA1 region of the HA genes of 15 H3N2 strains between 1987–9. The nucleotide bases are numbered according to the positive strand sequence of the A/Aichi/2/63(H3N2) HA gene [27]. Only positions which drifted from the HA gene of BK1/79 [6] are shown. All mainstream nucleotide changes after BK1/79 are included. The position and the direction of the amino-acid change from BK1/79 are also shown in the last two columns. One letter codes for amino acids are used.

away from NN1046/87$_5$, while two strains (KB/88$_2$, NN184/88$_2$) were located on the same branch derived from the stem, one mainstream change away from Sichuan/87$_4$. Three strains isolated during 1988–9 influenza season (AI/89$_1$, KT13/89$_1$, NN1749/89$_3$) were located on the same branch seven mainstream changes further away from the former strains.

Nucleotide sequence changes of the HA genes of H1N1 strains isolated between 1986–9 from that of USSR/77 sequence

Figure 5 shows the nucleotide changes in the HA genes of nine human influenza A(H1N1) strains isolated between 1986–9 in Japan from that of USSR/77 [16] along with the amino-acid changes. The amino-acid changes in the HA1

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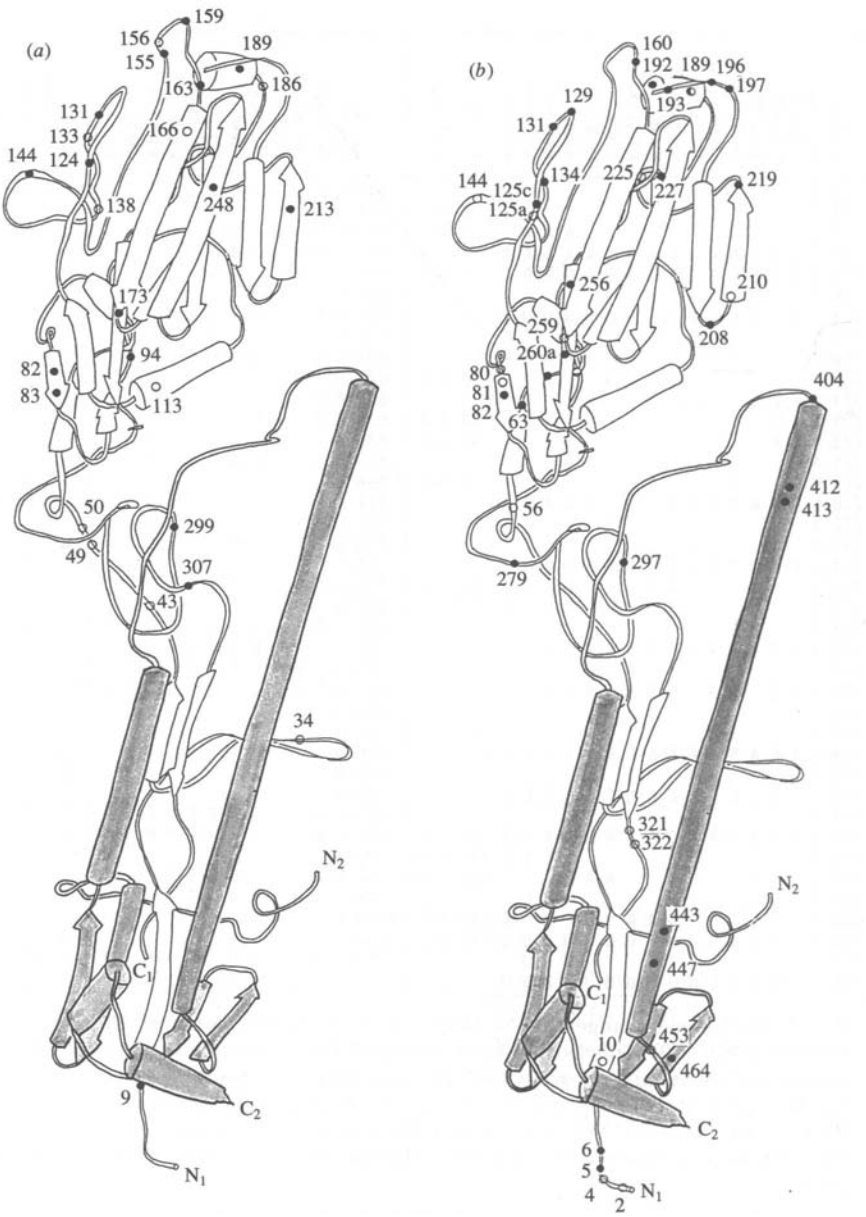

