Genetic analysis of porcine H3N2 viruses originating in southern China

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From immunological and phylogenetic analyses of H3 influenza viruses isolated from pigs and ducks in the People's Republic of China (China), Hong Kong, Taiwan and Japan, between 1968 and 1982, we arrived at the following conclusions. The H3 haemagglutinin and N2 neuraminidase genes from swine isolates can be segregated into four mammalian lineages, including: (i) the earliest human strains; (ii) early swine strains including Hong Kong isolates from 1976–1977; (iii) an intermediate strain between the early swine and recent human strains; and (iv) recent human strains. In this study we found an unusual swine strain (sw/Hong Kong/127/82) belonging to the third lineage which behaved like those of the early swine-like lineage in the haemagglutination inhibition test; but neuraminidase inhibition profiles with monoclonal antibodies indicated that this virus is related to late human strains. On the basis of pairwise comparisons of complete or partial nucleotide sequences the genes encoding the three polymerase proteins (PB2, PB1, PA), the nucleoprotein, the membrane protein and possibly the nonstructural proteins of sw/Hong Kong/127/82 are of the swine H1N1 lineage, whereas genes encoding the two surface glycoproteins belong to the human H3N2 lineage. In contrast, all RNA segments of one swine isolate (sw/Hong Kong/81/78) are similar to those of recent human H3N2 viruses. This study indicated that frequent interspecies infections between human and swine hosts appeared to occur during 1976–82. Although the evolutionary rates of human (0·0122/site/year), swine (0·0127/site/year) and avian (0·0193/site/year) virus genes are similar when based upon synonymous substitutions, nonsynonymous substitutions indicated that viral genes derived from human and swine viruses evolved about three times faster (0·0026–0·0027/site/year) than those of avian viruses (0·0008/site/year). Furthermore, the evolutionary mechanism by which human and swine H3 haemagglutinin genes evolve at a similar rate, based on nonsynonymous substitutions, appeared to be quite different from previous evidence which showed that human H1 haemagglutinin genes evolved three times faster than those of swine viruses. However, comparison of the number of nonsynonymous substitutions in the antigenic sites (A–E) of haemagglutinin molecules demonstrated that swine viruses evolve at a rate that is about one fifth to one tenth that of human viruses, reflecting the conservative nature of the antigenic structure in the former.

Introduction

The haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins of Hong Kong (H3N2) influenza A viruses have been studied by several investigators (Laver & Webster, 1973; Shortridge *et al.*, 1976; Shortridge & Webster, 1979; Nerome *et al.*, 1981). These reports still have considerable impact upon the direction of epizootiological studies since they suggested that the H3N2 pandemic strain was derived from avian and human viruses through a reassortment event. Competitive RNA–RNA hybridization revealed that the HA gene of the 1968 H3N2 virus originated from an avian virus and that the remaining seven RNA segments may have been derived from a previously circulating Asian (H2N2) virus (Scholtissek *et al.*, 1978). The PB1 genes of the 1957 and 1968 pandemic strains are also derived from avian viruses (Kawaoka *et al.*, 1989). By subsequent virological surveillance and genomic analyses of swine isolates, swine are recognized as having been a potential reservoir of the 1968 pandemic strain (Shortridge *et al.*, 1976; Nerome *et al.*, 1983, 1985; Castrucci *et al.*, 1993).

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Table 1. *Influenza virus strains and nucleotide sequences determined in this study*

Year of isolation	Test viruses	Place of origin	Antigenic* group	Sequenced in this study	Accession no. HA	Accession no. NA
	Human H2N2 virus					
	Kumamoto/5/65 (Kum65)			+		D21186
	Human H3N2 virus					
	Sichuan/2/87 (Sic87)			+	D21173	
	Swine H3N2 viruses					
1969	sw/Wadayama/5/69 (swWad69)	Japan		+	D21183	
1976	sw/HK/1/76 (swHK176)	China	II	+	D21174	D21187
	sw/HK/2/76 (swHK276)	China	II	−		
	sw/HK/3/76 (swHK376)	China	I	+	D21175	D21188
	sw/HK/4/76 (swHK476)	China	II	+		D21189
	sw/HK/5/76 (swHK576)	China	II	−		
	sw/HK/6/76 (swHK676)	China	I	+	D21176	
	sw/HK/7/76 (swHK776)	China	I	−		
	sw/HK/8/76 (swHK876)	China	I	−		
	sw/HK/9/76 (swHK976)	China	I	−		
	sw/HK/10/76 (swHK1076)	China	II	−		
	sw/HK/11/76 (swHK1176)	China	I	−		
	sw/HK/12/76 (swHK1276)	Hong Kong	I	−		
1977	sw/HK/13/77 (swHK1377)	Hong Kong	I	+	D21177	D21190
	sw/HK/14/77 (swHK1477)	Hong Kong	I	−		
	sw/HK/15/77 (swHK1577)	Hong Kong	I	−		
1977	sw/HK/16/77 (swHK1677)	Hong Kong	I	−		
	sw/HK/17/77 (swHK1777)	Hong Kong	I	−		
	sw/HK/18/77 (swHK1877)	Hong Kong	I	−		
	sw/HK/19/77 (swHK1977)	Hong Kong	I	−		
	sw/HK/20/77 (swHK2077)	Hong Kong	I	−		
	sw/HK/21/77 (swHK2177)	Hong Kong	I	+	D21178	D21191
	sw/HK/22/77 (swHK2277)	China	I	−		
	sw/HK/23/77 (swHK2377)	China	I	−		
	sw/HK/24/77 (swHK2477)	China	I	−		
	sw/HK/35/77 (swHK3577)	China	II	−		
	sw/HK/72/77 (swHK7277)	China	II	+	D21179	D21192
	sw/HK/77/77 (swHK7777)	China	II	−		
1978	sw/HK/81/78 (swHK8178)	China	II	+	D21180	
	sw/HK/82/78 (swHK8278)	China	II	+	D21181	D21193
1982	sw/HK/125/82 (swHK12582)	Taiwan	I†	−		
	sw/HK/126/82 (swHK12682)	Taiwan	I†	−		
	sw/HK/127/82 (swHK12782)	Taiwan	I†	+	D21182	D21194
	Avian H3N2 viruses					
1976	dk/HK/24/76 (dkHK2476)	China	AV‡	+		D21184
1977	dk/HK/245/77 (dkHK24577)	China	AV	+	D21171	D21185
1980	dk/HK/940/80 (dkHK94080)	China	AV	+	D21172	

* Antigenic groups were determined on the basis of HI reactions presented in Table 2 and they were further divided into different evolutionary lineages.

† Three avian isolates seemed to belong to antigenic group I based on the HI reaction with anti Aichi/2/68 serum absorbed by Kumamoto/22/76 antigen but their HI titres were slightly lower than those of antigenic group I.

