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Evolution of Influenza A Virus Nucleoprotein Genes: Implications for the Origins of H1N1 Human and Classical Swine Viruses

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A phylogenetic analysis of 52 published and 37 new nucleoprotein (NP) gene sequences addressed the evolution and origin of human and swine influenza A viruses. H1N1 human and classical swine viruses (i.e., those related to Swine/Iowa/15/30) share a single common ancestor, which was estimated to have occurred in 1912 to 1913. From this common ancestor, human and classical swine virus NP genes have evolved at similar rates that are higher than in avian virus NP genes (3.31 to 3.41 versus 1.90 nucleotide changes per year). At the protein level, human virus NPs have evolved twice as fast as classical swine virus NPs (0.66 versus 0.34 amino acid change per year). Despite evidence of frequent interspecies transmission of human and classical swine viruses, our analysis indicates that these viruses have evolved independently since well before the first isolates in the early 1930s. Although our analysis cannot reveal the original host, the ancestor virus was avianlike, showing only five amino acid differences from the root of the avian virus NP lineage. The common pattern of relationship and origin for the NP and other genes of H1N1 human and classical swine viruses suggests that the common ancestor was an avian virus and not a reassortant derived from previous human or swine influenza A viruses. The new avianlike H1N1 swine viruses in Europe may provide a model for the evolution of newly introduced avian viruses into the swine host reservoir. The NPs of these viruses are evolving more rapidly than those of human or classical swine viruses (4.50 nucleotide changes and 0.74 amino acid change per year), and when these rates are applied to pre-1930s human and classical swine virus NPs, the predicted date of a common ancestor is 1918 rather than 1912 to 1913. Thus, our NP phylogeny is consistent with historical records and the proposal that a short time before 1918, a new H1N1 avianlike virus entered human or swine hosts (O. T. Gorman, R. O. Donis, Y. Kawaoka, and R. G. Webster, J. Virol. 64:4893–4902, 1990). This virus provided the ancestors of all known human influenza A virus genes, except for HA, NA, and PB1, which have since been reassorted from avian viruses. We propose that during 1918 a virulent strain of this new avianlike virus caused a severe human influenza pandemic and that the pandemic virus was introduced into North American swine populations, constituting the origin of classical swine virus.

The influenza virus pandemic of 1918 claimed more than 20 million lives (9). Resolving the origin of this virulent virus has been a major focus of virological research for more than 60 years. Antigenic and seroarcheological studies since the 1930s (see, e.g., references 2, 11, 12, 17, 18, and 27) have indicated that the early human and classical swine influenza A H1N1 viruses (i.e., those related to Swine/Iowa/15/30) were very similar. Swine are susceptible to human influenza viruses and vice versa (19, 20), and avianlike H1N1 and H3N2 viruses have been isolated from pigs (23, 38, 44). Scholtissek et al. (39) proposed that swine may provide an efficient "mixing vessel" for the introduction of reassortant viruses into the human population. This hypothesis may explain the appearance of new pandemic viruses in the human population (40).

The nucleoprotein (NP) gene has been chosen for evolutionary studies of influenza A viruses (see, e.g., references 13 and 15) because its purported role as a determinant of host range (39, 45, 47) predicts that NP gene evolution should be host specific. Thus, the NP gene may serve as a model for host-specific evolution of influenza viruses. Using RNA hybrization techniques, Bean (3) showed that NP genes fall into five host-specific groups. This finding was confirmed by showing that NP genes have evolved into five major host-specific lineages that correspond to Bean's RNA hybrization groups (15). The inclusion of both human and classical swine virus NPs into one lineage suggests that they share a common ancestor. The above-mentioned antigenic studies of H1N1 human and classical swine viruses predict the same close relationship for surface proteins, hemagglutinin (HA) and neuraminidase (NA). Evolutionary analyses of other internal protein genes of influenza A viruses also show a close relationship between human and classical swine viruses (M and NS [31], PB1 [22], PA [35], PB2 [16]), and these viruses appear to be evolving away from avianlike ancestors (15, 16). This common pattern of evolution and origin for all virus genes suggests that the common ancestor was an avian virus and not a reassortant derived from previous human or swine influenza A viruses (16).

To date, evolutionary analyses of influenza A virus genes include sequences for only a few classical swine viruses, and human virus gene sequences are heavily biased toward recent virus isolates. Clearly, the lack of representative sequence data for swine viruses precludes an evolutionary analysis of classical swine viruses, and the bias in human

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virus sequence data weakens the accuracy of published evolutionary analyses. For example, Gammelin et al. (13) and Gorman et al. (15) show NP genes of recent classical swine virus isolates as diverging before the common ancestor of human and the earliest classical swine viruses. We believe that this portrayal of two separate lineages of classical swine virus NPs is an artifactual result of not having representative classical swine virus NPs between 1931 and 1977. These deficiencies hamper our understanding of the origin of human influenza viruses and the potential role of swine hosts in that origin.

Our aim has been to determine the evolutionary relationships of human and swine influenza viruses. Specifically, we have addressed the following questions. (i) Do human and classical swine viruses share a single common ancestor? If so, what was the host of this common ancestor and what was the date of divergence into human and swine virus lineages? (ii) How does virus evolution differ in the two host species? Have H1N1 human and classical swine viruses evolved independently; i.e., is there any evidence for a common gene pool for human and swine viruses? (iii) What can the evolution of new avianlike viruses in swine reveal about the early evolution of H1N1 human and classical swine viruses? (iv) What is the relationship between the origin of H1N1 human and classical swine viruses and the 1918 influenza pandemic? To address these questions, we have included 37 additional sequences that address the deficiencies in previous analyses. The present expanded evolutionary analysis of 89 NP genes is intended to complement our previous analysis of 41 NP genes (15).

MATERIALS AND METHODS

We selected 37 influenza A virus isolates (16 swine, 7 human, 13 avian, and 1 equine) from which to clone and sequence NP genes (Table 1). Many of the early swine virus isolates (SWOH35, SW29-37, SWJMS42, SWIA46, SW41-49, SWMAY54, and SWWIS57) were kindly provided by H. F. Maassab and were from the Francis Historical Influenza Collection at the University of Michigan. Remaining isolates were selected to complement 24 NP genes that we previously sequenced (15) and 28 others from literature and databank sources (Table 1).

Molecular cloning, sequencing, and sequence analysis were performed as previously described (15, 16). Briefly, cDNAs of viral NP RNAs were ligated into plasmid vectors and were then transfected into competent *Escherichia coli*. For each virus strain, two to five NP clones were sequenced. Phylogenetic analysis of sequence data was performed with PAUP (Phylogenetic Analysis Using Parsimony) software, version 2.4 (David Swofford, Illinois Natural History Survey, Champaign, Ill.).

