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Evolutionary characteristics of influenza B virus since its first isolation in 1940: dynamic circulation of deletion and insertion mechanism*

R. Nerome[1], **Y. Hiromoto**[1,2], **S. Sugita**[3], **N. Tanabe**[1], **M. Ishida**[1], **M. Matsumoto**[1], **S. E. Lindstrom**[1], **T. Takahashi**[2], and **K. Nerome**[1]

[1]Department of Virology I, National Institute of Infectious Diseases, Tokyo, Japan
[2]Hoshi University, Tokyo, Japan
[3]Epizootic Research Station, Equine Research Institute, Japan Racing Association, Tochigi, Japan

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Summary. New antigenic variants of B/Yamagata/16/88-like lineage which appeared in the season of 1997 as a minor strain tended to predominate in the following season. Also, we could observe for the first time, three peaks of activity caused by H3N2 virus and two variants of B influenza virus. Antigenic and phylogenetic analyses revealed that B/Victoria/2/87-like variants appeared again in Japan in 1997 after a nine-year absence. Influenza B viruses evolved into three major lineages, including the earliest strain (I), B/Yamagata/16/88-like variants (II), which comprised of three sublineages (II-(i), II-(ii), II-(iii)), and B/Victoria/2/87-like variants (III). Evolution of influenza B virus hemagglutinin was apparently distinguishable from that of influenza A virus, showing a systematic mechanism of nucleotide deletion and insertion. This phenomenon was observed to be closely related to evolutionary pathways of I, II-(i), II-(ii), II-(iii) and III lineages. It was noteworthy to reveal that the nucleotide deletion and insertion mechanism of influenza B virus completed one cycle over a fifty-year period, and that a three nucleotide deletion was again observed in 1997 strains

*The nucleotide sequence data of the HA genes of B/Mie/1/93, B/Osaka/c19/93, B/Kobe/1/94, B/Tokushima/101/93, B/Kagoshima/15/94, B/Hebei/3/94, B/Hebei/19/94, B/Beijing/184/93, B/Guandong/08/93, B/Guandong/05/94, B/Harbin/07/94, B/Alaska/12/96, B/Tokyo/942/96, B/Beijing/243/97 and B/Osaka/491/97 influenza viruses have been assigned the accession numbers D38643, D38644, D38645, D38646, D38647, D38648, D38649, AF050061, AF050064, AF050063, AF050065, AF050060, AF050067, AF050062 and AF050066.

belonging to lineage II-(iii). It was evident that amino acid substitutions accompanying nucleotide insertions were highly conserved.

Introduction

Unlike influenza A viruses which have been subdivided into 15 hemagglutinin and 9 neuraminidase subtypes [12, 17], influenza B viruses have comprised of a single group of hemagglutinin and neuraminidase antigens since their first isolation in 1940. Regarding this difference, it has been shown that numerous influenza A viruses circulate in wild and domestic animals such as a wide variety of bird species, swine, equine, seals, whales and mink. Indeed, since the early 1970s, there has been extensive virological surveillance for influenza viruses in birds and mammals, and these studies revealed a further addition to the number of hemagglutinin subtypes [5, 17, 21]. Subsequently, influenza A viruses, in addition to swine and horses, were also reported to be prevalent in seals, whales and mink [4, 6, 9]. This is in sharp contrast to influenza B viruses which have not been isolated from animals other than humans. These differences in epizootic background may lead to evidence that no antigenic shift has been observed in influenza B viruses.

Indeed, genetic reassortment between antigenic variants of influenza B viruses have occurred in the laboratory with high frequency [16, 22]. Despite the lack of antigenic shift, a number of antigenic variants of B viruses have been isolated during periods of widespread influenza virus activity since its first isolation in 1940 [2, 20]. Particularly, it is noteworthy that a higher number of monoclonal variants of influenza B viruses have been detected in the same epidemic season than A viruses [11]. For example, in this study twelve antigenic variants distinguished by a panel of monoclonal antibodies appeared to ciruclate in the 1981–1982 epidemic season in Japan. Thus, evolution of influenza B virus is definitely characterized by coexistence of several antigenic variants in the same epidemic period which is different from that of influenza A virus in which a single dominant virus predominates in a single epidemic period [1,15].

In agreement with the above reports, Yamashita et al. [28] first demonstrated that influenza B viruses have evolved in multiple lineages which can cocirculate for considerable periods of time. The continuing analysis of recent epidemic viruses from an evolutionary point of view showed that there have been two distinct evolutionary lineages of influenza B viruses since 1988, being represented by the two epidemic strains B/Victoria/2/87 and B/Yamagata/16/88, respectively [7, 19]. The evolutionary rate of influenza B viruses was reported to be lower than that of human influenza A viruses, thus, it is still uncertain why the former virus is able to survive for a long period of time without antigenic drift in the hemagglutinin glycoprotein. Recently, in the course of antigenic analysis along with investigation of genomic structure, the authors revealed that systematic deletion and insertion mutations have occurred in the same nucleotide regions of hemagglutinin molecule of influenza B viruses. This mechanism will be analyzed in relation to the differentiation of evolutionary lineages. The present study will

describe epidemic and evolutionary patterns unique to influenza B viruses isolated in Japan since 1988.