Fig. 3. Schematic drawing of the Hong Kong (H3) HA monomer (Wilson, Skehel & Wiley [15]) showing locations of the amino-acid substitutions on the HA molecule. HA2 region is shadowed. (*a*) Amino-acid changes from BK1/79 to KT248/89$_5$ (H3N2). All mainstream amino-acid changes after BK1/79 and strain-specific amino-acid changes from Sichuan/87$_4$ to KT248/89$_5$ in Fig. 2 are plotted. (*b*) Amino-acid changes from USSR/77 to NN1669/89$_1$. All mainstream amino-acid changes after USSR/77 and strain-specific amino-acid changes from YG120/86$_4$ to NN1669/89$_1$ in Fig. 5 are plotted. ● Mainstream amino-acid changes; ○ strain-specific amino-acid changes.

polypeptides of A/Taiwan/1/86(TW/86) and A/Singapore/17/86 (SP/86) [9, 13, 17] are also shown. The first 26 bases were not determined. Nucleotide changes occurred at least at 96 positions in the 9 HA genes when compared with the HA gene sequence of USSR/77. There were 56 mainstream, 35 strain-specific,

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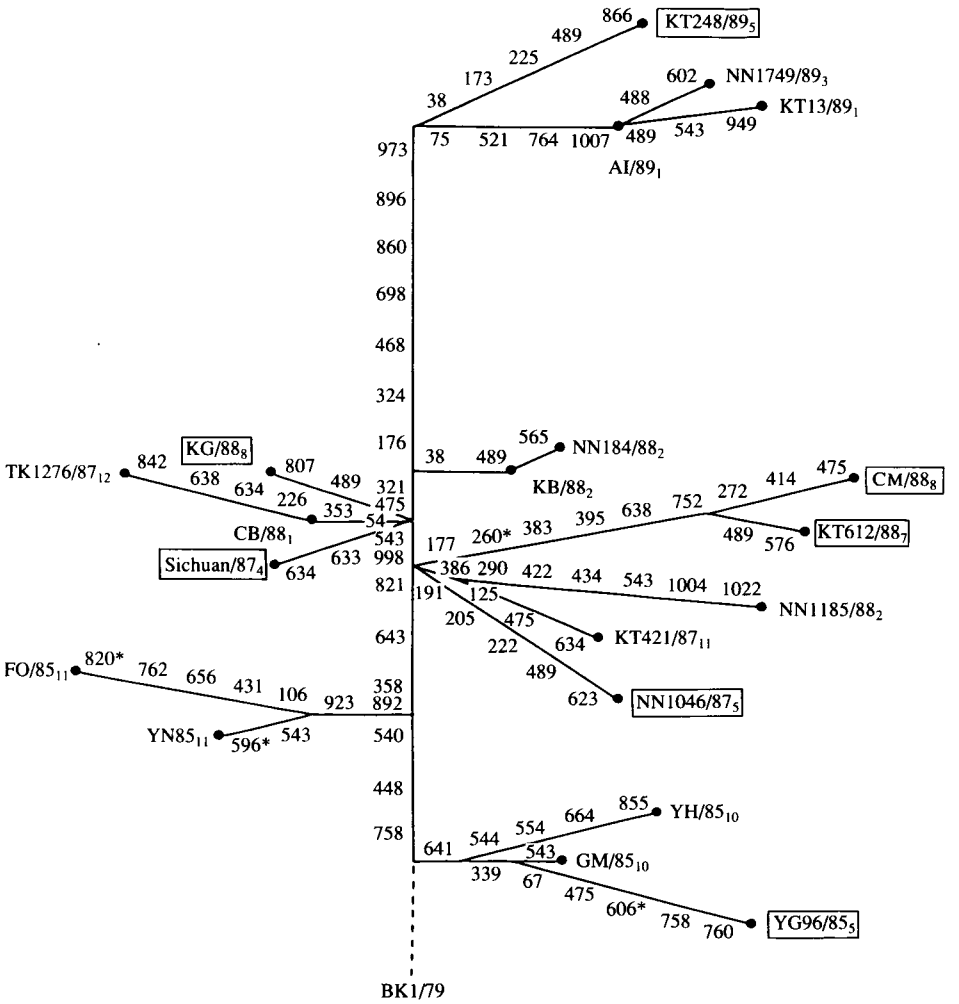