RESULTS

Evolutionary tree of NP gene nucleotide sequences. A phylogenetic analysis of 89 influenza A virus NP gene sequences is presented as an evolutionary tree rooted to an aligned influenza B virus NP (Fig. 1A). The general topology of the tree is similar to that of our previous analysis (15), except that now the human and classical swine virus NPs are shown sharing a single common ancestor (upper star in Fig. 1A) and EQPR56 is slightly closer to other NP lineages. The human lineage contains three examples of humanlike viruses isolated from swine: SWCAM35, SWHK76, SWDAN83. It is notable that SWCAM35 is not a classical swine virus and is most closely related to WS33, the first human virus isolate. The isolates SWHK76 and SWDAN83 represent humanlike swine viruses from China. Within the classical swine virus NP lineage are two examples of swinelike viruses isolated from humans, NJ76 and WIS88. These two viruses are independently derived and are closely related to contemporary strains isolated from pigs (SWTN77 and SWIA88, respectively). This pattern emphasizes the susceptibility of swine to human viruses and vice versa, but these interspecies transmissions appear to represent evolutionary dead ends since there is no evidence that they circulate extensively or leave descendant viruses.

The classical swine NP lineage contains a subgroup of isolates that have not evolved significantly for 19 years relative to the original SWIA30 isolate (SWJMS42, SWIA46, and SW41-49). The lack of accompanying data for these three isolates precludes any explanation for this pattern. However, the remaining isolates fall in the expected chronological order within the lineage. The only Asian isolate represented in the H1N1 classic swine virus lineage, SWHK82, appears to be a reassortant containing avian H3N2 surface proteins (23). The classical swine virus NP of this isolate is related to North American strains in the mid-1970s. This agrees with the appearance of classical swine viruses antigenicly similar to North American strains in the late 1970s in Southeast Asia (43) and Japan (34, 51).

A distinct sublineage of Italian swine virus isolates (SWIT76, SWIT79, SWIT41-81, and SWIT47-81) supports the reported introduction of classical swine viruses into Italy in 1976 (32). However, antigenic analyses of Italian swine virus isolates (12a) indicates that classical swine viruses disappeared from Italy after 1986 and have been replaced by avianlike H1N1 swine viruses. These new avianlike swine viruses first appeared in Europe in 1979 (38), but antigenic analyses suggest that they began to circulate in Italy in 1985 (12a). The timing of the appearance of the new avianlike H1N1 viruses in Italy (1985) and the disappearance of classical swine viruses (1986) suggests that the new swine virus in some way hastened the extinction of classical swine viruses in Italy.

Within the avian NP lineage, only one Asian swine isolate is represented (SWHK2-82) and is closely related to a H3N2 Asian duck isolate (DKHK75). Kida et al. (23) provide additional examples of H3N2 avianlike viruses isolated from pigs in southern China. Antigenic studies of swine virus isolates from southern China suggest that pigs are commonly infected with H3N2 human and avian viruses (43). However, there are no data to indicate whether these H3N2 avian viruses circulate and evolve in the Asian swine host reservoir. In contrast, the new avianlike European swine viruses form a distinct sublineage within the avian lineage (SWGER81, SWNED85, and SWIT89 [Fig. 1A]). The formation of a swine-specific lineage indicates that these new swine viruses are circulating in the swine host reservoir and have begun to evolve separately from their avian ancestor.

Other examples of avianlike swine viruses isolated from mammals include those isolated from whales (WHALEM84 and WHALEP76), seals (SEAL80), and mink (MINKSW84) (Fig. 1). In every case these isolates appear to be independently derived from avian viruses. The inability to isolate descendant strains indicates that transmissions of avian viruses to marine mammals represent evolutionary dead ends (25).

Evolutionary tree of amino acid sequences. To evaluate the effect of gene evolution on NP proteins, nucleotide sequences were translated and a phylogenetic tree of amino

TABLE 1. Influenza virus strains used in phylogenetic analyses

Strain	Abbreviation	GenBank accession no.	Reference
Equine strains			
A/Equine//Prague/1/56 (H7N7)	EQPR56	M63748	This report
A/Equine/Miami/63 (H3N8)	EQMI63	M22575	14
A/Equine/London/1416/73 (H7N7)	EQLON73	M30750	15
A/Equine/Kentucky/2/86 (H3N8)	EQKY86	M30751	15
A/Equine/Tennessee/5/86 (H3N8)	EQTN86	M30758	15
Human strains			
B/Lee/40	BLEE40	K01395	4
A/Wilson-Smith/33 (H1N1)	WS33	M30746	15
A/Puerto Rico/8/34 (H1N1)	PR834	NA[a]	50
A/Puerto Rico/8/34 (H1N1) (Cambridge)	PR8CAM34	J02147	48
A/Hickox/40 (H1N1)	HICKOX40	M63749	This report
A/Ft. Monmouth/47 (H1N1)	FM47	M63750	This report
A/Ft. Warren/50 (H1N1)	WARREN50	NA	1
A/England/19/55 (H1N1)	ENG55	M63751	This report
A/Singapore/1/57 (H2N2)	SING57	M63752	This report
A/Ann Arbor/6/60 (H2N2)	AARBOR60	M23976	8
A/Victoria/5/68 (H2N2)	VIC68	M63753	This report
A/NT/60/68 (H3N2)	NT60-68	J02137	21
A/Udorn/307/72 (H3N2)	UDORN72	M14922	5
A/USSR/77 (H1N1)	USSR77	NA	1
A/Brazil/78 (H1N1)	BRAZIL78	NA	1
A/Texas/1/77 (H3N2)	TEXAS77	NA	1
A/California/10/78 (H1N1)	CALIF78	NA	1
A/Hong Kong/5/83 (H3N2)	HK83	M22577	14
Human strains, swinelike viruses			
A/New Jersey/8/76 (H1N1) (Ft. Dix)	NJ76	M63754	This report
A/Wisconsin/3523/88 (H1N1)	WIS88	M63755	This report
Swine strains, classical viruses			
A/Swine/Iowa/15/30 (H1N1)	SWIA30	M30747	15
A/Swine/1976/31 (H1N1)	SW31	M22578	14
A/Swine/Ohio/23/35 (H1N1)	SWOH35	M63756	This report
A/Swine/29/37 (H1N1)	SW29-37	M63757	This report
A/Swine/Jamesburg/42 (H1N1)	SWJMS42	M63758	This report
A/Swine/Iowa/46 (H1N1)	SWIA46	M63759	This report
A/Swine/41/49 (H1N1)	SW41-49	M63760	This report
A/Swine/May/54 (H1N1)	SWMAY54	M63761	This report
A/Swine/Wisconsin/1/57 (H1N1)	SWWIS57	M63762	This report
A/Swine/Wisconsin/1/61 (H1N1)	SWWIS61	M63763	This report
A/Swine/Italy/437/76 (H1N1)	SWIT76	M63764	This report
A/Swine/Tennessee/24/77 (H1N1)	SWTN77	M30748	15
A/Swine/Italy/2/79 (H1N1)	SWIT79	M63765	This report
A/Swine/Italy/141/81 (H1N1)	SWIT141-81	M63766	This report
A/Swine/Italy/147/81 (H1N1)	SWIT147-81	NA	13
A/Swine/Ontario/2/81 (H1N1)	SWONT81	M63767	This report
A/Swine/Hong Kong/127/82 (H3N2)	SWHK82	M22570	14
A/Swine/Iowa/17672/88 (H1N1)	SWIA88	M63768	This report
Swine strains, humanlike viruses			
A/Swine/Cambridge/1/35 (H1N1)	SWCAM35	M63769	This report
A/Swine/Hong Kong/6/76 (H3N2)	SWHK76	M22571	14
A/Swine/Dandong/9/83 (H3N2)	SWDAN83	M63770	This report
Swine strains, avianlike viruses			
A/Swine/Hong Kong/126/82 (H3N2)	SWHK2-82	M63771	This report
A/Swine/Germany/2/81 (H1N1)	SWGER81	M22579	14
A/Swine/Netherlands/12/85 (H1N1)	SWNED85	M30749	15
A/Swine/Italy/839/89 (H1N1)	SWIT89	M63772	This report
Miscellaneous mammalian strains, avianlike viruses			
A/Whale/Maine/328/84 (H13N2)	WHALEM84	M30759	15
A/Seal/Massachusetts/1/80 (H7N7)	SEAL80	M27518	25
A/Whale/Pacific Ocean/76 (H1N3)	WHALEP76	M27517	25
A/Mink/Sweden/84 (H10N4)	MINKSW84	M24454	36