‡ Lower HI titres with absorbed antiserum to AIC268 and the genetic analyses indicate that these viruses belong to the avian variant group (AV).

In recent studies, Air *et al.* (1990) and Nerome *et al.* (1991) compared the evolutionary pathways of N2 NA genes and showed that the NA of the 1968 H3N2 virus was introduced from an Asian strain. However, NA genes of two viruses isolated from pigs in 1976 and 1980 appeared to be highly conserved in the swine population and to have evolved independently from those of the human H3N2 viruses (Nerome *et al.*, 1991); they were shown to be on the swine lineage of an evolutionary tree constructed by the neighbour-joining method. We further demonstrated that all the NA genes from swine H3N2 viruses isolated in China and Japan were distantly related to those of avian viruses (unpublished data). This result differs from previous reports which suggested that the HA (Kida *et al.*, 1988) and PA (Okazaki *et al.*, 1989) genes of swine H3N2 viruses isolated in Hong Kong between 1978 and 1982 are genetically closer to those of avian viruses than to those of human and swine viruses.

In particular, the evolutionary tree indicated that the HA and PA genes of two H3N2 viruses (swHK8178 and swHK12682) originating in Hong Kong were closely related to duck viruses isolated in Hokkaido (Bean *et al.*, 1992; Okazaki *et al.*, 1989). However, the nucleoprotein (NP) gene of a swHK12782 strain is reported to be on a swine H1N1 lineage (Gorman *et al.*, 1990; Gammelin *et al.*, 1990). In addition, our antigenic and genetic analyses reported here suggest that the HA and NA genes of the above swine viruses are related to swine and human viruses but not to avian viruses. Even though the incompatibility observed between HA and NP genes may be explained by genetic reassortment between swine and avian viruses, the antigenic and genomic characterization revealed a clear inconsistency.

Large-scale virological surveillance in southern China, Hong Kong and Japan has provided ample evidence for the prevalence in the swine population of numerous H3N2 viruses that are antigenically and genetically related to the earliest human H3N2 viruses (Shortridge *et al.*, 1987; Nerome *et al.*, 1985, 1991). To provide further evidence in regard to interspecies infection among avian, swine and human hosts, H3 influenza viruses obtained from China, Hong Kong, Taiwan and Japan were analysed by antigenic and evolutionary means. In this study, we defined the characteristic evolutionary patterns of avian and swine influenza viruses in relation to those of human viruses, which were distinguishable according to previous reports (Kida *et al.*, 1988; Okazaki *et al.*, 1989).

Methods

Viruses. The antigenicity and the nucleotide sequences of the HA and NA genes of viruses originating from China, Hong Kong, Taiwan and Japan are listed in Table 1. Those nucleotide sequences that are not shown here are available from the sequence databases, as listed in Table 1. They were analysed from an evolutionary perspective along with 17 viruses, the HA genes of which have been sequenced. The names of the latter strains and their abbreviations are presented in Fig. 2. All viruses were grown in 11-day-old fertile hen's eggs and purified as described previously (Nerome *et al.*, 1983). Virus RNA was extracted with hot phenol as described by Palese & Schulman (1976).

Antigenic analysis. HA antigens of the isolates were characterized by means of the haemagglutination-inhibition (HI) test with absorbed post-infection ferret sera. The HI test was undertaken in small volumes using disposable microtitre plates with round-bottomed wells and 0·5% chicken red blood cells. The absorbed antisera were prepared as described by Nerome *et al.* (1982). The neuraminidase inhibition assay was performed as described in the WHO Report (1973).

Nucleotide sequencing. The nucleotide sequences of 13 HA genes were directly determined as previously described from virion RNA using a series of synthetic oligodeoxynucleotide primers (Sanger *et al.*, 1977; Kanegae *et al.*, 1990). Primers corresponding to nucleotides 11–29, 149–165, 277–295, 449–465, 578–595, 770–787 and 883–900 of the HA gene of the Aichi/2/68 strain were synthesized in our laboratory. In order to sequence NA genes we also synthesized primers corresponding to nucleotides 8–40, 345–362, 487–504, 664–682, 823–841, 1153–1170 and 1353–1370 of the NA gene of the A/sw/Ehime/1/80 (H1N2) strain. In order to determine the partial nucleotide sequences of the other six genes, we synthesized the following oligonucleotide primers which are complementary to the PB1 gene (16–32) of sw/TN/24/77 (H1N1), the PB2 gene (18–35) of sw/Iowa/15/30 (H1N1), the PA gene (15–32) of sw/Iowa/15/30, the NP gene (15–30) of Iowa/15/30, the M gene (23–40) of sw/1976/31 and the NS gene (9–29) of PR/8/34 (H1N1).

Analysis of evolutionary patterns. The total number of substitutions, as well as the number of synonymous (silent) and nonsynonymous (amino acid changing) substitutions between the nucleotide sequences of 30 HA and 22 NA genes were used to construct phylogenetic trees according to the neighbour-joining method (Nei & Gojobori, 1986).

Results

Antigenic analysis of the isolates derived from domestic poultry, swine and humans

HI tests with absorbed antisera defined two (or three) antigenic variants in the swine population between 1976 and 1982 (Table 2). For example, anti-Aichi/2/68 serum absorbed with Kumamoto/22/76 virus antigen inhibited haemagglutination by the group I variants, and failed to react with group II variants. Three swine strains isolated in 1982 and two avian viruses reacted to a lower titre with the above antiserum. Conversely, the swine group II variants reacted to a high titre with the anti-Kumamoto/22/76 serum absorbed with Aichi/2/68 antigen but not with anti-Aichi/2/68. Thus, the swine isolates originating in southern China and Taiwan can be segregated into two groups according to their HI reactions: one group is related to the earliest human H3N2 virus and a second group is similar to a recent human strain. Viruses belonging to the two antigenic groups were isolated from the swine population in 1976 and 1977. The two avian H3 influenza viruses, which possess similar HAs, react only with the absorbed Aichi/2/68 serum.