Fig. 4. The evolutionary tree for the HA genes of the 1987–9 H3N2 strains from BK1/79 based on the nucleotide changes. Only the tree after YG96/85$_5$ is shown. Numbers refer to the mainstream nucleotide changes which become fixed in most of the subsequent strains (vertical line); or to strain-specific nucleotide changes on the side-branches. Off-season viruses are boxed. * Denotes the same nucleotide as that in BK1/79.

and 5 undetermined changes. Thirty-eight mainstream changes occurred in the HA1 and 18 in the HA2 regions. These nucleotide changes resulted in the amino-acid changes at 43 residues, 36 in the HA1 and 7 in the HA2 polypeptides. The location of the changed amino acids were plotted on the three-dimensional model of the HA molecule (Fig. 3*b*). As in the H3N2 viruses, the nucleotide changes were scattered all over the HA gene, whereas most amino-acid changes were clustered between residues 125a and 227 in the HA1 polypeptide; amino acids which were present in H1 but not in H3 subtype HAs were numbered using alphabetic suffixes. An evolutionary tree for which shows how the HA genes of the 1986–9 H1N1 were derived strains from that of USSR/77, based on nucleotide changes, is shown in Fig. 6. There were 54 mainstream changes between the HA genes of

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Base No.	USSR/77	YG120/86$_4$	NN238/86$_4$	TK770/86$_4$	NN38/86$_{12}$	NN174/87$_3$	NN1396/88$_5$	NN1605/88$_{12}$	HS/88$_{12}$	NN1669/89$_1$	A.A. No.	A.A. change	TW/86	SP/86
50	G	A	A	A	A	A	A	A	A	A				
53	C						U							
57	U				A						2	L-I		
64	C						U				4	A-V		
66	C	U	U	U	U	U	U	U	U	U	5	L-F	F	F
69	G	A	A	A	A	A	A	A	A	A	6	A-T	T	T
81	G				A						10	A-T		
143	C	U	U	U	U	U	U	U	U	U				
146	A						G	G	G					
203	A	G												
209	C	U	U	U	U	U	U	U	U	U				
210	A	C	C	C	C	C	C	C	C	C				
224	A									G	56	I-M		
245	A	U	U	U	U	U	U	U	U	U	63	K-N	N	N
250	A	G	G	G	G	G	G	G	G	G	65	N-S	S	S
252	A					G					66	I-V		
266	C				U	U	U							
297	U								A		80	S-T		
302	G									U	81	K-N		
303	A						G	G	G	G	82	K-E		
362	A	G	G	G	G	G	G	G	G	G				
371	C					U								
384	U	C	C	C	C	C	C	C	C	C				
401	C	U	U	U	U	U	U	U	U	U				
437	C				A									
439	A						G				125a	K-E		
440	G	A	A	A	A	A	A	A	A	A				
446	A	C	C	C	C	C	C	C	C	C	125c	R-S	S	S
458	A	C	C	C	C	C	C	C	C	C	129	K-N	N	N
463	A	C	C	C	C	C	C	C	C	C	131	N-T	T	T
472	G	A	A	A	A	A	A	A	A	A	134	R-K	K	K
476	C	A	A	A	A	A	A	A	A	A				
500	G							U	U	U				
505	A	G	G	G							144	K-R	K	R
545	A	G	G	G	G	G	G	G	G	G				
553	C						U	U	U	U	160	S-L		
620	U					C								
639	G								A		189	G-R		
640	A	G	G	G	G	G	G	G	G	G	189	E-G	G	G
649	A	G	G	G	G	G	G	G	G	G	192	K-R	R	R
651	A	G	G	G	G	G	G	G	G	G	193	T-A	A	A
661	G	A	A	A	A	A	A	A	A	A	196	R-Q		
662	G	U	U	U	U	U	U	U	U	U	196	H	H	H
664	A	C	C	C	C	C	C	C	C	C	197	K-T	T	T
696	A	C	C	C	C	C	C	C	C	C	208	N-H	H	H