Continued on following page

TABLE 1—*Continued*

Strain	Abbreviation	GenBank accession no.	Reference
H13 gull virus strains			
A/Gull/Maryland/704/77 (H13N6)	GULMD77	M30754	15
A/Gull/Maryland/1824/78 (H13N9)	GULMD78	M30755	15
A/Gull/Maryland/1815/79 (H13N6)	GULMD79	M30756	15
A/Gull/Minnesota/945/80 (H13N6)	GULMN80	M30757	15
A/Gull/Massachusetts/26/80 (H13N6)	GULMA80	M30752	15
A/Gull/Astrakhan/227/84 (H13N6)[b]	GULAST84	M30753	15
Avian strains			
A/Duck/New Zealand/31/76 (H4N6)	DKNZ76	M30760	15
A/Grey Teal/Australia/2/79 (H4N4)	GTAUS79	M30761	15
A/Duck/Manitoba/1/53 (H10N7)	DKMAN53	M63773	This report
A/Turkey/Ontario/7732/66 (H5N9)	TYONT66	M63774	This report
A/Duck/Pennsylvania/1/69 (H6N1)	DKPEN69	M63775	This report
A/Duck/Memphis/928/74 (H3N8)	DKMEM74	M63776	This report
A/Gull/Maryland/5/77 (H11N9)	GULM5-77	M63777	This report
A/Mallard/NY/6750/78 (H2N2)	MLRDNY78	M14921	5
A/Turkey/Minnesota/833/80 (H4N2)	TYMN80	M30769	15
A/Turkey/Minnesota/1661/81 (H1N1)	TYMN81	M63778	This report
A/Chicken/Pennsylvania/1/83 (H5N2)	CKPENN83	M30768	15
A/Ruddy Turnstone/NJ/47/85 (H4N6)	RTNJ85	M30766	15
A/FPV/Dobson/"Dutch"/27 (H7N7)	FPV27	M63779	This report
A/FPV/Rostock/34 (Giessen) (H7N1)	FPV34	M24456	26
A/Chicken/Germany/"N"/49 (H10N7)	CKGER49	M24453	36
A/Duck/Czechoslovakia/56 (H4N6)	DKCZ56	M30762	15
A/Duck/England/1/56 (H11N6)	DKENG56	M63780	This report
A/Duck/Ukraine/2/60 (H11N8)	DKUK60	M30763	15
A/Tern/South Africa/61 (H5N3)	TERNSA61	M30767	15
A/Duck/England/1/62 (H4N6)	DKENG62	M63781	This report
A/Shearwater/Australia/72 (H6N5)	SHWTR72	M27298	13
A/Parrot/Ulster/73 (H7N1)	PARU73	M22344	46
A/Duck/Hong Kong/7/75 (H3N2)	DKHK75	M22573	14
A/Duck/Bavaria/2/77 (H1N1)	DKBAV77	M22574	14
A/Budgerigar/Hokkaido/1/77 (H4N6)	BUDHOK77	M30765	15
A/Duck/Beijing/1/78 (H3N6)	DKBEI78	M63782	This report
A/Duck/Australia/749/80 (H1N1)	DKAUS80	M63783	This report
A/Teal/Iceland/29/80 (H7N7)	TEALIC80	M63784	This report
A/Mallard/Astrakhan/244/82 (H14N6)[b]	MAST82	M30764	15
A/Mallard/Astrakhan/263/82 (H14N5)[b]	MAST2-82	M63785	This report

[a] NA, not applicable.
[b] Astrakhan locality is synonymous with Gurjev.

acid sequences was constructed based on the branching topology of the nucleotide tree (Fig. 1B). This approach permits direct comparison of the corresponding branches in nucleotide and amino acid trees for differences in genetic versus protein evolution (15). In essence, the amino acid tree represents a phylogeny based on nonsilent changes; the collapse of internal branches in the avian lineage indicates that nearly all the homologous internal branches in the nucleotide tree are composed of silent changes. A comparison of human and classical swine virus sublineages reveals that at the nucleotide level they have evolved roughly the same amount from their common ancestor (i.e., the two branches are similar in length [Fig. 1A]). However, at the amino acid level the classic swine virus NPs are evolving much more slowly (Fig. 1B), which suggests that swine viruses are subjected to less selective pressure than are human viruses.

The avian lineage is characterized by evolutionary stasis of NPs (Fig. 1B) as noted previously (15). SWHK2-82, an Asian avianlike swine virus isolate, is very similar to an Asian duck virus isolate, DKHK75 (three amino acid differences [Fig. 1B]). In contrast to other NPs of the avian lineage, those from avianlike European swine virus isolates (SWGER81, SWNED85, and SWIT89) form a distinct sublineage that shows a progressive accumulation of amino acid changes consistent with dates of isolation.

Comparison of amino acid sequences. To detect patterns of derived (synapomorphic) amino acid changes for each lineage, we compared NP sequences with baseline sequences that are closest to the root of the phylogenetic tree (a table of aligned sequences is available upon request). The DKBAV77, MAST82, and MAST2-82 isolates are one amino acid change from the root node of the avian lineage, which represents the hypothetical ancestor for all avian NPs (Fig. 1B, lower star). This analysis permits identification of the specific amino acid changes that have occurred in the evolution of host-specific NPs and permits reconstruction of sequences for hypothetical ancestral NPs (Fig. 2). Two possible sequences for hypothetical ancestral avian NPs (Fig. 2, AVIAN 1 and AVIAN 2) differ at amino acid positions 105 and 450. These amino acid differences distinguish the common ancestors for H13 gull and North American avian from Old World avian groups. The common ancestor of human and classical swine NP lineages (Fig. 2, HUM-SWINE) is characterized by five