Materials and methods

Viruses

In antigenic and sequencing analyses, the following isolates from Japan and China were used: B/Aichi/5/88, B/HongKong/22/89, B/Bangkok/163/90, B/Beijing/184/93, B/Guangdong/05/94, B/Mie/1/93, B/Osaka/c19/93, B/Tokushima/101/93, B/Harbin/07/94, B/Hebei/3/94, B/Hebei/19/94, B/Kagoshima/15/94, B/Kobe/1/94, B/Yamagata/16/88, B/SendaiH/1/96, B/Tokyo/942/96, B/Shiga/56/97, and B/Osaka/491/97. The above strains were compared from antigenic and evolutionary points of view with viruses isolated in many parts of the world. Strain abbreviations are indicated in Table 1. All reference strains and isolates were grown in the allantoic cavity of 11-day-old hen's embryonated eggs or MDCK cells.

Table 1. Antigenic characterization of the hemagglutinin of influenza B viruses variants isolated between 1992 and 1997 season in Japan and China

	HI titer with following ferret anti-sera to								
Virus antigens	1	2	3	4	5	6	7	8	9
Reference strain									
B/Victoria/2/87 (VIC287)-lineage									
1. Aichi/5/88 (AIC588)	80	320	160	–[a]	–	–	–	–	–
2. Osaka/c19/93 (OSA1993)	40	160	160	–	–	–	–	–	–
3. Guangdong/05/94 (GUA594)	10	40	160	–	–	–	–	–	–
B/Yamagata/16/88 (YAM1688)-lineage									
4. Yamagata/16/88 (YAM1688)	–	10	10	1280	80	80	160	160	1280
5. Hong Kong/22/89 (HK2289)	10	40	–	80	80	80	20	10	640
6. Bangkok/163/90 (BK16390)	–	40	–	80	40	160	20	10	640
7. Mie/1/93 (MIE193)	–	20	10	640	20	40	320	160	640
8. Beijing/184/93 (BEI18493)	–	–	–	320	10	20	160	80	640
9. Harbin/07/94 (HAR794)	–	–	10	1280	10	20	80	80	1280
1992–1997 isolates									
Kagoshima/15/94 (KAG1594)	10	160	320	–	–	–	–	–	–
Osaka/491/97 (OSA49197)	–	10	40	–	–	–	–	10	–
Shiga/56/97	–	10	20	–	–	–	–	–	–
Tokushima/101/93 (TOK10193)	–	40	10	160	80	320	160	20	80
Kobe/1/94 (KOB194)	–	20	10	80	40	40	320	40	320
Hebei/3/94 (HEB394)	–	20	10	40	20	20	640	10	40
Hebei/19/94 (HEB1994)	–	–	10	20	10	20	320	80	320
Tokyo/942/96 (TOK94296)	–	–	–	80	20	40	40	40	80

Values represent the reciprocals of initial serum dilution inhibiting haemagglutining activity of test viruses
[a]– HI titler less than 10

Antigenic analysis

Hemagglutinin-inhibition (HI) tests of virus isolates were undertaken in microtitre plates using post-infection ferret sera to reference strains and 0.5% chicken erythrocytes. Prior to use, all antisera were treated with RDE and non-specific hemagglutinin in the sera was removed by addition of packed chicken erythrocytes.

Plaque cloning

Plaque production was undertaken in MDCK cells as described previously [23]. Mouse and human antisera to B/Bangkok/163/89 were used in the plaque cloning test using MDCK cells. B/Bangkok/163/90 virus was subjected to the plaque test in the presence of 4-fold-diluted antisera. The resultant plaques were picked up and purified through plaque formation in MDCK cells.

Nucleotide sequences

Viral RNAs were extracted as described previously [3]. The HA1 coding domain of the hemagglutinin genes were amplified in overlapping cassettes using the reverse transcription-polymerase chain reaction (RT-PCR). The resulting RT-PCR products were directly sequenced using an ABI 373 autosequencer. All nucleotide sequences used in this study are listed in Fig. 3.

Phylogenetic analysis

An evolutionary tree of the HA1 domain of the hemagglutinin gene was constructed by the neighbor-joining (N-J) method based on total nucleotide substitutions [13, 14].