With the exception of three isolates from 1982, the antigenic analyses of HA with absorbed antisera matched with evidence obtained from neuraminidase inhibition patterns obtained with a panel of monoclonal antibodies. For example, four monoclonal antibodies to the Tokyo/3/67 (H2N2) virus inhibited the NA activity of the viruses belonging to the first variant group (I), and failed to react with those of group II (Table 2). In contrast, the NA activity of the viruses belonging to the latter group was inhibited by four or five monoclonal antibodies to the recent human H3N2 strains, Vict/3/75 and Texas/1/77. Thus, two H3N2 variant groups had co-circulated in the swine population. It is evident therefore that the neuraminidases from the first and second variant groups were closely related to those of

Table 2. *Antigenic characterization of haemagglutinin and neuraminidase from human, swine and avian H3N2 viruses using cross-absorbed antisera and monoclonal antibodies*

Hosts	Virus antigens	Antigenic group HA	Antigenic group NA	HI titre with absorbed antisera*: Aichi minus Kumamoto	HI titre with absorbed antisera*: Kumamoto minus Aichi	NI profiles with monoclonal antibodies to: Jap/305 78.4	Jap/305 102/2	Jap/305 136/5	Jap/305 117/2	Tokyo/3/67 S10/1	Tokyo/3/67 16/8	Tokyo/3/67 23/9	Tokyo/3/67 25/4	Vic/3/75 27/3	Vic/3/75 21/3	Texas/1/77 18/3	Texas/1/77 19/1	Texas/1/77 67/3
Human	Aichi/2/68	I	I	4096	–	–	–	–	–	+	+	+	+	–	–	+	+	+
	Tokyo/6/73	II	II	ND	ND	–	–	–	–	–	–	–	–	+	+	+	+	+
	Kumamoto/22/76	II	II	–	512	–	–	–	–	–	–	–	–	+	+	+	+	+
Swine	*1976 isolates*																	
	HK/3/76 HK/6/76 HK/7/76 HK/8/76 HK/9/76 HK/11/76 HK/12/76	I	I	2048	–	–	–	–	–	+	+	+	+	–	–	+	–	–
	HK/1/76 HK/2/76 HK/4/76 HK/5/76 HK/10/76	II	II	–	2048	–	–	–	–	–	–	–	–	+	+	+	+	+
	1977 isolates																	
	HK/13/77 HK/14/77 HK/15/77 HK/16/77 HK/17/77 HK/18/77 HK/19/77 HK/20/77 HK/21/77 HK/22/77 HK/23/77 HK/24/77	I	I	2048	–	–	–	–	–	+	+	+	+	–	–	+	–	–
	HK/72/77 HK/77/77 HK/35/77	II	II	–	2048	–	–	–	–	–	–	–	–	+	+	+	–	+
	1978 isolates																	
	HK/81/78 HK/82/78	II	II	–	2048	–	–	–	–	–	–	–	–	+	+	+	–	+
	1982 isolates																	
	HK/125/82 HK/126/82 HK/127/82	I	II	512–1024	–	–	–	–	–	–	–	–	–	–	+	+	–	+
Avian	duck/HK/24/76 duck/HK/245/77	AV†	AV	512	–	+	+	+	+	–	+	–	+	–	–	–	–	–

* Values given represent the reciprocal of the terminal serum dilution inhibiting haemagglutination of test antigens. A positive NI profile represents the inhibition of neuraminidase activity of test antigen at an antibody dilution of 1:4. –, HI titres less than 32. ND, Not done.

† AV, antigenic type belonging to avian viruses.

early and recent human H3N2 viruses, respectively. HI and NI tests also revealed that the HAs of three swine isolates from 1982 are similar to those of the first variant group, whereas their neuraminidases are apparently similar to those of the second variant group, implying antigenic reassortment between the two variant groups. Avian (N2) antigens were further characterized by reactivity with four monoclonal antibodies to the neuraminidase of Jap/305/57 (H2N2).

Nucleotide sequence analysis

To confirm the antigenic differences among the isolates derived from the above hosts, the complete nucleotide sequences of the HA1 domains of the HA genes of avian, swine and human H3N2 viruses were determined and pairwise comparisons were made, which included sequences previously determined (Both & Sleigh, 1980; Fang *et al.*, 1981; Kida *et al.*, 1987, 1988; Nakajima *et al.*, 1988; Min Jou *et al.*, 1980; Laver *et al.*, 1980; Bean *et al.*, 1992).

For certain viruses, the nucleotide sequences determined in this study were markedly different from those described previously by Kida *et al.* (1988). To illustrate this, the nucleotide sequences of the HA1 domains of swHK8178, swHK8278 and swHK12782 are shown in Fig. 1 and compared with those reported by Kida *et al.* (1988). For example, there are 32 and 30 amino acid differences between the HA genes of swHK8178 and swHK8278 determined in this study and that previously reported for swHK8178. In the study of Kida *et al.* (1988) the nucleotide sequences of the HA genes of three swine isolates (sw/HK/125, 126 and 127/82) were identical, except for one change at position 989, and they differed from the sequence of swHK12782 HA1 reported here by 99 nucleotides and 32 amino acids.

The similarities and differences in nucleotide and amino acid sequences of the HA1 domains of the HA genes of human, swine and avian viruses are summarized in Table 3. On the basis of the highest nucleotide sequence similarity (99·5%), the earliest swine strain swWAD69 is thought to be derived directly from the earliest human virus (AIC268). With the exception of swHK12782, swWAD69 was also genetically closer to the strains belonging to group I than to group II antigenic variants, since the nucleotide and amino acid sequence similarities of the HA gene of swWAD69 and group I variants containing swHK376, swHK676, swHK1377 and swHK2177 were relatively high (95·2–96·4% and 93·0–94·5%, respectively) when compared with those of group II variants. In contrast, the nucleotide and amino acid sequence similarities of the HA gene of swWAD69 and group II variants were 93·4–94·2% and 91·2–92·4%, respectively. The HA genes of swHK176, swHK7277 and swHK8278 belonging to group II appeared to be closely related to that of VIC375 based on their high nucleotide sequence identity (97·2–97·9%). It was noteworthy that despite belonging to antigenic group I, the HA gene of swHK12782 is distantly related to those of both swWAD69 and VIC375

(*a*)

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                10        20        30        40        50        60        70        80        90       100
swHK8178        HDLPKNDNSTATLCLGHHAVPNGTLVKTITTDQIEVTNATELVQSSSMNKICNKPHRILDGVNGTLIDALLGDPHCDGFQNEKWDLFVERSKAFSNCYPY
swHK8278        ..................................................................C.................................
swHK8178(*)     Q...GT.................I.....D.............TG....N..K....IDC.............V..D.T........N........

               110       120       130       140       150       160       170       180       190       200
swHK8178        DVPDYASLRSLVASSGTLEFINEGFNWTGVTQNGGSGACKKGPDNGFFSRLNWLYKSGSTYPVQNVTMPNNGNSDKLYIWGVHHPSTDKEQTDLYVQASG
swHK8278        ...................................R..................................................................
swHK8178(*)     ...................T...T.........N...R..A.........T........L.......D...............NQ...N.......

               210       220       230       240       250       260       270       280       290       300
swHK8178        KVTVSTKRSQQTVIPNVGLRPWVRGLSSRISIYWTIVKPGDILVINSNGNLIAPRGYFKMRTGKSSIMRSDAPIGTCSSGCITPNGSIPNDKPFQNVNKI
swHK8278        ..................................................................................................
swHK8178(*)     R...........M...A.S.....................................................E.....................