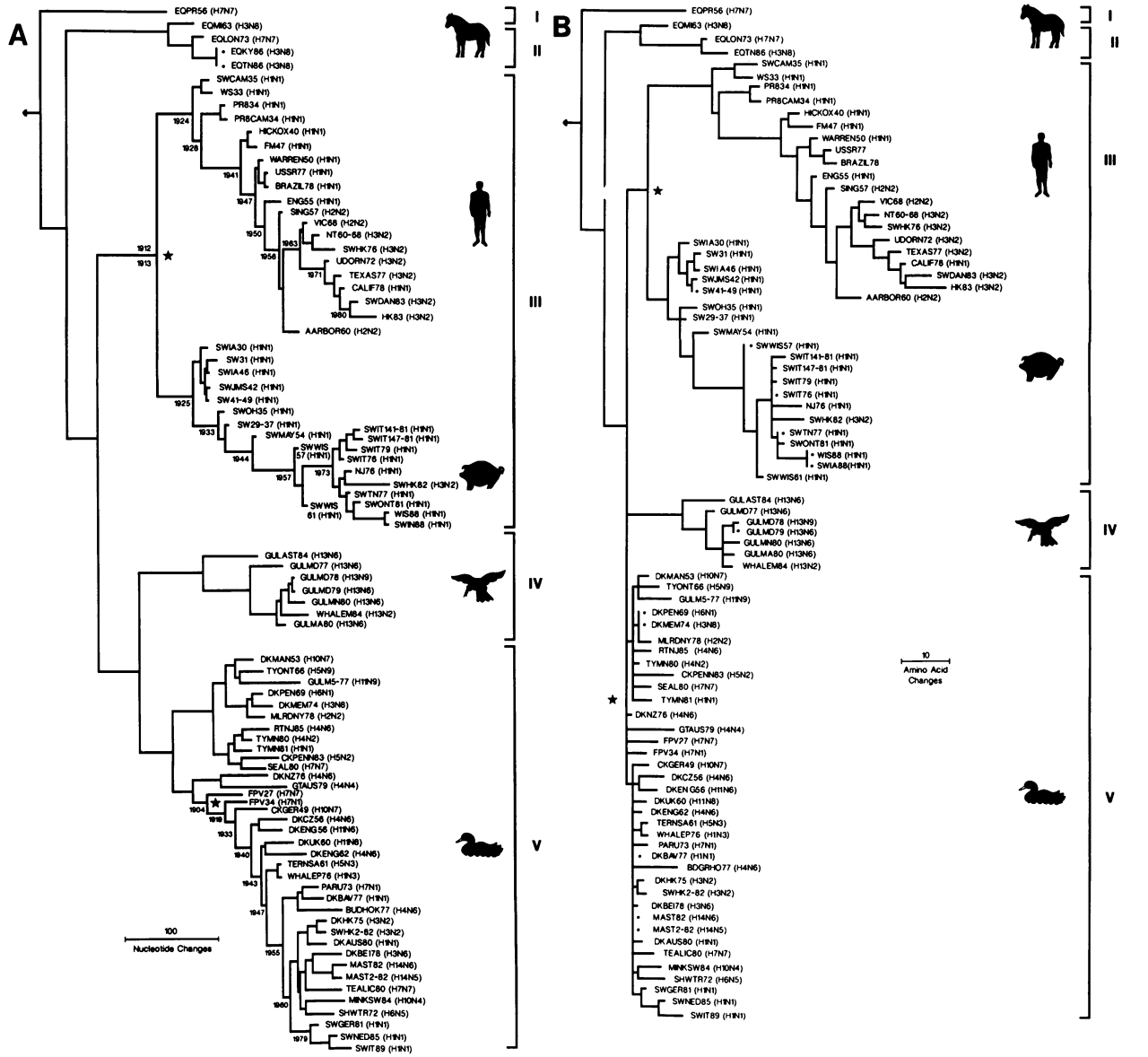


FIG. 1. (A) Phylogenetic tree of 89 influenza A virus NP gene nucleotide sequences rooted to the NP of B/Lee/40 (37 sequences from this study plus 52 published sequences). Sequences were analyzed with PAUP software, which uses a maximum parsimony algorithm to find the shortest trees (MULPARS, SWAP=GLOBAL, and HOLD=10 options). The arrow indicates the direction of the B/Lee/40 NP from the root node. Horizontal distance is proportional to the minimum number of nucleotide differences to join nodes and NP sequences. Vertical lines are for spacing branches and labels. Roman numerals indicate Bean's (3) RNA NP hybridization groups: I, Equine/Prague/56; II, recent equine; III, human and classical swine; IV, H13 gull; V, avian. Animal symbols indicate host specificities of the lineages. Dates for hypothetical ancestor nodes were derived by dividing branch distance by evolutionary rate estimates (see Fig. 4). Stars indicate the hypothetical ancestors for the human-swine NP lineage (upper) and avian NP lineage (lower). (B) Phylogenetic tree of influenza A virus NP amino acid sequences. The amino acid phylogeny conforms to the topology of the nucleotide tree (panel A). Sequences represented in these trees are listed in Table 1.

amino acid changes relative to either of the ancestral avian NPs (positions 33, 136, 351, 425, and 473). Two of these changes (positions 351 and 473) occur as unique convergent characters in some avian NPs (GULM5-77 and FPV34 [see Fig. 3 in reference 15]). This suggests that the three changes at positions 33, 136, and 425 are unique in the evolution of the human-swine NPs. Furthermore, the small number of unique amino acid changes emphasizes the avian character of the human-swine NP ancestor.

Specific amino acid changes that are synapomorphic for post-1930 human and classical swine NPs can be identified. For the classical swine NP lineage these include five amino acid changes at positions 100, 105, 289, 350, and 447 (Fig. 2, C-SWINE). For the human NP lineage, 13 changes occur at positions 16, 31, 61, 100, 127, 253, 283, 313, 357, 375, 408, 421, and 472 (Fig. 2, HUMAN). Recent human and classical swine virus NPs (HK83 and SWIA88) show an accumulation of 34 and 14 additional amino acid changes, respectively.