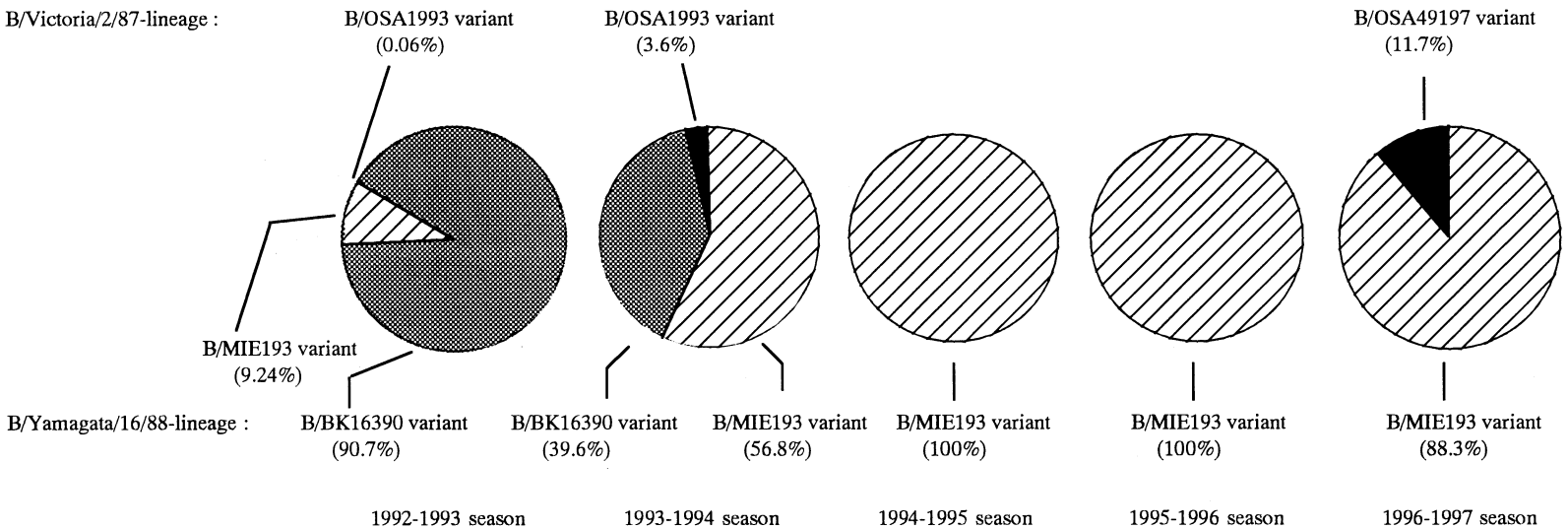
Results and discussion

Antigenic transition of influenza B virus in Japan

In the past five influenza seasons in Japan since 1992, a total of 19, 167 influenza viruses have been isolated (Fig. 1), and antigenic characterization was done at all prefectural and municipal institutes of hygiene as well as the WHO Collaborating Center for Influenza and Respiratory Viruses, National Institute of Infectious Diseases, Tokyo. Using post-infection ferret sera it was defined that H3N2, B and H1N1 viruses were the first major, second major and minor epidemic viruses respectively, based on the numbers of virus isolations, influenza-like illnesses and class closures in schools (data not shown). Also, it was evident from the numbers of influenza-like cases and virus isolation that five large outbreaks of influenza since 1989 were seen in the seasons of 1989–90, 1990–91, 1992–93, 1994–95 and 1996–97 which were always associated with a combination of H3N2 and B viruses. As shown in Fig. 1, MIE193-like strains of the YAM1688 lineage were isolated in Japan in five consecutive influenza seasons from 1992–93 to 1996–97. On the basis of virus isolations, influenza B virus activity tended to peak at a later period than H3N2 virus, as observed in five recent seasons in which influenza activity was predominantly caused by H3N2 virus in the former half of the season while B virus predominated in the latter half.

For example, H3N2 virus predominated the first and second peaks of the 1996–97 epidemic season in Japan. Interestingly, however, a third peak caused

Major Epidemic viruses :	H3N2+B	H3N2+B	H3N2+B	H1N1	H3N2+B
No. of viruses isolates:					
H3N2	2394	1566	3979	295	3222
H1N1	2	0	77	3624	2



No. of B virus isolates :	2449	129	217	8	1203

Fig. 1. Antigenic transition of epidemic strains of influenza B viruses, and major epidemic viruses during a six-year period, from 1992 to 1997 in Japan. Strain abbreviations are as indicated in Table 1