703	A							G	G	G	210	N-S		
729	G	A	A	A	A	A	A	A	A	A	219	E-K	K	K
748	G		A	A	A	A	A	A		A	225	G-D		
754	C	A	A	A	A	A	A	A	A	A	227	A-E		
758	G	A	A	A	A	A	A	A	A	A				
761	A	G												
780	C						U							
840	C	U	U	U	U	U	U	U	U	U	256	H-Y	Y	Y
849	G						U				259	A-S		
856	A	G	G	G	G	G	G	G	G	G	260a	N-S	S	S
890	C	U					U							
896	G	A	A	A	A	A	A	A	A	A				
912	A	G	G	G	G	G	G	G	G	G	279	T-A	A	A
966	A	G	G	G	G	G	G	G	G	G	297	I-V	V	V
986	G	A	A	A	A	A	A	A	A	A				
992	C							U	U	U				
998	A	G	G	G	G	G	G	G	G	G				
1001	C	U	U	U	U	U	U	U	U	U				
1022	G		A											
1039	G	A									321	R-K		
1041	A	G									322	N-D		
1067	U								C					
1068	C	U	U	U	U	U	U	U	U	U				
1103	A	G	G	G	G	G	G	G	G	G				
1139	U						C							
1154	G			A	A	A		A						
1172	G			A										
1205	G							A	A	A				
1223	C	U	U	U	U	U	U	U	U	U				
1229	U	A	A	A	A	A	A	A	A	A				
1259	U								C					
1265	U	C	C	C	C	C	C	C	C	C				
1288	A	G	G	G	G	G	G	G	G	G	404	K-R		
1312	A	G	G	G	G	G	G	G	G	G	412	K-R		
1314	G	U	U	U	U	U	U	U	U	U	413	V-F		
1322	U						C							
1405	G	A	A	A	A	A	A	A	A	A	443	S-N		
1416	G	C	C	C	C	C	C	C	C	C	447	A-L		
1435	G		A								453	S-N		
1448	U	C	C	C	C	C	C	C	C	C				
1466	A			G										
1468	A	C	C		C	C	C	C	C	C	464	T-N		
1472	A	G	G		G	G	G	G	G	G				
1496	U						C							
1520	A	G	G	G	G	G	G		G	G	G			
1553	A	C	C	C	C	C	C	C	C	C				
1565	G	A												
1622	U	C	C	C	C	C	C	C	C	C				
1628	U	G	G	G	G	G	G	G	G	G				
1685	C	U	U	U										
1718	C	U	U	U	U	U	U	U	U	U				

Fig. 5. Nucleotide changes in the HA genes of 9 H1N1 strains between 1986–9 compared to that of USSR/77. The nucleotide bases are numbered according to the positive strand sequence of the A/PR/8/34(H1N1) HA gene [12]. Only positions which drifted from the HA gene of USSR/77 [15] are shown. All mainstream changes after USSR/77 are included. The position and the direction of the amino-acid change from

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USSR/77 and YG120/86_4, while only two mainstream changes occurred between YG120/86_4 and NN1396/88_5. We did not regard the change at position 748, which resulted in the amino-acid change at residue 225 from glycine to asparatic acid, as a mainstream change because it was shown to be a change due to adaptation to growth in the chick embryo [17]. Spring isolates (YG120/86_4, NN238/86_4, TK770/86_4) in 1986 and winter isolates (NN38/86_{12}, NN174/87_3) from the 1986–7 influenza season were located on two different branches derived from the same point in the stem. NN1396/88_5 which was the first H1N1 isolate after the end of the 1986–7 influenza season in Japan acquired two more mainstream changes away from YG120/86_4, and three strains isolated during the 1988–9 influenza season (NN1605/88_{12}, HS/88_{12}, NN1669/89_1) were located on the same branch which was different from that of NN1396/88_5.