               310       320
swHK8178        TYGACPKYVKQNTLKLATGMRNVPEKQT
swHK8278        ............................
swHK8178(*)     ............................
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(*b*)

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                10        20        30        40        50        60        70        80        90       100
swHK12782       QDLPGNDNSKATLCLGHHAVPNGTLVKTITDDQTEVTNATELVQSSSTGKICNSPHRILDGMNCTLIDALLGDPHCDVFQNKTWDLFVERSKAFSNCYPY
swHK12682(*)    .........T.............I........I..................N..K....RD.................DE.........N...S....

               110       120       130       140       150       160       170       180       190       200
swHK12782       DVPDYASLRSLAASSGTLEFTIEGFTWTGVTQNGGSNACKRGSDRGFFSKLNWLTKSGNTYPVLNVTMPNNDNFDKLYIWGVHHPSTDQEQTSLYVQASG
swHK12682(*)    ..........V........IT.............S.....PAS....R........S.............................N....N.......

               210       220       230       240       250       260       270       280       290       300
swHK12782       RVTVSTSRGQQTIIPNIGPRPWVRGLSSRISIYWTIVKPGDVLVISSNGNLIAPRGYFKMRTGKSSIMRSDAPIDSCISECITPNGSIPNDKPFQNINKI
swHK12682(*)    ......R.S.........S......Q.G.................N.............................T.V.................V...

               310       320
swHK12782       TYGACPKYVKQNTLKLATGMRNVPEKQT
swHK12682(*)    ............S...............
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Fig. 1. Comparison of deduced amino acid sequences of the HA1 domains of swine influenza virus HAs with those previously reported for swine viruses originating in China. Influenza swHK8178 and swHK8278 (*a*) and swHK12782 (*b*) virus HA proteins determined in this study were used for comparison with amino acid sequences of reference strains. The sequences of swHK8178 and swHK12682 that are different from those of the present study are shown in the lower position (indicated by asterisks; Kida *et al.*, 1988).

and showed 93·8% and 91·4% nucleotide sequence identity with those of swWAD69 and VIC75, respectively. On the basis of this evidence, swHK12782 seemed to be an intermediate virus between antigenic groups I and II. This result led to the suggestion that swHK12782 may be genetically separate from group I variants.

The degree of genetic identity of the HA genes was high within a group: with the exception of swHK12782, nucleotide sequence similarities among group I or group II variants amounted to 95·2–99·5% and 97·1–98·6%, respectively. As a result, on the basis of pairwise comparisons of HA genes, swine viruses belonging to group I and group II appeared to be closely related to the earliest human strain, AIC268 and a recent VIC375, respectively. It was of particular interest that the nucleotide sequence identities (95·1–96·4%) between the earliest human strain, AIC268, and four recent avian viruses isolated between 1977 and 1980 were higher than with the earliest avian strain dkUKR (90·6%), suggesting the possible derivation of the HA gene of the earliest human H3 virus from a duck virus in China.

Phylogenetic analysis of HA genes of human, swine and avian viruses

As shown in Fig. 2, the phylogenetic tree showed that H3HA genes of swine isolates can be segregated into four host-specific lineages, including (i) the earliest human strains, (ii) early swine strains, (iii) an intermediate strain between the earliest swine and recent human strains and (iv) recent human strains since 1972 (Fig. 2). The evolutionary tree suggests that the HA genes of the earliest human and early swine lineages diverged from a common ancestral virus that was prevalent around 1967 (indicated by P in Fig. 2). Branching patterns in the tree indicated that the HA gene of the latter lineage might have evolved in the swine population since 1967. The evolutionary analysis indicates that swHK12782 is a

Table 3. *Comparison of nucleotide and amino acid similarities among the HA1 domain of HA genes from influenza A (H3N2) viruses derived from human, swine and avian hosts**

			Sequence similarity (%) of the HA1 domain of HA genes from:																
			Human virus				Swine virus									Avian virus				
Host	Strain		AIC 268	VIC 375	BK 179	PHI 82	swWAD 69	swHK 376	swHK 676	swHK 1377	swHK 2177	swHK 12782	swHK 176	swHK 7277	swHK 8278	dkUKR 63	dkHOK 577	dkHK 24577	dkHOK 880	dkHK 94080
Human	AIC 268		**	95·4	93·3	92·5	99·5	96·5	95·6	95·6	95·3	93·9	93·9	93·4	93·1	90·6	96·2	96·4	94·8	95·1
	VIC 375		93·6	**	95·7	94·5	95·7	93·1	92·7	92·7	92·4	91·4	97·9	97·6	97·2	85·3	92·5	92·5	91·3	91·3
	BK 179		89·7	93·3	**	98·4	93·8	91·6	91·1	91·2	91·1	89·4	95·1	94·3	93·7	87·5	90·7	90·9	89·7	89·7
	PHI 282		88·5	91·2	97·3	**	92·6	90·7	90·2	90·3	90·2	88·2	94·0	93·3	92·7	86·4	89·5	89·7	88·7	88·5
Swine	swWAD569	I	99·1	94·5	90·6	88·5	**	96·4	95·5	95·5	95·2	93·8	94·2	93·7	93·4	90·4	96·0	96·2	94·6	95·1
	swHK376	I	94·8	89·7	86·3	85·7	94·5	**	96·3	96·3	95·8	91·2	87·8	91·3	91·1	88·2	93·3	93·3	92·3	92·5
	swHK676	I	93·3	89·7	86·9	86·0	93·0	93·9	**	98·9	99·0	91·0	88·2	90·7	90·6	87·8	92·3	92·7	91·6	91·9
	swHK1377	I	93·3	89·7	86·9	86·0	93·0	93·9	98·29	**	99·5	90·6	88·2	90·7	90·3	87·7	92·3	92·7	91·5	91·9
	swHK2177	I	93·3	89·7	87·5	86·6	93·0	93·3	98·5	99·1	**	90·5	87·6	90·4	90·4	87·7	92·0	92·4	91·2	91·5
	swHK12782	I	93·9	90·0	87·8	85·7	93·9	89·1	89·1	89·1	88·5	**	89·4	88·2	87·8	86·7	91·3	91·3	90·1	90·5
	swHK176	II	91·5	97·6	93·5	91·5	92·4	91·7	91·6	91·2	90·9	90·3	**	97·1	97·2	87·1	91·1	91·3	90·1	90·1
	swHK7277	II	90·3	96·1	91·5	90·0	91·2	86·6	86·6	86·6	86·3	89·8	96·4	**	98·6	86·6	90·8	91·0	98·5	90·0
	swHK8278	II	90·3	95·7	91·2	90·0	91·2	86·6	86·6	86·6	86·3	90·0	96·4	97·6	**	86·5	90·4	90·4	89·0	89·2
Avian	dkUKR63		95·4	91·5	89·1	87·2	95·4	90·9	90·6	90·6	90·6	90·6	90·0	88·5	88·5	**	90·4	91·0	90·8	89·8
	dkHOK577		96·4	92·1	88·5	86·6	96·4	91·8	91·2	91·2	91·2	91·5	90·0	88·8	88·8	96·1	**	96·0	93·5	95·5
	dkHK24577		96·7	91·5	88·2	88·3	96·7	92·1	92·1	92·1	92·1	91·5	89·7	88·5	88·5	96·4	97·9	**	93·7	97·3
	dkHOK880		95·7	91·5	87·8	86·0	95·7	91·2	90·5	90·6	90·6	90·3	89·4	88·2	88·2	96·1	96·7	97·0	**	94·3
	dkHK94080		96·7	92·1	85·5	86·6	96·7	92·1	92·1	92·1	92·1	91·5	90·0	88·8	88·8	96·4	97·9	98·8	97·6	**