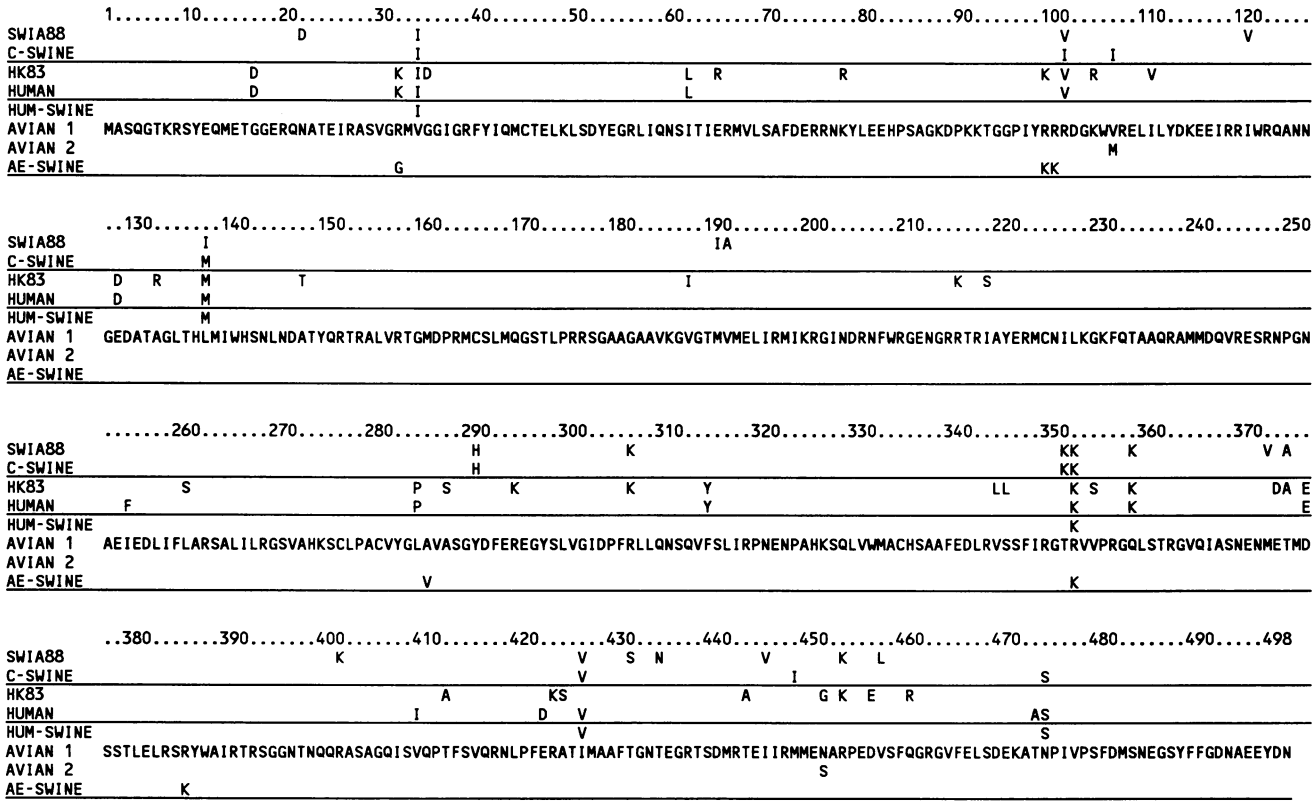


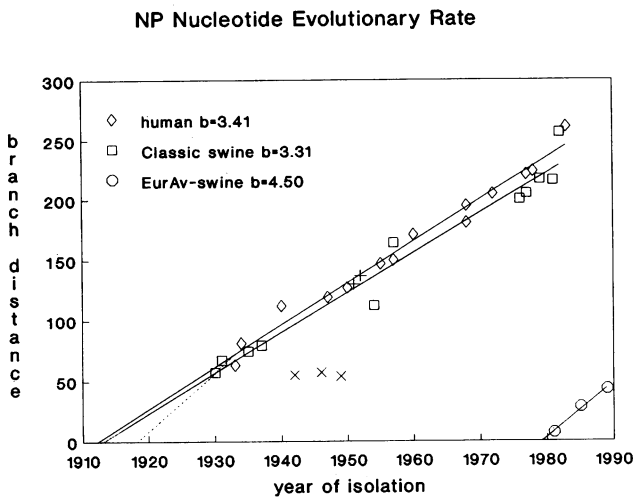
FIG. 2. Predicted amino acid sequences of hypothetical ancestor NPs. The ancestor sequences show the synapomorphic characters that define each lineage relative to the avian ancestral root NP (lower star in Fig. 1B) and are derived from analysis of amino acid sequences grouped by phylogenetic relationship (Fig. 1; see text). AVIAN 1 and AVIAN 2 represent one of two possible sequences for the avian ancestral root NP and differ by two amino acids (positions 105 and 450). Amino acid differences at these positions distinguish H13 gull and North American avian (AVIAN 1) from Old World avian (AVIAN 2) groups. AVIAN 2 is equivalent to baseline Old World avian NP sequences DKBAV77, MAST82, and MAST2-82 (Fig. 1B). Other ancestor NP sequences shown are human-swine lineage (HUM-SWINE; indicated by the upper star in Fig. 1), ancestor of classical swine NPs (C-SWINE; 1925 node in Fig. 1), a recent classical swine virus NP (SWIA88), the ancestor of human NPs (HUMAN; 1924 node in Fig. 1), and a recent human virus NP (HK83). The sequence shown for the European avianlike swine virus NPs (AE-SWINE) represents a consensus sequence of characters that are shared in at least two isolates. The only character that is shared by all AE-SWINE isolates and distinguishes them from the ancestral avian baseline is a valine at position 284.

Recent H1N1 avianlike swine viruses can be compared for parallel patterns of evolution with early classical swine virus NPs. The common ancestor of the new H1N1 avianlike swine NP lineage shows two changes relative to the avian baseline at positions 31 and 284; by 1985, four additional changes had appeared, at positions 98, 99, 351, and 384 (Fig. 2, AE-SWINE); and the 1989 isolate SWIT89 shows four more changes at positions 49, 323, 377, and 497 (not shown). With the exception of the lysine at position 351, none of these amino acids are found in classical swine or human virus NPs. The lysine at position 351 appears to be an example of convergence with human and classical swine NPs.

Evolutionary rate analysis. A regression of branch distances of NP isolates from common ancestor nodes permits estimation of evolutionary rates for the human lineage and, for the first time, the classical swine virus lineage (Fig. 3). At the nucleotide level, human and swine virus NP genes are evolving at nearly identical rates (3.31 to 3.41 nucleotide changes per year [Fig. 3]), but at the protein level the swine virus NPs are evolving at half the rate of human virus NPs (0.66 versus 0.34 amino acid change per year [Fig. 3]). The per-nucleotide annual rate of change is 2.12×10^{-3} to 2.18×10^{-3} change per year for swine and human virus NPs, respectively. These values are higher than our previous estimate of 1.62×10^{-3} change per year for the human NP lineage (15), which was based on fewer human virus NPs (6 versus 20 isolates), but are very close to the 2.2×10^{-3} change per year estimated for human virus NPs (1) and are similar to the estimate of 2.0×10^{-3} change per year for the NS gene (6). Our estimate of evolutionary rate for Old World avian NPs (1.90 changes per gene per year, 1.21×10^{-3} change per nucleotide per year [Fig. 4]) is lower than previously reported (2.17 changes per gene per year; 1.39×10^{-3} change per nucleotide per year) (15). The new estimate may be regarded as more accurate since it is based on a larger sample of avian NPs (22 versus 13 NPs).

NPs of reemergent H1N1 human viruses (USSR77 and BRAZIL78) share a common ancestor with the WARREN50 isolate as previously shown (1) or predicted (29, 41). Assuming a 1950 origin, we treated these reemergent isolates as appearing in 1951 (USSR77) and 1952 (BRAZIL78). The close position of these isolates to the regression line supports the interpretation that they had not been in circulation for 27 years.