DISCUSSION

From January 1985 to March 1989, off-season viruses of H1N1 and H3N2 subtypes of influenza A viruses were isolated on five occasions in Japan. Typical herald waves were observed in two of them, one in 1985 for H3N2 viruses, and the other in 1986 for H1N1 viruses. The number of off-season isolates was small (1–6 in sporadic cases and about 30–180 in typical herald waves) compared to that of the corresponding winter isolates (about 600–1600) in Japan [14]. The virus strains used in the present study were mainly chosen by the following criteria: (1) viruses isolated in Nagano or Kamata area in Tokyo, (2) vaccine strains used in Japan (FO/85_{11}, Sichuan/87_4; H3N2, YG120/86_4; H1N1), (3) viruses isolated from different part of Japan, and (4) antigenically drifted strains (GM/85_{10}, YN/85_{11}, FO/85_{11}). We compared the nucleotide sequences of the HA genes of type A influenza viruses isolated during off-seasons and later epidemic seasons and constructed evolutionary trees of the HA gene of the influenza A(H1N1) and A(H3N2) viruses to determine the genetic relationship between off-season viruses and later epidemic viruses. When we compared the viruses isolated during a short period, the rate of the nucleotide substitutions was different in each virus and it was difficult to estimate genetic relationship among them by the number of changed bases. Consequently we estimated the genetic relationship between the HA genes of the two viruses by mainstream changes.

It has been pointed out that growth in embryonated eggs selects genetically different viruses from clinical samples than does growth in mammalian cells [17–19]. Typical substitutions due to egg adaptations are glutamic acid (E) to lysine (K) at amino-acid residue 189, aspartic acid (D) to asparagine (N) at residue 190, and aspartic acid to glycine (G) or asparagine at residue 225 in influenza A(H1N1) viruses [17], or asparagine to lysine at residue 145, glutamic acid to lysine at residue 156, asparagine to lysine at residue 193, and arginine (R) to

USSR/77 are shown together with the changed amino acids in TW/86 or SP/86 which was taken from Cox, Black & Kendal [9]. The amino acid at residue 315 (R) was taken from Robertson [17] and Stevens and colleagues [13] and is the same as that of USSR/77. Therefore, it is not seen in the figure. The amino acids are numbered corresponding to the numbering for the H3 subtype according to the alignment of Winter, Fields & Brownlee [12]. The amino acids which were absent in the H3 subtype were numbered using alphabetic suffixes. One letter codes for amino acids are used.