* A pairwise comparison of nucleotide sequences determined in this study was performed including the following reported sequences: AIC268 (Verhoeyen *et al.*, 1980), VIC375 (Min Jou *et al.*, 1980), BK179 (Both & Sleigh, 1981), PHI282 (Nakajima *et al.*, 1988), dkUKR63 (Fang *et al.*, 1981), dkHOK577 (Kida *et al.*, 1987) and dkHOK880 (Kida *et al.*, 1987).

swine virus that is located between early swine and recent human lineages. The HA gene of the avian viruses falls into three lineages which appear to branch from a common ancestor around the mid-1950s.

According to the estimated date of the common ancestor, the human and swine H3 isolates had already divided off from dkUKR63 around 1958. Even though current human strains isolated between 1982 and 1987 form one branch cluster, they had previously divided off (around 1972) from the recent human lineage containing VIC75, swHK176, swHK8178 and swHK8278 viruses. It is of particular interest that a single branch including swHK12782 seems to have evolved in the swine population since dividing from the recent human lineage around the early 1970s. The evolutionary tree also showed that the mammalian H3N2 viruses circulating since 1968 are descended from the avian lineage containing viruses similar to dkHK2457, dkHOK577 and dkHK94080.

Phylogenetic analysis of NA genes

To compare the evolutionary patterns of the NA gene with that of the HA gene we constructed a phylogenetic tree from synonymous substitutions by the neighbour-joining method (Fig. 3). In accordance with evolutionary pathways of the HA gene, N2 NA genes of swine and human H3N2 viruses were divided into (i) the earliest human strains, (ii) early swine strains, (iii) an intermediate strain between recent human and early swine lineages and (iv) a recent human lineage. The evolutionary location of NA genes indicated that the HA and the NA genes of all swine isolates evolved in a parallel fashion but at different evolutionary rates. This analysis indicated that the NA gene of the earliest human H3N2 virus had already divided off from that of the late Asian virus (KUM 65) around the mid-1960s. From an evolutionary perspective they were distinguishable from avian strains.

Evolutionary rate of the HA gene

The neighbour-joining method yielded two phylogenetic trees based upon synonymous and nonsynonymous substitutions (data not shown), which allowed a direct comparison of the evolutionary rates within each host-specific lineage. This was accomplished by plotting the year of isolation for a virus against numbers of synonymous and nonsynonymous substitutions (branch length to each ancestral node) (Fig. 4). The HA genes of three swine isolates (swHK176, swHK7277 and swHK8278) were omitted from the calculation because they belong to the recent human lineage. Fig. 4(*a*, *b*) shows that the slopes of the regression lines for synonymous and nonsynonymous substitutions of the human and swine HAs are very similar and that for the human and swine virus lineages the rates were 0·0122 and 0·0127 synonymous substitutions/site/year, respectively. The rate of nonsynonymous substitutions for human and swine virus genes is 0·0026 and 0·0027/site/year, respectively, indicating that even at the protein level human and swine viruses are evolving at similar rates. In