Dates for common-ancestor nodes can be derived from estimates of evolutionary rates for each lineage. Independent estimates from the human and classical swine virus



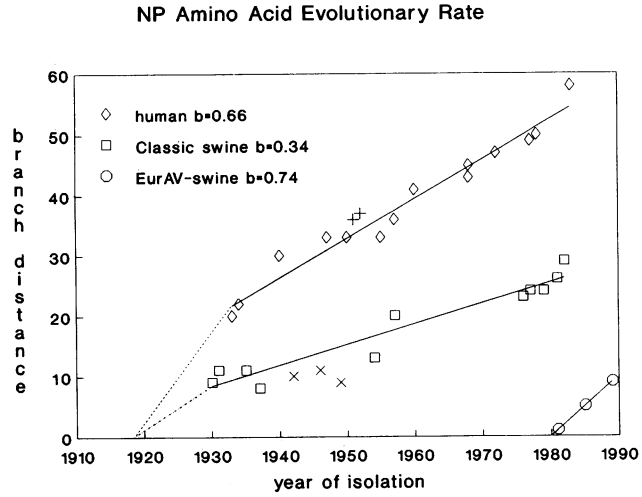
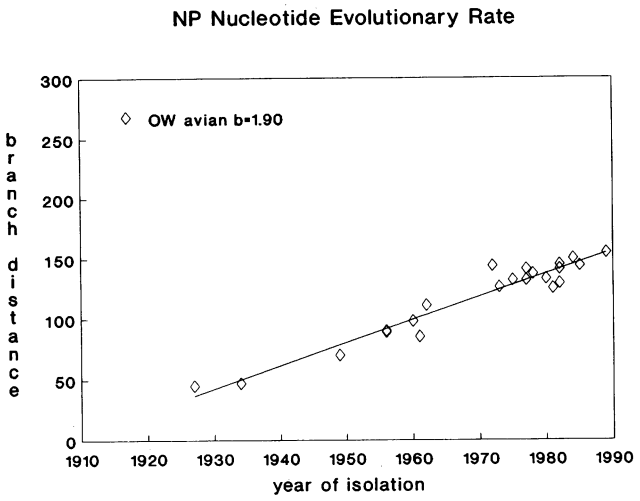


FIG. 3. Evolutionary rates for NP genes and proteins for human and swine virus isolates. The evolutionary rate is estimated by regression of the year of isolation against the branch distance from the common ancestor node of the nucleotide and amino acid phylogenetic trees (Fig. 1). Regression statistic *b* provides rate estimates. Shown are regressions for NPs of human viruses, classical swine viruses, and European avianlike swine viruses. The reemergent H1N1 human virus isolates (USSR77 and BRAZIL78, indicated by +) are not included in the regression but are shown for reference (they are treated as appearing 27 years earlier). Three classical swine virus isolates (SWIA46, SWJMS42, and SW41-49, indicated by ×) that show no evolution are not included in the regression but are shown for reference. Nucleotide regressions have been extrapolated beyond the first isolates and show estimated dates of origin for human (1912), classical swine (1913), and European avianlike swine (1979) virus lineages. Similar treatment of amino acid regressions yields earlier estimates for human (1900) and classical swine (1905) virus lineages (not shown). Dotted lines show average estimated evolutionary rates of human and classical swine NPs if 1918 is assumed to be the date of origin.



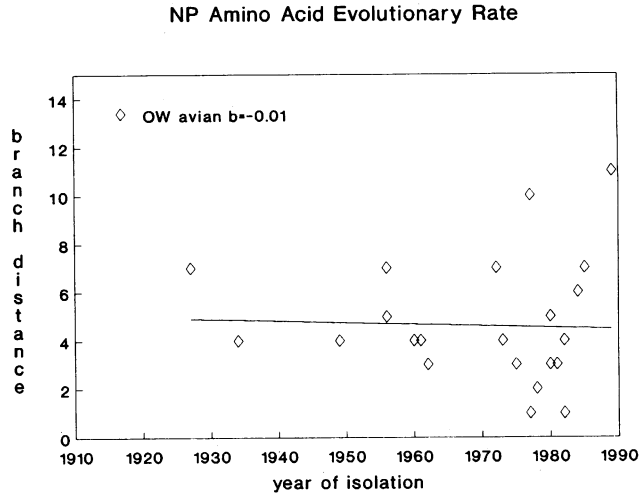


FIG. 4. Evolutionary rates for NP genes and proteins for avian virus isolates. The evolutionary rate is estimated by regression of the year of isolation against the branch distance from the common ancestor node of the nucleotide and amino acid phylogenetic trees (Fig. 1). Regression statistic *b* provides rate estimates. Extrapolation of the nucleotide regression estimates 1904 as the date of the ancestor of Old World avian virus NPs related to FPV27.

lineages agree on 1912 to 1913 as the date of the common ancestor (Fig. 1A and 3). This common ancestor is relatively close to the hypothetical ancestor of the avian lineages (five amino acid changes) and is well within the range of distances for all avian NPs (Fig. 1B). The date for the hypothetical ancestor of the Old World avian lineage is estimated at 1904 (Fig. 1A).

Evolutionary rate analysis of the avianlike swine virus NPs indicates that they are evolving at a higher rate than classical swine virus NPs (Fig. 3): 4.5 versus 3.31 changes per year at the nucleotide level, and 0.74 versus 0.34 change per year at the amino acid level. We estimate 1979 as the date for the common ancestor of these avianlike swine virus NPs, which matches the date when the first avianlike swine virus was isolated in Europe (38). The new lineage shows relatively rapid, divergent evolution away from an avianlike ancestor within the avian lineage (Fig. 1).

DISCUSSION

Common ancestry for human and classical swine viruses. Our evolutionary analysis of influenza A virus NP nucleotide sequences shows that human and classical swine viruses

(i.e., those related to Swine/Iowa/15/30) share a single common ancestor and that this ancestor is estimated to have emerged in 1912 to 1913. Moreover, our analysis shows that NP genes in human and swine virus lineages have evolved at similar rates that are higher than for avian virus NP genes (Fig. 1A, 3, and 4). It is not possible from this analysis to determine the host in which the virus first appeared. If there had been some asymmetry in the lineages, e.g., early swine virus NPs forming a sister group to NPs of later human and classical swine viruses, this would have provided some evidence that pigs were the original host. Instead, the two nucleotide lineages are highly symmetrical and estimate virtually the same date for a common ancestor.

Gammelin et al. (13) have proposed that the common ancestor for avian and human virus NPs may have existed as far back as 1837. In their analysis, human virus NP amino acid evolutionary rates over the past 50 years were extrapolated back 150 years to the common ancestor with the avian virus NP, DKBAV77. Such lengthy extrapolations may be misleading (15, 16). The disparity in our estimates arises because (i) our estimates are based on nucleotide phylogenies and (ii) we attempt to estimate only the date of the immediate common ancestor for human and classical swine virus NPs and then evaluate the relationship of that ancestor to those of avian virus NPs. We believe that more accurate rates of evolution and dates of common ancestors can be estimated from nucleotide phylogenies. This is because protein evolution is dependent on nucleotide evolution and nucleotide sequences contain much more information to resolve ambiguous relationships, particularly when a high proportion of silent mutations occurs (e.g., in avian virus lineages). Moreover, because selection acts directly on the proteins (the phenotype) whereas the genetic code is degenerate, amino acid evolutionary rates may be less constant over time, particularly after a virus is introduced into a new host. During this early adaptation phase, selection pressure on proteins and their evolutionary rates are expected to be relatively high. Thus, linear extrapolation of long-term amino acid evolutionary rates is expected to provide earlier estimates of dates of common ancestors. For example, on the basis of amino acid evolutionary rates in our analysis, the common ancestor of human and classical swine virus NPs is estimated to have appeared in 1900 or 1905, 7 to 13 years earlier than estimates based on nucleotide evolutionary rates (Fig. 3).