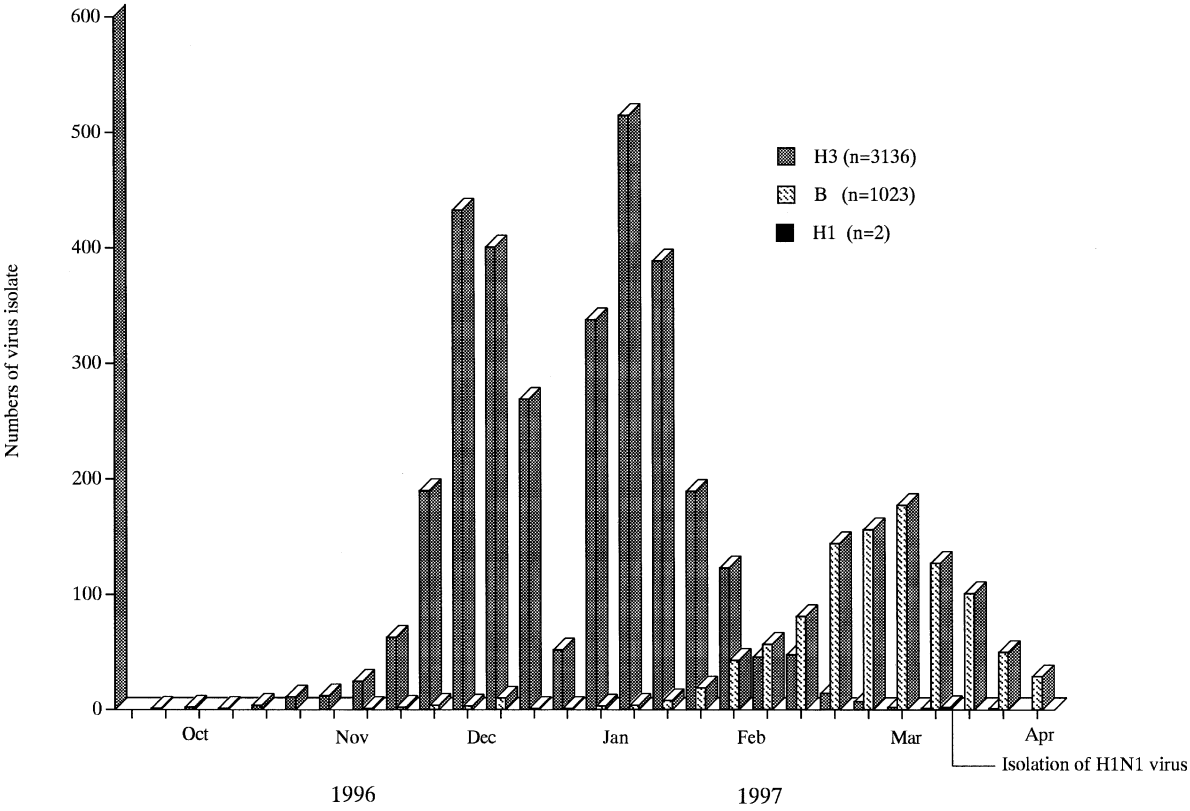


Fig. 2. Epidemic patterns of the 1996–1997 influenza season in Japan based on the numbers of virus isolations

by two antigenic variants of B virus was also observed in this season (Fig. 2). Antigenic analysis of these viruses indicated that they were divided into two groups, including MIE193-like and VIC287-like strains. In this period a total of 1203 influenza viruses were isolated, and of them, 141 (11.7%) were identified as VIC287-like variants which caused a small peak of activity in April of 1997. It was of particular interest to know that VIC287-like variants cocirculated in the outbreak for the first time since 1989. In addition, antigenicity of this variant (OSA49197) was apparently different from OSA1993 virus which was isolated in the 1993 season in Japan (Table 1). It was worth notice that viruses antigenically identical to OSA49197 already circulated in China in 1994. As seen in Fig. 1, a YAM1688-like variant (MIE193) prevailed at two-year intervals by repeating antigenic change, whereas a VIC287-like variant caused an outbreak in the 1997 epidemic season after a 9 year absence. Furthermore, influenza B viruses which were prevalent in the above six epidemic seasons appeared to undergo changes in their hemagglutinin antigens. Although the reasons why large outbreaks are always associated with H3N2 and B viruses are still uncertain, this epidemic pattern may continue until the appearance of a new pandemic strain. Having considered the constant appearance of a number of antigenic variants of influenza

B viruses [19], this virus may continue to play an important role in influenza outbreaks in the future.

Antigenic characteristics of epidemic strains

YAM1688-like viruses first became the major epidemic strain in the 1988–89 influenza season in Japan together with Hong Kong (H3N2) viruses, although HK2289-like variants, which were apparently different antigenically from the former, were also isolated in the same epidemic season. For example, antiserum to YAM1688 reacted to high titer (1280) with homologous virus and lower titer (80) with HK2289 (Table 1). Although HK2289 was nearly identical to BK16390, MIE193 was distinguishable from the former two variants. Furthermore, VIC287-like variants were not detected in Japan during a four-year period, from 1989 to 1992, whereas the 1993–94 influenza season was characterized by the reappearance of three antigenic variants (OSA1993 and KAG1594) belonging to the VIC287-like lineage along with a new variant, MIE193 that was on the YAM1688 lineage. However, TOK94296 and two Chinese isolates (HEB394 and HEB1994) reacted to lower titres (20–80) with anti-YAM1688 serum. As shown in the HI table, MIE193 virus was similar to reference strains HAR794 and BEI18493, suggesting the same antigenic group.

To investigate the background in which VIC287-like and YAM1688-like variants appeared, a number of epidemic B viruses collected in the 1992–93 season were classified on the basis of their HI patterns. As seen in Fig. 1, of a total of 2449 isolates, 2221 (90.7%) were identified as BK16390-like variants in the 1992–93 season, while 226 (9.2%) were MIE193-like variants. In the latter period of this season, one OSA1993-like variant belonging to the VIC287 lineage was isolated, and antigenic analysis clearly revealed its antigenicity to be closer to the VIC287-like Japanese reference strain, AIC588. Particularly, it was noteworthy to know that the proportions of the above three variants were quite different in the following season (1993–94). In fact, of a total of 129 B strains, 73 (56.8%) were MIE193-like variants, and the proportion of BK16390-like variants which predominated in the former season of 1993–94, showed a dramatic replacement by the former MIE193-like variant. Activity caused by OSA1993-like variants (3.6%) tended to increase slightly along with the majority of activity being caused by MIE193-like viruses. The former VIC287-like variants were continuously isolated in the United States and European countries [8,19] in the early 1990s, whereas, this virus reappeared in 1993 in Japan after a four year absence. Interestingly, VIC287-like strain, KAG1594, was antigenically different from the international reference strain, GUA594 (Data not shown). Subsequently, a total of 119 VIC287-like variants were isolated at the end of 1996–97 season.