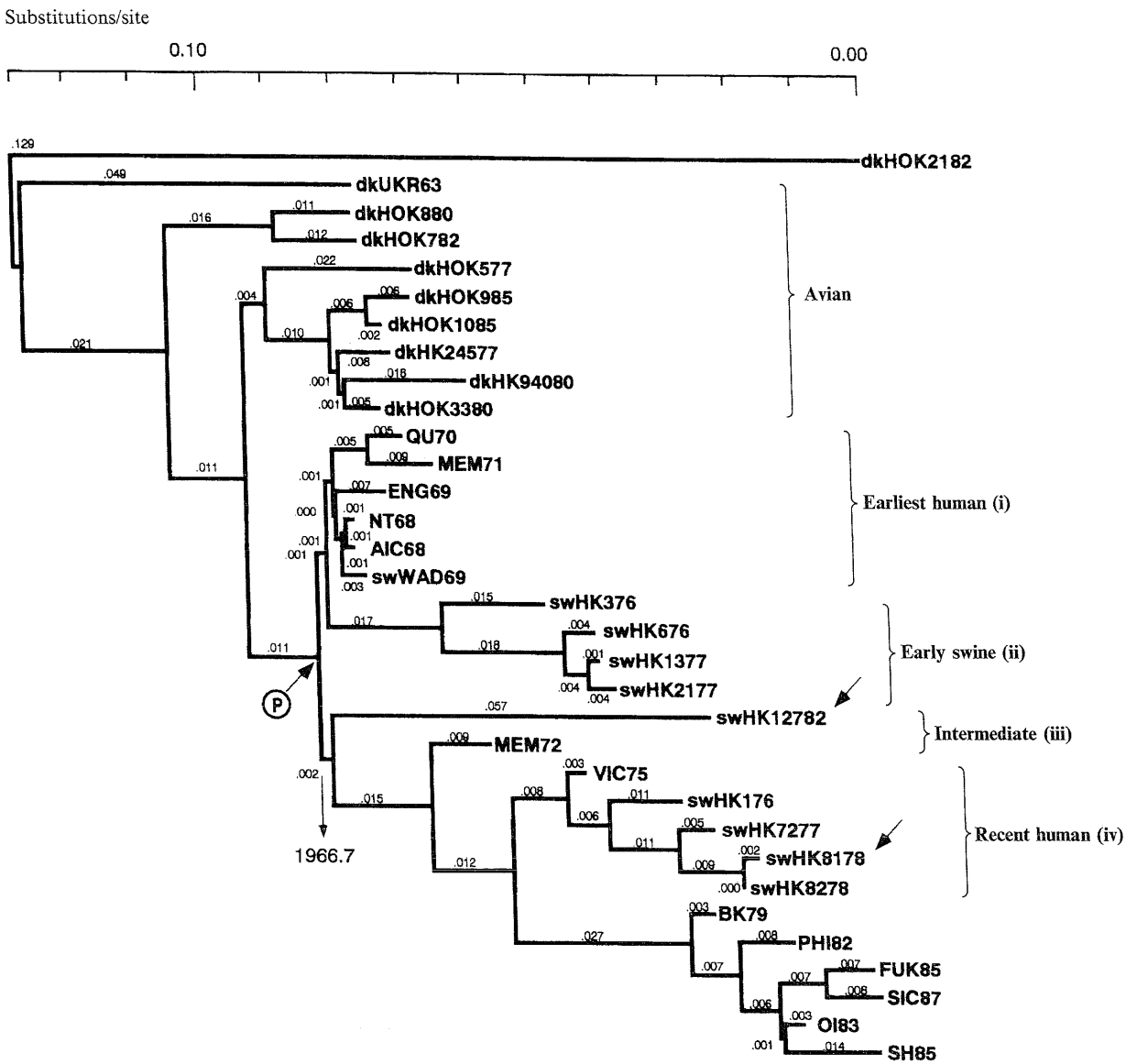

Fig. 2. Evolutionary profiles of H3HA genes from human, swine and avian influenza viruses based on the calculated mutational distances between HA1 domains. Distance matrices of all pairs for positions 76–1061 of the HA1 gene were estimated by the six parameter method (Gojobori *et al.*, 1982) and a phylogenetic tree was constructed by the neighbour-joining method (Nei & Gojobori, 1986; Saitou & Nei, 1987). Branch lengths were estimated based upon minimum evolution. The assumption of minimum evolution is based upon the best tree so that erroneous calculations for the construction of the tree are minimal. Ⓟ marks the position of a putative ancestor. The date (year) of divergence of the earliest human and an ancestral virus of early swine strains is indicated by the downward pointing arrow. Arrows indicate the positions of the HA gene of swHK8178, which is on a human lineage, and the HA gene of swHK12782, which belongs to a human lineage located between the early swine and recent human lineages. The scale shown at the top of the figure represents the number of silent substitutions/site from the root. In addition to the nucleotide sequences determined in this study, the following reported sequences were also used for comparisons (Min Jou *et al.*, 1980; Verhoeyen *et al.*, 1980; Fang *et al.*, 1981; Sleigh *et al.*, 1981; Both & Sleigh, 1981; Newton *et al.*, 1983; Kida *et al.*, 1987, 1988; Nakajima *et al.*, 1988). FUK85 (Fukuoka/c-29/85), SH85 (Stockholm/4/85), OI83 (Oita/3/83), PH182 (Philippines/2/82), BK179 (Bangkok/1/79), VIC375 (Vict/3/75), MEM72 (Memphis/102/72), AIC (Aichi/2/68), NT68 (NT/68), MEM71 (Memphis/1/71), QU (QU/7/70) and ENG69 (Eng/878/69) were reported by Sleigh *et al.* (1981). The nucleotide sequences of the HA genes of dkHOK1085 (dk/Hokkaido/10/85), dkHOK985 (duck/Hokkaido/9/85), dkHOK3380 (duck/Hokkaido/33/80), dkHOK577 (duck/Hokkaido/5/77), dkHOK880 (duck/Hokkaido/8/80), dkHOK782 (duck/Hokkaido/7/82) and dkHOK2182 (duck/Hokkaido/2/82) were reported by Kida *et al.* (1987).

contrast, the rate of nonsynonymous substitutions of the avian virus (0·0008) was quite different from those in the above mammalian viruses (Fig. 4*b*).

Interestingly, it was evident that the rate of nonsynonymous substitutions in the antigenic sites A to E (Wiley *et al.*, 1981) of the human virus HA appeared to