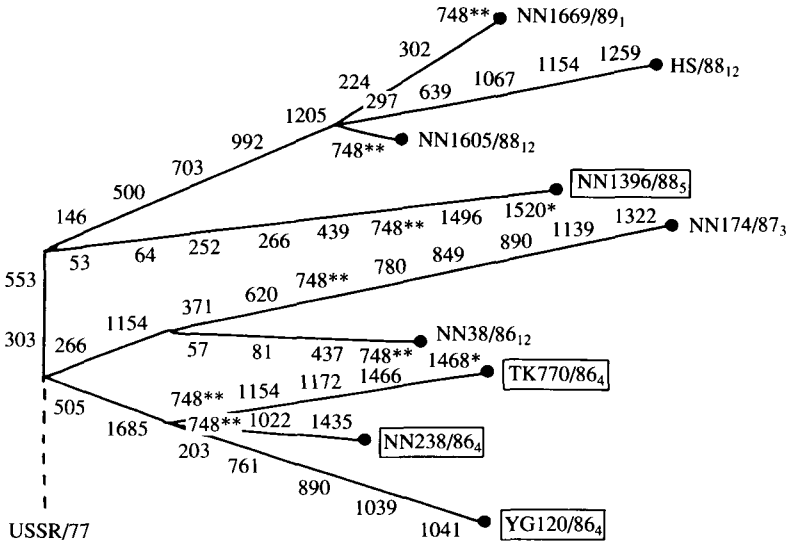

Fig. 6. The evolutionary tree for the HA genes of the 1986–9 H1N1 strains from USSR/77 based on the nucleotide changes. Only the tree after YG120/86$_4$ is shown. Numbers refer to the mainstream nucleotide changes on the vertical line; or to strain-specific nucleotide changes on the side-branches. Off-season viruses are boxed. *denotes the same nucleotides as those in USSR/77. **Denotes the nucleotide correlated with egg adaptation-dependent change.

glycine at residue 229 in influenza A(H3N2) viruses [18, 19]. In the present study, the nucleotide change at position 543 from G to A which resulted in the amino-acid change at residue 156 from glutamic acid to lysine in H3N2 viruses were observed in a few strains, while the nucleotide change at position 748 from G to A which resulted in the amino-acid change at residue 225 from glycine to aspartic acid in H1N1 viruses were observed in most of the strains. Because the nucleotide sequence of USSR/77 strain was obtained from egg-grown viruses [6], the nucleotide A at position 748 was thought to reflect the nucleotide in mammalian cell-grown viruses.

When H3N2 strains are considered, the three potential herald isolates can be compared with isolates made during the subsequent influenza season.

Comparison of a spring isolate from a herald wave (YG96/85$_5$) with the winter isolates (YH/85$_{10}$, GM/85$_{10}$, YN85$_{11}$ & FO/85$_{11}$). Here a herald virus constituted the same lineage as YH/85$_{10}$ and GM/85$_{10}$, and these viruses were genetically close. On the other hand, YN/85$_{11}$ and FO/85$_{11}$ constituted one lineage and branched off the same point in the stem three mainstream changes away from YG96/85$_5$; these two strains were thought to have a different origin. The epidemic H3N2 influenza viruses in the world in 1984–5 season were A/Philippines/2/82-like or A/Caen/1/84-like viruses [20]. The antigenicity of YG96/85$_5$ was close to A/Philippines/2/82-like but rather closer to A/Mississipi/1/85-like viruses [8]. The antigenicity of GM/85$_{10}$, YN/85$_{11}$, and FO/85$_{11}$ was clearly distinguished from that of YG96/85$_5$ and YH/85$_{10}$ [8].

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Comparison of a spring sporadic isolate (NN1046/87$_5$) with the winter epidemic viruses (KT421/87$_{11}$, TK1276/87$_{12}$, CB/88$_1$, NN1185/88$_2$, NN184/88$_2$, KB/88$_2$). NN1046/87$_5$ was the first H3N2 strain detected in Japan after 1985–6 influenza season and acquired three mainstream changes compared to YN/85$_{11}$. The winter isolates were divided into three groups. Group 1 includes KT421/87$_{11}$ and NN1185/88$_2$. These shared one strain-specific change at position 386 and branched off the same point in the stem as NN1046/87$_5$. Therefore, the herald virus NN1046/87$_5$ was genetically close to KT421/87$_{11}$ and NN1185/88$_2$. On the other hand, TK1276/87$_{12}$ and CB/88$_1$ (group 2) were located on a branch one mainstream change away from NN1046/87$_5$ and shared two strain-specific changes at positions 54 and 353. A Chinese strain Sichuan/87$_4$ which was isolated in the spring was genetically close to group 2 viruses. NN184/88$_2$ and KB/88$_2$ (group 3) branched off the stem point one mainstream change further away from Sichuan/87$_4$, and shared two strain-specific changes at positions 38 and 489. Antigenically, all these viruses were close to Sichuan/87$_4$ which had the same antigenicity as YN/85$_{11}$ (data not shown).