Cloning of MIE193-like variant from BK16390 virus-mixed populations

A series of antigenic analyses increased the possibility that MIE193-like variants may appear from a mixed population of the previous major epidemic strain,

Table 2. Antigenic analysis of the antigenic variants cloned from Bangkok/163/90 in the presence of antisera by post-infection ferret sera and specific antiserum

Virus antigens	HI titre with the following antisera		
	Bangkok/163/90	Mie/1/93	Antiserum specific for Mie/1/93[a]
Reference strain			
Bangkok/163/90	2048	32	NT
Mie/1/93	256	512	+[b]
Cloned viruses			
Intermediate			
20-1	512	32	−[b]
20-2	512	64	−
20-3	512	32	−
Mie193-like			
20-15	256	256	+
20-16	512	256	+
20-21	256	256	+
20-22	1024	512	+
20-23	256	128	+
20-31	256	256	+
20-32	512	256	+

[a] Absorbed antiserum was prepared as described previously [16]

[b] and −[b] represent HI titre less than 32 and 64 or greater, respectively

+*NT* Not tested

Each cloned virus were obtained from plaques produced in the presence of mouse antiserum to B/Bangkok/163/90 virus

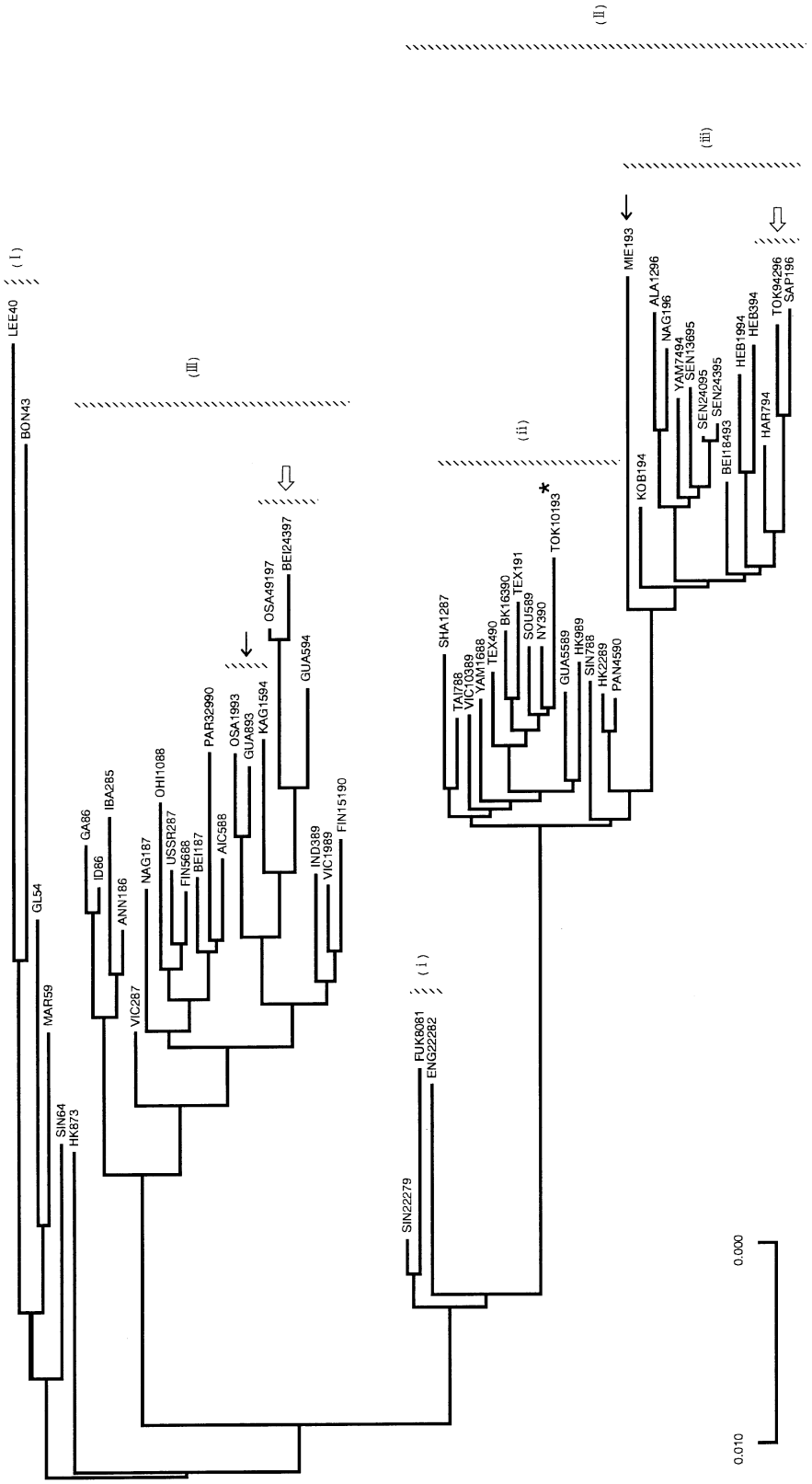
BK16390-like virus. To confirm this speculation, plaque formation by the latter virus was undertaken in the presence of antiserum to BK16390. Results are presented in Table 2. Of 10 cloned viruses, 7 showed high HI titres with antiserum to MIE193 virus and 3 reacted to lower titres with the above antiserum, respectively, On the basis of higher HI titres, 70% of cloned viruses appeared to the MIE193-like variants, suggesting the existence of a mixed population in B/Bangkok/163/90 viruses. Absorbed antiserum specific for MIE193 virus further demonstrated that the above 7 cloned viruses were identified as MIE193 virus. Also, the remaining three variants (30%) were intermediate strains between BK16390 and MIE193 viruses. Partial nucleotide sequences of the HA genes of the former MIE193-like viruses defined their similarity to the latter reference strain (data not shown). These results suggest that MIE193-like variants appeared from a mixed population of BK16390 viruses.