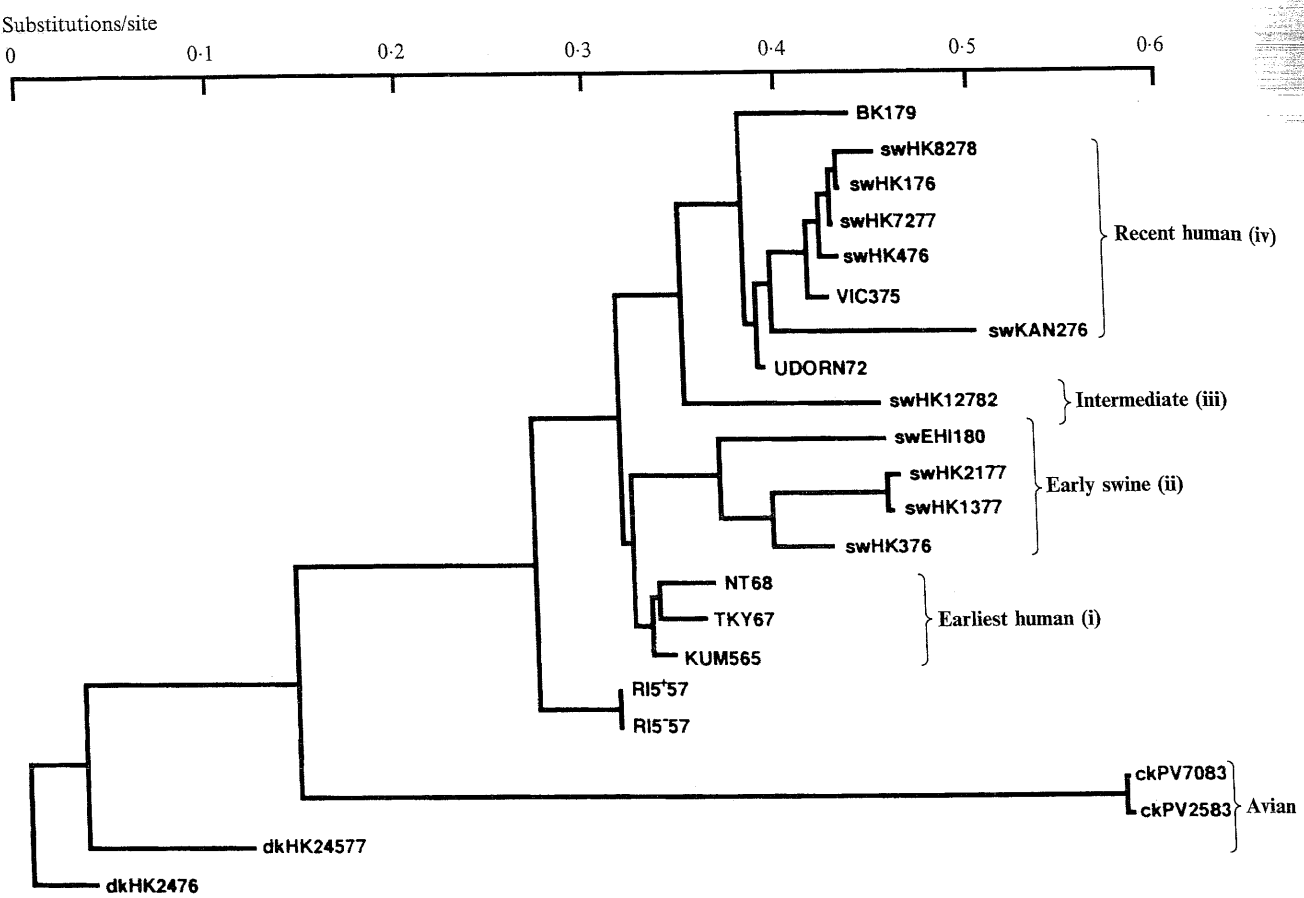

Fig. 3. Evolutionary tree of the N2 NA genes of human, swine and avian influenza viruses. The tree was constructed from synonymous substitutions by the neighbour-joining method as previously described (Nei & Gojobori, 1986; Saitou & Nei, 1987). As described in Fig. 2, the best tree was constructed to minimize erroneous calculations. We examined nine NA sequences determined here and ten that were published previously (Bently & Brownlee, 1982; Elleman *et al.*, 1982; Markoff & Lai, 1982; Rompuy *et al.*, 1982; Martinez *et al.*, 1983; Lentz *et al.*, 1984; Air *et al.*, 1985; Deshpande *et al.*, 1985; Kawaoka *et al.*, 1989; Nerome *et al.*, 1991).

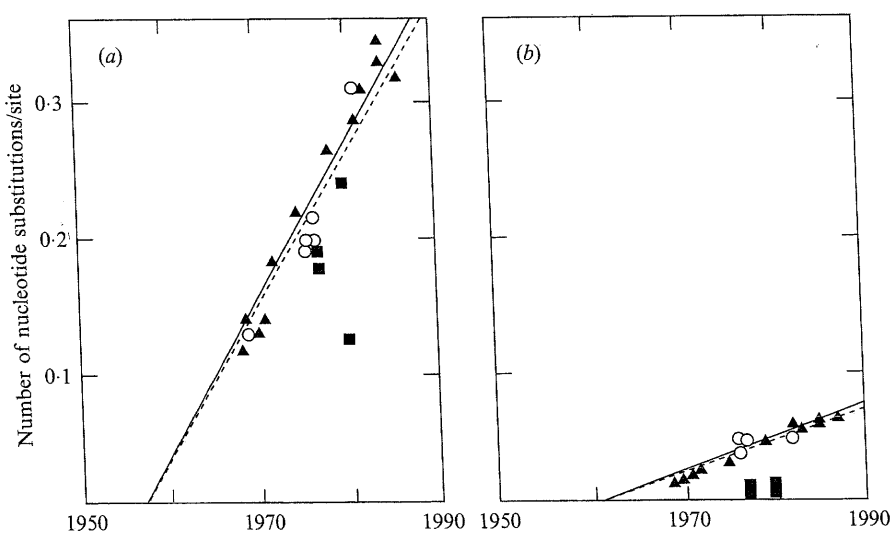

Fig. 4. Synonymous (*a*) and nonsynonymous (*b*) rates of the evolution of the HA1 domain of HA genes from human, swine and avian influenza viruses. The branch lengths were estimated from two trees constructed from synonymous and nonsynonymous substitutions, and these values were plotted against the year of virus isolation. ▲, Human strains; ○, swine strains; ■, avian strains. Solid and dotted lines represent regression lines of human and swine virus genes, respectively.

Table 4. *Comparison of gene derivations of swine H3N2 viruses isolated from pigs in Hong Kong with that obtained from previously reported results*

		Gene derivation of virus				
		This study			Previous studies	
Virus	Gene	Host origin*	Region of sequences or other analyses†	Homology (%)‡	Host origin	References
swHK8178	PB1	H (H3)	Part (51–200)	97·3 (MEM88)		
	PB2	H (H3)	Part (51–200)	96·0 (MEM88)		
	PA	H (H3)	Part (51–200)	96·0 (NT68)	AV (H3)	Okazaki *et al.* (1989)
	HA	H (H3)	Whole (HA1 domain)	97·2 (VIC75)	AV (H3)	Kida *et al.* (1988); Bean *et al.* (1992)
	NP	H (H3)	Part (51–200)	98·0 (UDO72)		
	NA	H (H3)§	NI test	ND		
	M	H (H3)	Part (51–200)	99·7 (UDO72)		
	NS	H (H3)	Part (51–200)	95·3 (UDO72)		
swHK12782	PB1	SW (H1)	Part (51–200)	96·7 (TEN77)		
	PB2	SW (H1)	Part (51–200)	95·3 (TEN77)		
	PA	SW (H1)	Part (51–200)	94·7 (TEN77)		
	HA	H (H3)	Whole (HA1 domain)	93·9 (AIC268)	AV (H3)	Kida *et al.* (1988)
	NP	SW (H1 or H3)	Part (51–200)	98·7 (TEN77, HK126)		
	NA	H (H3)	Whole	94·8 (UDO72)		
	M	SW (H1)	Part (51–200)	98·0 (TEN77)	AV	Ito *et al.* (1991)
	NS	SW (H1)‖	Part (51–200)	95·7 (NAG89)		
swHK12682	PA	ND	ND	ND	AV (H3)	Okazaki *et al.* (1989)
	HA	H or SW (H3)§	HI test	ND	AV (H3)	Kida *et al.* (1988); Bean *et al.* (1992)
	NA	H (H3)§	NI test	ND		

* H, SW and AV represent human, swine and avian virus origins.

† Partial nucleotide sequences of six genes encoding the internal proteins were determined and their nucleotide sequence similarities were compared with those of the following genes: PB2 (Gorman *et al.*, 1990); PB1 (Kawaoka *et al.*, 1989); PA (Okazaki *et al.*, 1989); NP (Gorman *et al.*, 1990); M (Schultz *et al.*, 1991); NS (Buonagurio *et al.*, 1986).

‡ Abbreviation of strains: MEM88, A/Memphis/8/88 (H3N2); NT68 (Fig. 2); VIC75 (Fig. 2); UDO72, A/Udorn/72; TEN77, A/sw/Tennessee/24/77 (H1N1); AIC68 (Fig. 2); HK126 (Table 1); HK127 (Table 1); IO30, A/sw/Iowa/15/30.

§ The host derivation was determined by HI test with absorbed antisera and NI test with monoclonal antibodies.

‖ Even though the NS gene of swHK12782 could not be compared with that of swine H3N2 virus, this gene had the highest nucleotide sequence homology with sw/Nagasaki/1/89 (H1N2: NAG89).

ND, Not determined.

be about five times higher than that of the swine virus (data not shown). For example, the mean evolutionary rates based on the number of nonsynonymous substitutions per site in the antigenic sites of human and swine H3 HAs of viruses isolated between 1968 and 1982 were estimated to be 0·312 and 0·062, respectively.

