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Evolutionary Analysis of the Influenza A Virus M Gene with Comparison of the M1 and M2 Proteins

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Phylogenetic analysis of 42 membrane protein (M) genes of influenza A viruses from a variety of hosts and geographic locations showed that these genes have evolved into at least four major host-related lineages: (i) A/Equine/Prague/56, which has the most divergent M gene; (ii) a lineage containing only H13 gull viruses; (iii) a lineage containing both human and classical swine viruses; and (iv) an avian lineage subdivided into North American avian viruses (including recent equine viruses) and Old World avian viruses (including avianlike swine strains). The M gene evolutionary tree differs from those published for other influenza virus genes (e.g., PB1, PB2, PA, and NP) but shows the most similarity to the NP gene phylogeny. Separate analyses of the M1 and M2 genes and their products revealed very different patterns of evolution. Compared with other influenza virus genes (e.g., PB2 and NP), the M1 and M2 genes are evolving relatively slowly, especially the M1 gene. The M1 and M2 gene products, which are encoded in different but partially overlapping reading frames, revealed that the M1 protein is evolving very slowly in all lineages, whereas the M2 protein shows significant evolution in human and swine lineages but virtually none in avian lineages. The evolutionary rates of the M1 proteins were much lower than those of M2 proteins and other internal proteins of influenza viruses (e.g., PB2 and NP), while M2 proteins showed less rapid evolution compared with other surface proteins (e.g., H3HA). Our results also indicate that for influenza A viruses, the evolution of one protein of a bicistronic gene can affect the evolution of the other protein. It is apparent that conservation of the M1 protein places constraints on M1 gene evolution and in turn affects the evolution of the M2 gene and its product.

The genome of influenza A viruses consists of eight single-stranded RNA segments of negative sense (22). Influenza A viruses can infect a variety of avian and mammalian hosts, including humans. Mixed infection with different influenza virus strains can lead to reassortment of the genomic RNA segments (25, 26), a process that may be involved in the origin of new influenza pandemics (45). Hence, analysis of the nucleotide sequences of each of these gene segments may provide valuable information about the evolutionary relationships among influenza A viruses isolated from different hosts. Influenza A virus surface proteins (hemagglutinin [HA] and neuraminidase [NA]) have been widely studied (5, 12, 20, 21, 28) in contrast to the internal and nonstructural protein genes (PA, PB1, PB2, NP, and NS), whose evolutionary pathways were established only recently (13–15, 19, 32, 33).

The membrane protein (M) gene of influenza A viruses is 1,027 nucleotides long and encodes two proteins, M1 and M2, derived by splicing of mRNA (1, 22). An open reading frame, beginning at nucleotide 26 and extending to nucleotide 781 (M1 gene), encodes the M1 protein, while nucleotides 26 to 51 and 740 to 1,004 in a spliced +1 reading frame (M2 gene) encode the M2 protein. The M1 internal protein (252 amino acids long) is a major component of the virus particle with an essential role in virus assembly and budding (10). M2 (97 amino acids long) is a membrane-associated protein with an undefined role in virus replication (24). Recently, Sugrue et al. (44) suggested that M2 may function to protect the structural integrity of the acid-sensitive glycoprotein (e.g., HA) by modulating the effect of low pH encountered in the *trans*-Golgi network.

The available evidence indicates that M genes are highly conserved (22, 23, 39) and that the same M gene has been retained throughout the antigenic shift of HA and NA in human pandemics (17, 40). It has been reported that human and avian virus M genes can be distinguished by several amino acid substitutions in both M1 and M2 proteins (7). Otherwise, there is little information about the evolutionary relationships among M genes isolated from different host species.

The influenza A virus M gene is bicistronic, as described above. Other RNA viruses also have different overlapping cistrons (e.g., paramyxoviruses, rhabdoviruses, reoviruses, coronaviruses, etc. [43]). It is possible that the evolution of the products of polycistronic virus genes with overlapping regions are tightly linked, as has been shown before (42). However, the effect of this linkage on the evolution of polycistronic genes has not been investigated in detail. Because influenza A virus M1 and M2 proteins are obviously different in their structure and function, they might serve as evolutionary models of polycistronic genes. We therefore addressed the following questions in a phylogenetic study of influenza A virus M genes: (i) What are the evolutionary pathways of influenza A virus M genes isolated from a variety of hosts, and how do these pathways compare with those of other influenza virus genes? (ii) How do the evolution rates of M1 and M2 proteins, which are derived from the same RNA segment in different reading frames, compare?