Evolutionary analyses of recent epidemic strains

To investigate the evolutionary patterns of influenza B viruses isolated in Japan, China and the United States, a total of 56 hemagglutinin sequences, including previously reported genes, were analyzed from an evolutionary point of view. The phylogenetic tree constructed by the N-J method clearly revealed that influenza B viruses were divided into three lineages (Fig. 3) including the earliest strain LEE40 (I), recent YAM1688-like strains isolated between 1979 and 1996 (II) and VIC287-like strains isolated between 1986 and 1997 (III). Through a series of phylogenetic analyses, epidemic strains belonging to YAM1688-like variants (II) were thought to have evolved into three sublineages. The second sublineage (II-ii), containing fifteen YAM1688-like variants, divided off from the first sublineage (II-i), while the third sublineage (II-iii) originated from a putative ancestor common to HK2289 and PAN4590 of the second sublineage (II-ii).

In contrast, the branching pattern of third lineage (III) seemed to be more discrete, showing shorter evolutionary distances between viruses than those of the second lineage (II). Despite the slower evolution, the third lineage, which divided off from a putative origin common to HK873 virus, formed two minor branch clusters belonging to the VIC287-like lineage. As a result, it was apparent that influenza B viruses have evolved into five lineages, containing the earliest I, II-i, II-ii, II-iii and III lineages. Interestingly, only TOK10193 virus (indicated by an asterisk), isolated in Japan 1993, was distantly related to other 1993-isolates, being located phylogenetically in II-(ii) lineage. This result suggests the cocirculation of two distinct variants belonging to two evolutionary sublineages in the 1992–93 and 1993–94 seasons in Japan. For example, KAG1594 (OSA1993-type) was located on a minor branch cluster of the third lineage (indicated by closed arrows), although the above two strains, including one Chinese isolate, GUA893, appeared to have divided off from NAG187 virus. Also, despite being on the same evolutionary lineage, the branch containing MIE193 was distantly related to HAR794. Indeed, the branch length and location of MIE193 were characterized by separation from other branch clusters. From a series of evolutionary analyses, it was shown that three evolutionary lineages belonging to sublineage III (OSA1993 and KAG1594), II-ii (TOK10193) and II-iii (MIE193) coexisted in 1993 in Japan.

Moreover, the tree also contained three Chinese isolates (GUA594, BEI24397, and HAR794) as well as three Japanese isolates (OSA49197, TOK94296 and SAP196) which formed new minor branches in the second sublineage (II-ii), suggesting that new antigenic variants of influenza B virus may appear in Asian countries such as China or Japan. Indeed, YAM1688 was first detected in Japan in the spring of 1988, and it has since been shown that a number of variants have divided off from YAM1688 virus [7, 19]. The present study, based on the antigenic and phylogenetic analyses, also suggested that additional variants originating from MIE193 or KAGO1594 strains may appear in future influenza epidemics caused by B virus. This speculation was demonstrated in the 1996–97 season in Japan, when 88.3% (1062) and 11.7% (141) of isolates were identified as MIE193-


(I)
(II)
(III)
(i)
(ii)
(iii)
LEE40
BON43
GL54
MAR59
SIN64
HK873
GA86
ID86
IBA285
ANN186
NAG187
VIC287
OHI1088
USSR267
FIN5688
BEI187
AIC588
PAR32990
OSA1993
GUA893
KAG1594
OSA49197
BEI24397
GUA594
IND389
VIC1989
FIN15190
FUK8081
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SIN22279
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VIC10389
YAM1688
TEX490
BK16390
TEX191
SOU589
NY390
TOK10193
GUA5589
HK989
SIN788
HK2269
PAN4590
MIE193
KOB194
ALA1296
NAG196
YAM7494
SEN13695
SEN24095
SEN24395
BEI18493
HER1994
HEB394
HAR794
TOK94296
SAP196
0.000
0.010

like and GUA495-like (VIC287 lineage) viruses, respectively. Their evolutionary positions were located at the tops of the newest branch clusters of lineages III and II-(iii) (indicated by open arrows respectively), suggesting their predominance in future epidemic seasons.