The time frame given by Gammelin et al. (13) for NP evolution suggests that the virus may have been in humans for 75 years before our estimated divergence of human and classical swine viruses in 1912 to 1913. Our analysis does not support a long history of NP evolution in mammalian hosts prior to 1912. The internal branch that connects the human and swine NP amino acid lineages to the avian root (between the upper and lower stars in Fig. 1B) represents the changes in the common ancestor that are shared by human and swine virus NP lineages. The closeness of this common ancestor to the avian root (five amino acid changes, only three of which are not shared with any avian NP [Fig. 2]) suggests that this ancestor NP had recently been acquired from an avian virus. Comparison of the homologous internal branches in nucleotide and amino acid trees (Fig. 1) shows that the bulk of the nucleotide changes are silent coding changes (coding-to-noncoding ratio, 1:10.6). In this respect, the human-swine NP common ancestor is avianlike; i.e., evolutionary stasis of the protein limits coding changes at the nucleotide level (15, 16). This parallel suggests that the majority of the nucleotide changes in this common ancestor gene were inherited from an avian ancestor.

Differences in protein evolution between human and swine virus NPs are evident in the earliest virus isolates and represent host-specific signatures of virus evolution. After divergence of human and swine NP lineages, the ratio of coding to noncoding changes in the human virus NP increased and remained stable at 1:4.17 (1933 to 1983 mean; ratio of amino acid to nucleotide evolutionary rate regression slopes − 1 [Fig. 3]), which indicates a shift in gene evolution relative to the avianlike common ancestor (Fig. 1). Unlike evolutionary stasis among avian NPs, the human virus NP has undergone relatively rapid, divergent protein evolution which has not abated over the 50 or more years human viruses have been isolated. In comparison, evolution in the swine lineage remains more avianlike; i.e., the swine virus NP protein has evolved more slowly away from its avian ancestor, as indicated by a smaller ratio of coding to noncoding changes (1930 to 1988 mean, 1:8.74 [Fig. 4]).

Divergent evolution in human and classical swine viruses. The common ancestor for human and classical swine virus NPs marks the point where the evolution of the two viruses diverged. That divergence reflects a split in the virus gene pool into human and swine host reservoirs and shows that the viruses have evolved independently in each reservoir. It is apparent that regular interspecies transmissions of human and swine viruses (see, e.g., references 7, 10, 19, and 37) and examples from this report (i.e., NJ76, WIS88, SWCAM35, SWHK76, and SWDAN83) have not affected virus evolution in the two host species. Thus, the continued divergent evolution of the viruses in the two host reservoirs suggests that there are as yet unidentified factors that allow only certain viruses to persist, circulate, and evolve in each host species. The closeness of the human-swine common ancestor to avian NP proteins, the divergence and rapid parallel evolution of NP genes in human and swine viruses, and the different signatures of host-specific protein evolution in human and swine viruses suggest that the avianlike ancestor could have circulated for only a short time in one of the two host reservoirs before entering the other.

Evolutionary divergence in human and classical swine viruses is probably related to differences in immune protection as it is related to host population age structure. Human populations are characterized by older individuals (>10 years) with extensive immunological experience, whereas in swine populations young, immunologically naive individuals predominate. As a result of these differences, a newly established virus would be subjected to strong immune selection pressure in human populations which would result in rapid evolution of virus antigens; the reverse would be expected for a virus in swine populations. Following establishment of the same new virus in human and swine populations, only a small fraction of humans would continue to be susceptible to the swine virus strains, and such cases would not lead to epidemics. On the other hand, pigs could continue to be infected with human virus strains, but it is apparent that human-to-swine virus transmissions in North America over the past 60 years have not resulted in new persistent swine virus strains or lineages (Fig. 1). This evolutionary model is appropriate for the HA and NA surface proteins of influenza viruses because they are the principal targets of neutralizing antibodies (28). However, because the evolution of the internal NP protein is concordant with this model, it is possible that selection pressure via T-cell immune response has affected the evolution of NP genes. In contrast to virus protein evolution, host demo-

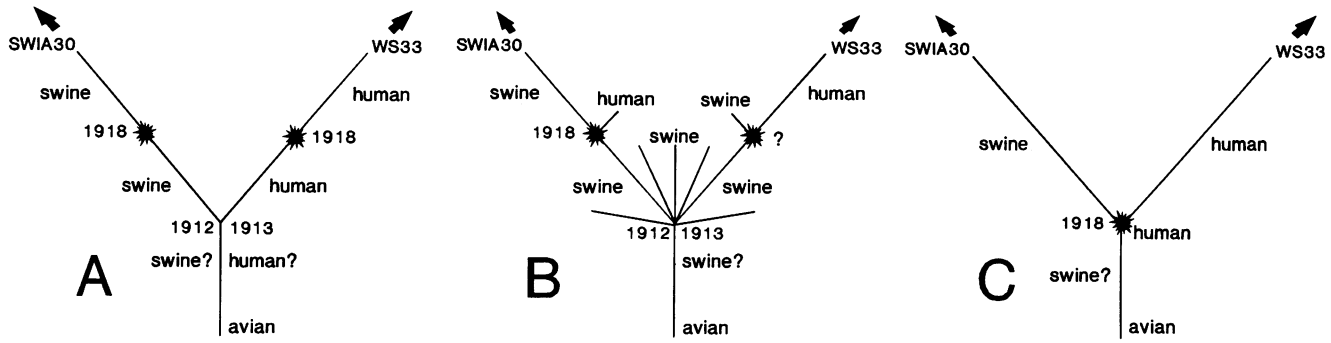


FIG. 5. Hypothetical phylogenies for early human and classical swine viruses adapted from Fig. 1. Names of earliest isolates identify lineages for classical swine (SWIA30) and human (WS33) viruses. All phylogenies show the common ancestor as being recently derived from an avian virus. (A) Divergence of human and classical swine viruses from a 1912 to 1913 common ancestor. To account for historical records, coincident pandemics and epizootics had to arise independently from virulent strains in 1918, which is not likely. Also, this phylogeny requires a new human virus to circulate and evolve for 6 years (1912 to 1918) without causing pandemics. (B) Divergence of multiple lineages of swine viruses from a 1912 to 1913 common ancestor. Of the two surviving lineages, the one that gives rise to classical swine viruses also caused the human influenza pandemic of 1918, but present-day human viruses are shown as being derived from a sister lineage of swine viruses. This model requires multiple strains of swine viruses to circulate and evolve independently for 6 years or more after 1912. This pattern of early evolution has not yet been demonstrated for recent avianlike swine viruses. The proposed post-1918 origin of human viruses from a sister swine virus strain would require higher pre-1933 evolutionary rates for human virus genes than is predicted by assuming a 1918 origin (Fig. 3). (C) Human and swine viruses diverging from a common ancestor in 1918. In this model the classical swine viruses are derived from the 1918 human pandemic virus. The pre-1918 host is unknown but could have been swine. This phylogeny requires higher evolutionary rates for human and classical swine virus over the period from 1918 to the early 1930s. However, these estimated rates are similar to those for the new avian-like H1N1 swine viruses.

graphic differences appear to have had little impact on the evolution of NP genes; the similar nucleotide evolutionary rates observed in the two host populations suggest that the viruses may have similar replication (and mutation) rates.