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TABLE 1. Influenza virus strains used in phylogenetic analyses

Strain	Abbreviation	Source or reference
A/Equine/Prague/1/56(H7N7)	EQPR56	This report
A/WS/33(H1N1)	WS33	50
A/WSN/33(H1N1)	WSN33	27
A/Puerto Rico/8/34(H1N1)	PR8-34	46
A/Fort Warren/1/50(H1N1)	FW50	50
A/USSR/90/77(H1N1)	USSR77	37
A/Singapore/1/57(H2N2)	SING57	50
A/Ann Arbor/6/60(H2N2)	AA60	11
A/Korea/426/68(H2N2)	KOREA68	This report
A/Aichi/2/68(H3N2)	AICHI68	This report
A/Udorn/307/72(H3N2)	UDORN72	23
A/Port Chalmers/1/73(H3N2)	PC73	50
A/Bangkok/1/79(H3N2)	BANG79	34
A/Memphis/8/88(H3N2)	MEM88	This report
A/Swine/Iowa/15/30(H1N1)	SWIA30	This report
A/Swine/29/37(H1N1)	SW29-37	This report
A/Swine/March/52(H1N1)	SWMAR52	This report
A/Swine/May/54(H1N1)	SWMAY54	This report
A/Swine/Wisconsin/1/61(H1N1)	SWWIS61	This report
A/Swine/Tennessee/24/77(H1N1)	SWTN77	This report
A/Swine/Ontario/2/81(H1N1)	SWONT81	This report
A/Wisconsin/3523/88(H1N1)	WIS88	This report
A/Swine/Iowa/17672/88(H1N1)	SWIA88	This report
A/Budgerigar/Hokkaido/1/77(H4N6)	BUDHOK77	This report
A/Chicken/Victoria/1/85(H7N7)	CKVIC85	This report
A/Duck/Czechoslovakia/56(H4N6)	DKCZ56	This report
A/Swine/Hong Kong/127/82(H3N2)	SWHK82	This report
A/Swine/Netherlands/12/85(H1N1)	SWNED85	This report
A/FPV/Dobson/27(H7N7)	FPVD27	This report
A/FPV/Weybridge/27(H7N7)	FPVW27	27
A/FPV/Rostock/34(H7N1)	FPVR34	29
A/Chicken/Pennsylvania/1370/83(H5N2)	CKPEN1370-83	4
A/Chicken/Pennsylvania/1/83(H5N2)	CKPEN1-83	This report
A/Turkey/Minnesota/833/80(H4N2)	TYMN80	This report
A/Equine/Tennessee/5/86(H3N8)	EQTN86	This report
A/Equine/Kentucky/2/86(H3N8)	EQKY86	This report
A/Mallard/NY/6750/78(H2N2)	MLRDNY78	7
A/Pintail/Alberta/119/79(H4N6)	PINALB79	30
A/Turkey/Minnesota/166/81(H1N1)	TYMN81	This report
A/Gull/Massachusetts/26/80(H13N6)	GULMA80	This report
A/Gull/Maryland/1824/78(H13N9)	GULMD78	This report
A/Gull/Maryland/1815/79(H13N6)	GULMD79	This report
B/Lee/40	B-LEE40	6