Analysis of deletion and insertion

Partial nucleotide sequences of 25 hemagglutinin genes of influenza B viruses since 1940 are shown in Fig. 4. Antigenic drift in influenza B viruses has been reported to occur in the HA1 domain through the accumulation of mutations in the hemagglutinin molecule [10]. In this report, it was suggested that deletions in the antigenic regions of the hemagglutinin of B viruses may be important in maintaining molecular structure. As shown in the figure, we also confirmed the occurrence of nucleotide deletions and insertions between positions 532 and 540 by analysis of hemagglutinin genes of the B viruses isolated during a fifty-seven-year period, from 1940 to 1997. The three deletions obseved at positions 532, 533 and 534 in the nucleotide sequence of LEE40 (evolutionary lineage I) increased to nine nucleotide deletions in the hemaggutinin of strain GL54. Thereafter, the extent of the deletions tended to decrease resulting in a complete replacement of nucleotides in the HA of VIC287 strain (lineage III). These insertions were subsequently maintained in OSA1993, KAG1594, OSA49197, and BEI24397 viruses (lineage III). Interestingly, a series of deletion and insertion occurred in strains isolated between 1940 and 1997 which generally correlated to the first, second and third lineages of the evolutionary tree, shown in Fig. 3. It was

Fig. 3. Evolutionary pathways of hemagglutinin genes of influenza B viruses. The best tree was constructed from total substitution analysis of nucleotide sequences as described previously [7, 13, 14]. Strain abbreviations used in evolutionary analysis were listed in Table 1. Closed arrows indicate hemagglutinin genes of viruses belonging to a new minor branch cluster of the third lineage, while open arrows show new variants belonging to the first lineage. The scale shown at the top of the figure represents the number of total substitutions/site from the root. In addition to the nucleotide sequences determined in the present study, sequence from the following reports were also used in construction of the evolutionary tree: [7, 8, 10, 18, 19, 24, 27] (M65174). Abbreviation of strain's name is as follow: LEE40, Lee/40; BON43, Bonn/54; GL54, GL/54; MAR59; Maryland/59, SIN64; Singapore/64, HK873, Hong Kong/8/73; SIN22279, Singapore/222/79; FUK8081, Fukuoka/80/81; ENG22282, England/222/82; IBA285, Ibaraki/2/85; GA86; GA/86; ID86, ID/86; ANN86, Ann Arbor/1/86; NAG187, Nagasaki/1/87; USSR287, USSR/2/87; BEI187, Beijing/1/87; SHA1287, Shanghai/2/87; FIN5688, Finland/56/88; OHI1088, Ohio/10/88; SIN788, Singapore/7/88; TAI788, Taiwan/7/88; IND389, India/3/89; VIC1989, Victoria/19/89; GUA5589, Guangdong/55/89; HK989, Hong Kong/9/89; SOU589, South Dakota/5/89; VIC10389, Victoria/103/89; PAR32990, Paris/329/90; FIN15190, Finland/151/90; TEX490, Texas/4/90; NY390, New York/3/90; PAN4590, Panama/45/90; TEX191, Texas/1/91; GUA893, Guandong/08/93; KOB194, Kobe/1/94; YAM7494, Yamagata/74/94; SEN13695, Sendai/136/95; SEN24095, Sendai/240/95; SEN24395, Sendai/243/95; ALA1296, Alaska/12/96; NAG196, Nagasaki/1/96; SAP196, Sapporo/1/96; BEI24397, Beijing/243/97. Remaining strain abbreviations are indicated in Table 1