An important characteristic of classical swine virus is that it has circulated and evolved in North American swine herds for more than 60 years. However, when classical swine viruses have been introduced into Europe or Asia, they usually have not persisted for long periods. The reasons for this pattern are not understood. The association of classical swine viruses with North America suggests a unique regional ecology which is probably related to differences in swine husbandry practices in North America, Europe, and Asia.

A 1918 origin for H1N1 human and classical swine viruses? The estimated date of 1912 to 1913 for the common ancestor of human and swine viruses (Fig. 5A) does not agree with results of seroarcheological studies that suggest that 1918 marked the appearance of a new H1N1 virus in the human population (see, e.g., reference 27), nor with 1918 historical records of a severe pandemic of human influenza and epizootics of swine virus in North America (9). An alternative explanation might be that a number of virus lineages may have diverged around 1912 to 1913 and the only surviving lineages are those that now represent swine and human hosts (e.g., Fig. 5B). A major assumption in estimating the date of the common ancestor is that evolutionary rates within a lineage remain constant from its origin. If 1918 is accepted as the date for the divergence of the human and swine virus NP lineage (Fig. 5C), evolutionary rates from 1918 to the early 1930s were significantly higher than after that period. Our evolutionary analysis suggests that human and classical swine viruses originated from a common event and that the ancestor virus had only a short prior existence in human or swine host reservoirs.

Model for evolution of early human and swine viruses. Evolution of recent avianlike H1N1 swine viruses in Europe may serve as a model for evolution of early human and classical swine viruses. The NP genes of these avianlike viruses have been evolving in the European swine host reservoir for some 10 years (1979 to 1989) at a higher rate than is observed for human or classical swine virus NPs (4.50 versus 3.41 and 3.31 nucleotide changes per year, respectively [Fig. 3]). Higher nucleotide evolutionary rates may be typical for an avian virus recently introduced into the swine host reservoir. If the evolutionary rate of the new avianlike swine virus NPs is applied to the human and classical swine virus lineages for the pre-1930s period, the estimated date of the common ancestor is 1918 to 1919. Conversely, if 1918 is assumed as the date of the common ancestor, the predicted initial evolutionary rate is 4.7 nucleotide changes per year for human NPs (between 1918 and 1933) and 4.75 changes per year for classical swine NPs (between 1918 and 1930). With this modification, the predicted amino acid evolutionary rate for 1918 to 1930 classical swine virus NPs becomes more similar to that of the recent avianlike swine virus NPs (0.9 versus 0.74 changes per year) than to that of the pre-1930s human NPs (1.35 changes per year). The much higher predicted evolutionary rate for early human virus NP proteins than for classical swine viruses mirrors the pattern for post-1930s human and classical swine viruses (Fig. 3). The parallel between pre-1930 classical swine virus NPs and the new avianlike swine virus NPs suggests that the SWIA30 isolate is nearly as avianlike as the recent SWIT89 isolate; from the root of the avian lineage, SWIA30 has diverged by 14 amino acid changes in 12 to 17 years and SWIT89 has accumulated 11 changes in 10 years. The unusually close agreement in the predicted evolution of these human and swine virus NPs makes a post-1912 date of origin for human and classical swine viruses plausible.

An unusual aspect of evolution in the new avianlike swine virus NPs is that the rate of amino acid evolution is twice as high as that in classical swine viruses while nucleotide

evolution is only 30 to 40% higher (Fig. 3). The disproportionally higher rate of NP protein evolution in the new avianlike swine viruses compared with the classical swine viruses suggests that they are under stronger selective pressure, which, as discussed above, is expected for new viruses. The same situation would apply to pre-1930 classical swine viruses if their date of origin is assumed to be 1918 (Fig. 3).

A proposal consistent with our evolutionary analysis and historical records would be that an H1N1 avianlike virus entered human or swine hosts a short time before 1918. In 1918 a virulent strain of this virus caused a severe human influenza pandemic, and, as suggested by Crosby (9), the human pandemic virus was subsequently introduced into North American swine populations, giving rise to the classical swine viruses (Fig. 5C). The suggestion that classical swine virus was derived from the human pandemic virus of 1918 does not imply that swine did not play a role in the appearance of this new virus in the human population. It is entirely possible that somewhere in the world, swine may have served as an intermediate host prior to the emergence of the 1918 pandemic virus. Clearly, such an event would be highly transitory and therefore impossible to detect in an evolutionary analysis. The potential of avian viruses to enter, circulate, and evolve in the swine host reservoir is demonstrated by the new avianlike swine viruses in Europe.

The possibility that the 1918 virus was not a reassortant virus but an entirely novel avianlike virus may be a contributing factor to its virulence, predicted rapid evolution, and entrance into and persistence in the swine host reservoir. A comparison of mortality figures shows that the reassortant pandemic viruses of 1957 and 1968 were apparently milder than the highly virulent 1918 virus (9). There is evidence that wide dissemination of human virus during the 1968 pandemic resulted in an increased incidence of human viruses isolated from pigs (see, e.g., references 24, 30, and 49). Unlike the 1918 pandemic virus, there is as yet no evidence that the pandemic viruses of 1957 or 1968 resulted in new swine-specific strains that have persisted to the present day. Also, there is no evidence that the pandemics of 1957 and 1968 had any detectable effect on the evolution of NPs of human or classical swine viruses (Fig. 1 and 4). Therefore, although new strains of swine viruses may be expected to appear following human pandemics, few would be expected to persist and evolve in the swine host reservoir.

The mode of interspecies transmission of influenza viruses or genes from the avian host reservoir to the human population remains unresolved. The 1957 and 1968 pandemic viruses originated from China, and there is anecdotal evidence that 19th century pandemic viruses also originated from China (28). Humanlike and avianlike viruses are regularly isolated from pigs in China, Taiwan, and Southeast Asia (24, 33, 42, 44), but persistent, widely circulating, swine-specific strains comparable to classical swine viruses in North America or the new avianlike swine viruses in Europe have not been found. Common Oriental agricultural practices place humans, swine, and domestic ducks in close association and enhance the likelihood of interspecies transmission of influenza viruses. Further understanding of the origin of human pandemic viruses will require detailed knowledge of the ecology and evolution of human, swine, and avian influenza viruses in a variety of situations around the world where interspecific exchanges are likely.

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