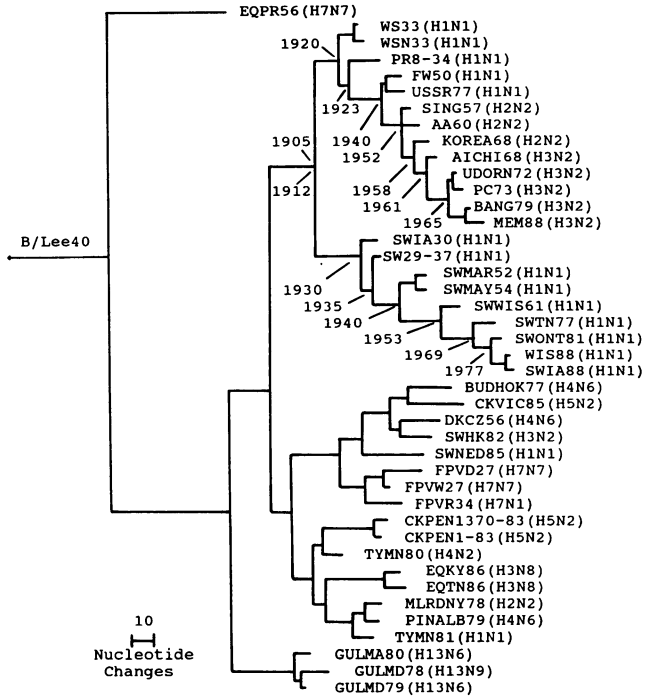

FIG. 1. Phylogenetic trees for influenza A virus M genes. Nucleotide tree rooted to B/Lee/40 M (left). Full-length sequences of 42 M genes were analyzed with PAUP, which relies on a maximum parsimony algorithm. The lengths of the horizontal lines are proportional to the minimum number of nucleotide differences required to join nodes and M gene sequences. Vertical lines are used to separate progeny virus lineages at the point where they branch off from the common ancestral virus lineage. Their lengths are not important. The nucleotide sequence of the B/Lee/40 M gene (6) was aligned with the influenza A virus M genes by use of the Needleman-Wunsch pairwise alignment algorithm. Alignment was achieved with no deletion from residue 1 to 756. The aligned B/Lee/40 M gene sequence shows 644 to 661 base differences (or 65.8 to 67.5% differences) compared with those for influenza A virus M genes. Amino acid trees of M1 (center) and M2 (right) proteins were generated by using the topology option of PAUP to make predicted amino acid sequences conform to the full-length nucleotide tree (left). Strain abbreviations are listed in Table 1.

MATERIALS AND METHODS

Viruses and viral RNA extraction. Twenty-seven viral isolates representing a spectrum of geographic locations, host species, and dates of isolation were drawn from the repository at St. Jude Children's Research Hospital, Memphis, Tenn. Fifteen additional influenza A virus and B/Lee/40 M gene sequences were taken from the literature and data bank sources (Table 1). The viral isolates were grown in 11-day-old embryonated chicken eggs at 35°C for 2 days and purified by differential sedimentation through 25 to 70% sucrose gradients in a Beckman SW28 rotor. Viral RNA was isolated by treatment of purified virus with proteinase K and sodium dodecyl sulfate, followed by extraction with phenol-chloroform (1:1), as described previously (3).

Molecular cloning of the M genes. Full-length cDNA was prepared by reverse transcription of virion RNA of each virus as previously described (18). Briefly, cDNA was synthesized from the viral RNA template by using a 12-base synthetic primer complementary to the 3′ terminus of the template in the presence of [α-^{32}P]dATP. Second-strand DNA synthesis was carried out with a 13-base synthetic primer complementary to the 3′ end of the cDNA and the Klenow fragment of *Escherichia coli* DNA polymerase I. Double-stranded cDNAs were then blunt-end ligated into the *Pvu*II site of pATX vector DNA (a derivative of pAT153, courtesy of Clayton Naeve, St. Jude Children's Research Hospital, Memphis, Tenn.).

Nucleotide sequencing. The nucleotide sequences of the cloned M cDNA were determined by the dideoxynucleotide chain termination method (38). Oligodeoxynucleotide primers were annealed to double-stranded template DNA denatured with NaOH as described by Chen and Seeburg (9) and extended with modified T7 DNA polymerase (Sequenase; U.S. Biochemical, Cleveland, Ohio).

Oligonucleotide primers complementary to the M gene segment were synthesized on an Applied Biosystems model 380A DNA synthesizer by the cold-phase phosphoramidite method. The reaction products were resolved on 6% polyacrylamide—7 M urea thin gels containing a 1 to 5× TBE (90 mM Tris-borate [pH 8.0], 1 mM EDTA) gradient. The nucleotide sequences of A/Equine/Prague/1/56 (H7N7) and