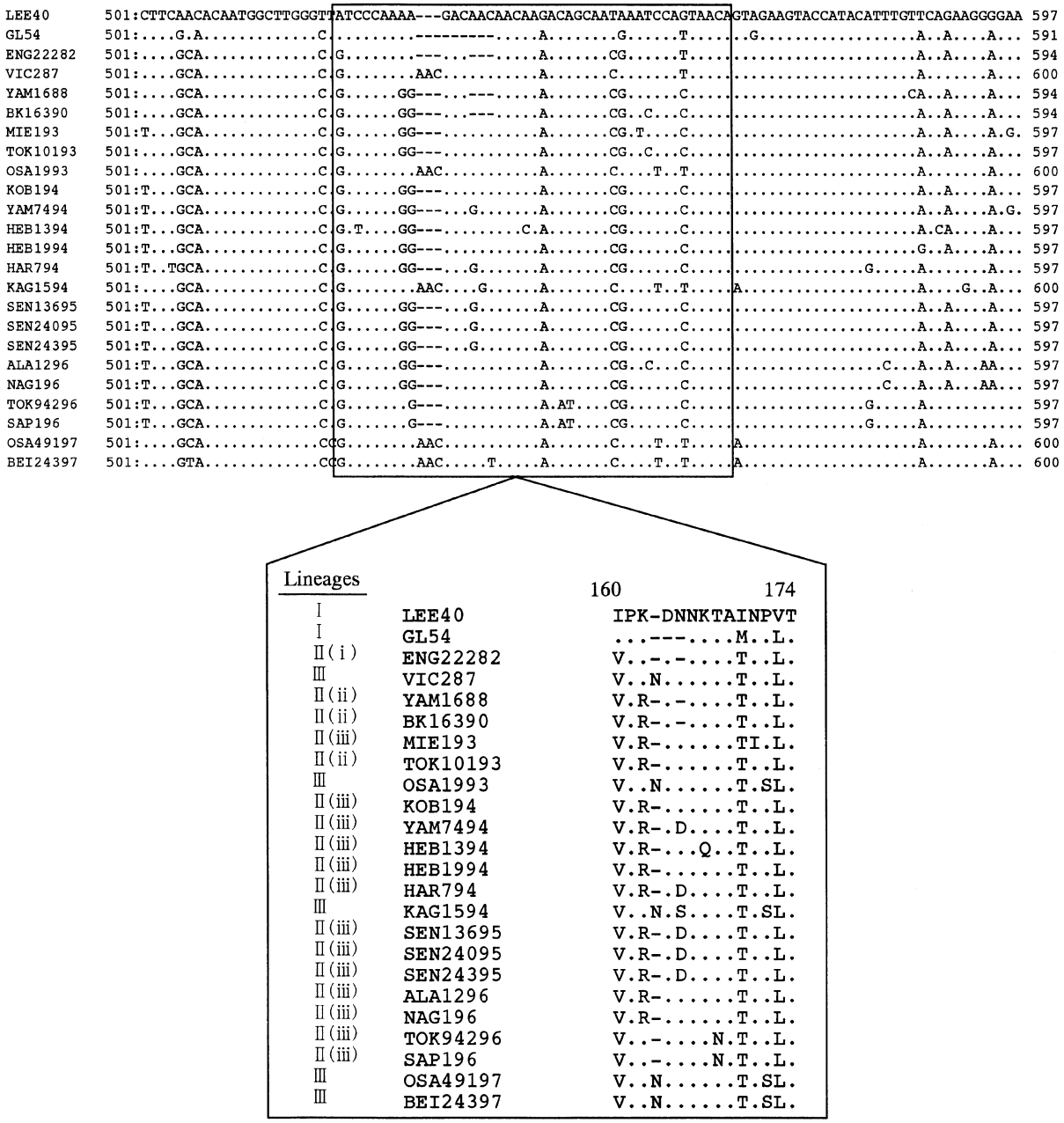

Fig. 4. Partial nucleotide and deduced amino acid sequences of the deletion and insertion region of the HA1 domain of the hemagglutinin genes of influenza B viruses and their relation to determined evolutionary pathways. Broken lines in the sequences indicate deletion positions

worth notice to know that, with two exceptions (Ser and Asp), amino acid changes resulting from nucleotide deletions and insertions were very limited during a fifty-six-year period, from 1940 to 1997. For example, amino acid residues such as Asp-Asn or Asn-Asp-Asn have been frequently observed in the region of nucleotide insertion and deletion in a number of strains. One possibility is that these sequences may play an important role in the maintenance of molecular structure of the hemagglutinin due to conformational changes resulting from amino acid substitutions in other domains.

Having considered the above evidence, the appearance of YAM1688 in Japan in 1988 was of particular interest, as nucleotide deletions were again revealed. In fact, the six deletions in the hemagglutinin gene of YAM1688 were also observed in BK16390, isolated in 1990. However, the isolation of MIE193 strain revealed only a three nucleotide deletion. Furthermore, the six nucleotide deletion that was again observed in 1988 was consistent with the evolutionary pathways of sublineage of the second lineage (II-iii), showing that the latter lineage containing YAM1688 apparently divided off from ENG2282 in which the six deletions were conserved. However, as mentioned above, isolate TOK10193 (indicated by an asterisk) was located in sublineage (ii) despite containing a nucleotide deletion pattern similar to viruses of sublineage (iii). Finally, it was noteworthy to reveal that, after 53 years, the nucleotide deletion pattern returned to that of the first strain isolated in 1940, as demonstrated in strains isolated in Japan in 1993 which belonged to the third lineage (iii). Since then, evolutionary pathways reflecting the deletion and insertion mechanism divided off into two patterns, showing that the six nucleotide deletion was maintained during an eight-year period, from 1988 to 1996 on lineage II-iii. In contrast, viruses containing a full insertion similar to that of VIC87 (lineage (iii)) reappeared in Japan in 1993 (OSA1993) and again in 1997.

From comparative analysis of the three dimensional structure of crystalized hemagglutinin (A/Aichi/1/68-H-3) from X-31 reassortant [10, 25, 26], amino acid changes resulting from the above nucleotide deletions and insertions may be considered on the globular head domain that corresponds to antigenic region B of the H3 hemagglutinin. This was the first evidence that a nucleotide deletion and insertion mechanism has caused viruses since 1993 to evolve into two lineages. Also, during a fifty-seven-year period, from 1940 to 1997, inserted and deleted amino acid residues at positions 163, 164, 165 were consistently Asn-Asp-Asn, respectively. The numbers of amino acid changes systematically alternated over time. Interestingly, alteration of the hemagglutinin molecule of influenza B virus by amino acid substitution in other regions may occur through the above deletion and insertion mechanisms. This may be one of the mechanisms by which influenza B virus is able to survive for long period of time without antigenic shift as seen in influenza A viruses.

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this activity several thousands of influenza viruses have been isolated throughout Japan in a single epidemic season. This work was supported in part by grants from the Pharmaceutical and Supply Bureau and International Affairs Division, Japanese Ministry of Health and Welfare.

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Authors' address: Dr. K. Nerome, Department of Virology I, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162, Japan.

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