Comparison of the summer sporadic isolates (KT612/88$_7$, KG/88$_8$) with the winter isolates (KT13/89$_1$, AI/89$_1$, NN1749/89$_3$). KT612/88$_7$ and KG/88$_8$ were located on different branches, each of them branched off the same point in the stem as group 1 or group 2 viruses of the former influenza season, respectively. CM/88$_8$ which was isolated in Thailand during the summer was located on the same branch as KT612/88$_7$. The three winter isolates were located on the same branch seven mainstream changes away from KB/88$_2$. This showed that the winter isolates had a different origin than the summer isolates and the summer isolates were genetically close to the viruses of the former influenza season. KT248/89$_5$ which was isolated in May in 1989 was analysed in order to determine the mainstream changes which were shared by winter isolates in the 1988–9 influenza season.

Few H1N1 influenza isolates were made in the world in the 1984–5 and 1985–6 seasons and the epidemic H1N1 viruses circulating in 1983–4 were A/Chile/1/83-like viruses [20–22]. The whole HA gene of the H1N1 viruses was analysed, because the antigenicity of the H1N1 viruses isolated after YG120/86$_4$, which cannot be distinguished from that of TW/86 or SP/86 [13] was similar and the number of nucleotide changes was small. Two candidate herald H1N1 strains can be compared with H1N1 strains isolated subsequently.

Comparison of the spring isolates from a herald wave in 1986 (YG120/86$_4$, TK770/86$_4$, NN238/86$_4$) and the winter isolates in 1986–7 (NN38/86$_{12}$, NN174/87$_3$). Three herald viruses shared two nucleotide changes at positions 505 and 1685, while two winter isolates also shared two nucleotide changes at positions 266 and 1154 (Fig. 6). The nucleotide change at position 1154 was found in one of the herald viruses (TK770/86$_4$). The herald viruses and the later epidemic viruses were branched off the same point in the stem and therefore these viruses were thought to be genetically close.

Comparison of a spring sporadic isolate in 1988 (NN1396/88$_5$) and the winter epidemic viruses (HS/88$_{12}$, NN1605/88$_{12}$, NN1669/89$_1$). No H1N1 viruses were

isolated in Japan in the 1987–8 influenza season, but a few were isolated in the spring of 1988. $NN1396/88_5$ newly acquired two mainstream changes at positions 303 and 553 from the isolates in the 1986–7 influenza season. The nucleotide changes at these positions were also observed by the analysis of the H1N1 strains from other countries (N. Cox, personal communication). The epidemic viruses in the 1988–9 influenza season shared five nucleotide changes at positions 146, 500, 703, 992, and 1205, however, none of these changes were shared by $NN1396/88_5$ which had eight strain-specific changes. Although the conclusion will not be drawn until the analysis of the H1N1 viruses of the next influenza season, 1988–9 epidemic viruses were unlikely to be direct descendents of the virus circulating in the preceding spring.

The seasonal epidemicity of the influenza raises the question of the persistence of the influenza viruses during the interpandemic seasons [23–25]. Usually there are one or two year intervals between the epidemic of each subtype virus in Japan. Previously, we reported that at least four types of H1N1 viruses, distinguished by oligonucleotide fingerprinting patterns, were involved in influenza outbreaks during the 1978–9 influenza season in Japan, and suggested that most epidemic strains of H1N1 viruses were introduced from outside the country [26]. At that time we did not get the spring or summer isolates, and therefore, could not exclude the possibility that the viruses introduced into Japan in the spring or summer caused epidemics in the following influenza seasons. In the present study, we studied this possibility on five occasions. In one instance with H1N1 viruses in 1986 and in two instances with H3N2 viruses in 1985 and 1987, the spring isolates were genetically close to some of the winter isolates and so could possibly become the parental viruses in the following influenza seasons. However, even so, influenza viruses of the same subtype but with different origins and which were not observed in off-seasons co-circulated in Japan. Our results demonstrate the difficulty of accurately predicting future influenza activity based on past events in one country, and emphasize the necessity of analysing influenza viruses worldwide along with the domestic isolates.

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