Derivation of eight RNA segments of two swine isolates

Since the HA and NA genes of swine isolates from China were of swine or human lineage, we also examined the host-specificity of the six remaining RNA segments coding for internal proteins. The partial nucleotide sequences of the genes of swHK8178 and swHK12782 were determined and a pairwise comparison was made with previously sequenced genes of human, swine and avian viruses. The host origin, the region of nucleotide sequences compared, the percentage similarity and the name of the strains examined are summarized in Table 4.

The PB2, PB1 and PA genes of swHK12782 virus showed high nucleotide sequence similarity (94·7–96·7%) with those of swine H1N1 viruses. The NP gene of swHK12782 was closely related to that of both swine H1N1 and H3N2 viruses: the nucleotide sequence similarity between swHK12782 and swine H1N1 and H3N2 viruses was 98·7%. On the basis of high nucleotide sequence similarity (98·0%) with that of TEN77, the M gene of swHK12782 appeared to be derived from a swine H1N1 virus. The remaining gene encoding the NS protein of this virus is also related to that of swine H1 viruses. The nucleotide sequence similarity of the NS genes of this virus and A/sw/Nagasaki/1/89 (H1N2) [the NS gene of which has already been identified in our laboratory (data not shown)] amounted to 95·7%. Furthermore, on the basis of pairwise comparisons of the nucleotide sequences and evolutionary trees, it was concluded that HA and NA genes of swHK12782 are derived from a human H3N2 virus. The nucleotide

sequence similarities of the six genes encoding internal proteins of strain swHK8178 compared with those of human H3N2 viruses (95·3–99·7%) was higher than with those of human H1N1 (85–95%), swine H1H1 (86–94%), swine H3N2 (83–96%) or avian (85–95%) viruses, suggesting the transfection of human H3N2 viruses into pig populations.

Discussion

The frequent isolation of H3N2 influenza viruses from the swine population of the southern region of China indicated that the earliest H3N2 virus, which suddenly appeared in man in 1968, still circulates with small changes in antigenicity. Using a panel of monoclonal antibodies to the HA of A/NT/60/68 virus, some recent swine isolates appeared to share most of the epitopes with the earliest human H3N2 virus (Shortridge *et al.*, 1987), supporting the above speculation.

The first aim of this study was to determine whether host specificity among the viruses isolated from humans, swine and avian species was detectable by antigenic analyses and pairwise comparisons of nucleotide and deduced amino acid sequences of their HA genes. Our antigenic analyses revealed that swine viruses could be divided into two antigenic groups, while nucleotide and amino acid sequence analyses show that three variant groups of swine isolates have possibly evolved since 1969. In a previous study we compared the evolutionary pathways of the N2 neuraminidase of human and swine viruses (Nerome *et al.*, 1991) and showed that two lineages have co-circulated in the swine populations since 1976.

Although human and swine viruses show nucleotide sequence similarities, the HA genes of swine and avian viruses are distinguishable. To investigate the host specific nature of the viruses, functional constraints operating on the amino acid sequences at the receptor-binding site (at positions 226–228) are good indices with which to differentiate between swine and avian lineages. The putative receptor-binding site on the HA gene of all swine viruses isolated in southern China is, without exception, Leu–Ser–Ser at residues 226–228. This is quite different from a previous report that three swine isolates had the amino acid sequence Gln–Ser–Gly at positions 226–228, similar to avian viruses (Kida *et al.*, 1988). This difference is attributable to differences in the viruses used in the two studies (Fig. 1). To investigate the possibility that both strains examined here (swHK8178 and swHK12782) already represent two distinct subpopulations, one including an avian-like H3 strain and the other including a swine H3N2-like strain, the original seed viruses, which are preserved in a lyophilized state in the laboratory where the above swine viruses were isolated, were revived under the close surveillance of two of the authors (K.F.S. and Y.K.) and grown in Madin-Darby canine kidney cells (MDCK) and 11-day-old fertile hen's eggs at a dilution ranging from 10^{-1} to 10^{-4}. Following distribution to three laboratories in Japan, USA and Hong Kong, the genes from the seed viruses were again analysed. The results indicated that the seed viruses were not likely to contain an avian H3 virus. The host origins of genes encoding the internal proteins of swHK8178 and swHK12782 viruses were not compatible with those reported previously (Okazaki *et al.*, 1989; Ito *et al.*, 1991) and discrepancies in the derivations of HA, PA and M genes are presented in Table 4. From comparative analyses based on antigenic characterization and nucleotide sequences, it was concluded that all genes of swHK8178 and swHK12782 are derived from human or swine viruses and not avian viruses. From antigenic analyses, it was shown that the HA and NA genes of swHK12682 are derived from human or swine H3N2 viruses. The results showing that swHK12582, swHK12682 and swHK12782 are re-assortants possessing the HA and NA of human-like viruses and the six genes coding for internal proteins derived from classical swine virus were confirmed in two independent laboratories (personal communication from R. G. Webster and K.F.S.).

Although Italian swine isolates were reported by Castrucci *et al.* (1993) to be genetic reassortants between human and avian viruses, the conclusion in their subsequent study (Castrucci *et al.*, 1994) concerning Hong Kong swine viruses and using previously reported sequences (Kida *et al.*, 1988) is not compatible with our results. Conversely, it is of interest to note that in 1992 a total of 73 swine H1N1 influenza viruses isolated in 11 states of the USA were genetically characterized in a detailed study by Wright *et al.* (1992), which demonstrated that all RNA segments of United States swine isolates were of a swine lineage.

With the assumption that the branching and the distances in the tree are correct, we can calculate the date for the putative ancestor nodes from the evolutionary rate. The branch giving rise to the non-avian lineages diverged from the avian lineage containing dkHOK94080, dkHK24577 and dkHOK577 viruses around 1963. In agreement with previous phylogenetic analyses of N2 NA genes (Nerome *et al.*, 1991), a human H3N2 virus might have already been present in some mammalian hosts for several years before its appearance in man in 1968. It may be that an ancestral virus of the early swine lineage was introduced into the swine population around 1967 and evolved independently in this host for about 10 years. Probably, the earliest human lineages originated from an ancestor of the above swine lineage. Based on the evolutionary rate, the swine lineage

was conserved in the swine population between 1967 and 1976–82. The longer the virus circulates in swine, the more it becomes characteristic of a swine lineage through functional constraints on the antigenic and the receptor-binding sites. Furthermore, the estimated evolutionary rates of amino acid-changing substitutions for the swine and human H3 lineages were nearly identical, whereas they can be separated by the degree of functional constraints in the antigenic site and by the branching of the evolutionary tree. In phylogenetic analyses of the NP genes, a large number of viruses with considerable variety of surface protein subtypes seemed to be distributed in different hosts (Gorman *et al.*, 1990), whereas the evolutionary tree constructed from H3HA genes showed predictable branch clusters unique to each host-specific lineage.

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