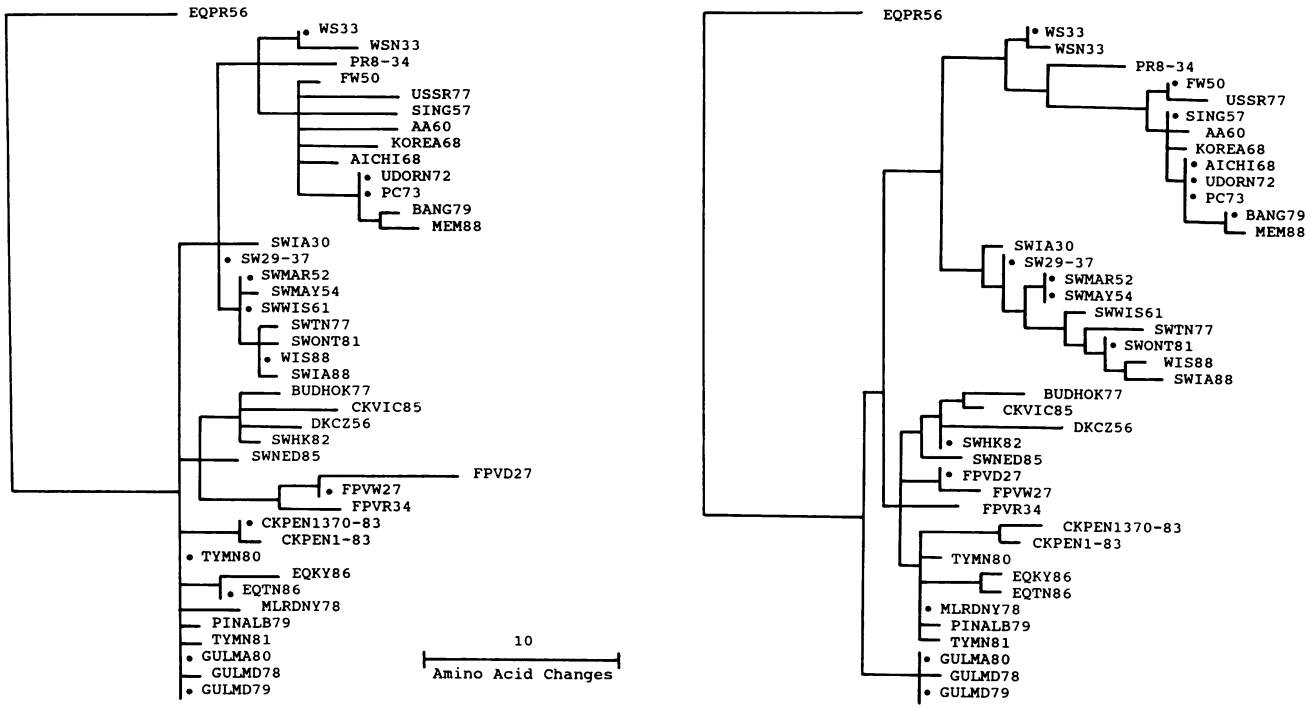

FIG. 1—*Continued.*

A/Swine/Wisconsin/1/61 (H1N1) were determined by direct sequencing of viral RNA. The primers were annealed to virion RNA and extended with avian myeloblastosis virus reverse transcriptase in the presence of [α-^{32}P]dATP.

Sequence analysis. Phylogenetic analysis of sequence data was performed with the PAUP software package version 2.4 (David Swofford, Illinois Natural History Survey, Champaign, Ill.). PAUP employs the maximum parsimony method to generate phylogenetic trees. Trees of the shortest length (most parsimonious) were found by implementing the MULPARS, SWAP=GLOBAL, and HOLD=10 options of PAUP.

Nucleotide sequence accession numbers. The sequences discussed here are available from the GenBank data base (accession numbers M63515 through M63540).

RESULTS AND DISCUSSION

Comparative analysis of M gene nucleotide sequences. Each of the cloned M genes comprised an identical number of nucleotides (1,027 bases); no insertions or deletions were found in any of the sequences.

A phylogenetic analysis of 42 influenza virus M gene segment nucleotide sequences is presented in Fig. 1. Excluding the B/Lee/40 sequence, PAUP identified nine evolutionary trees of equal length, consisting of 956 steps (nucleotide changes). The next-shortest trees were two steps longer. These nine trees varied only in the attachment of SING57 and AA60 with adjacent terminal branches. We chose one of them with a branching order for the two viruses that were most consistent with the sequence of isolation dates (Fig. 1, left). The aligned B/Lee/40 M sequence is separated from the root of influenza A virus M genes by a branch distance of 613 nucleotide changes (not shown).

Our analysis indicates that M genes have evolved into at least four major host-related lineages rooted at the deep forks of the tree. The first lineage is that of EQPR56 (Table 1), which contains the most-divergent M gene. The next major fork is represented by the split between H13 gull M genes and those of human, swine, and avian viruses. The third fork of the tree represents a separation of human and classical swine viruses (i.e., those related to Swine/Iowa/15/30) from avian lineages. M genes of the avian lineage are subdivided into Old World and North American groups, the latter containing representatives of recent equine virus M genes (EQKY86, EQTN86). Within the Old World avian group are M genes of avianlike swine viruses (SWHK82, SWNED85). The PA, PB1, and NP genes of these avianlike swine viruses show the same evolutionary patterns (14, 19, 33), demonstrating that H3N2 and H1N1 avian viruses can infect swine hosts.

The distant relationship of EQPR56 to other influenza viruses is also seen in the evolution of NP and PB2 genes (14, 15). The coupling of classical swine and human M gene lineages indicates that they share a common ancestor, which has been shown in previous evolutionary analyses of PA, PB2, and NP genes (13–15, 33). The M gene of the human virus isolate WIS88 is contained within the classic swine virus group and is consistent with its identification as a swine virus (36). A major difference in the evolutionary history of the M gene and that of the NP protein was seen. The NP gene of the equine H3 virus isolates form a divergent group with no close relatives in other species. The M genes of these viruses, however, appear to have been derived relatively recently from North American avian viruses. This indicates that these genes have been reassorted at different times.

Evolutionary relationships of M1 and M2 proteins. To investigate the evolutionary relationships between the two M gene products, we translated the M1 and M2 open reading frames and then analyzed their amino acid sequences (Fig. 2). Amino acid substitutions were observed at 62 (24.6%) positions among the 252 amino acid residues of the M1

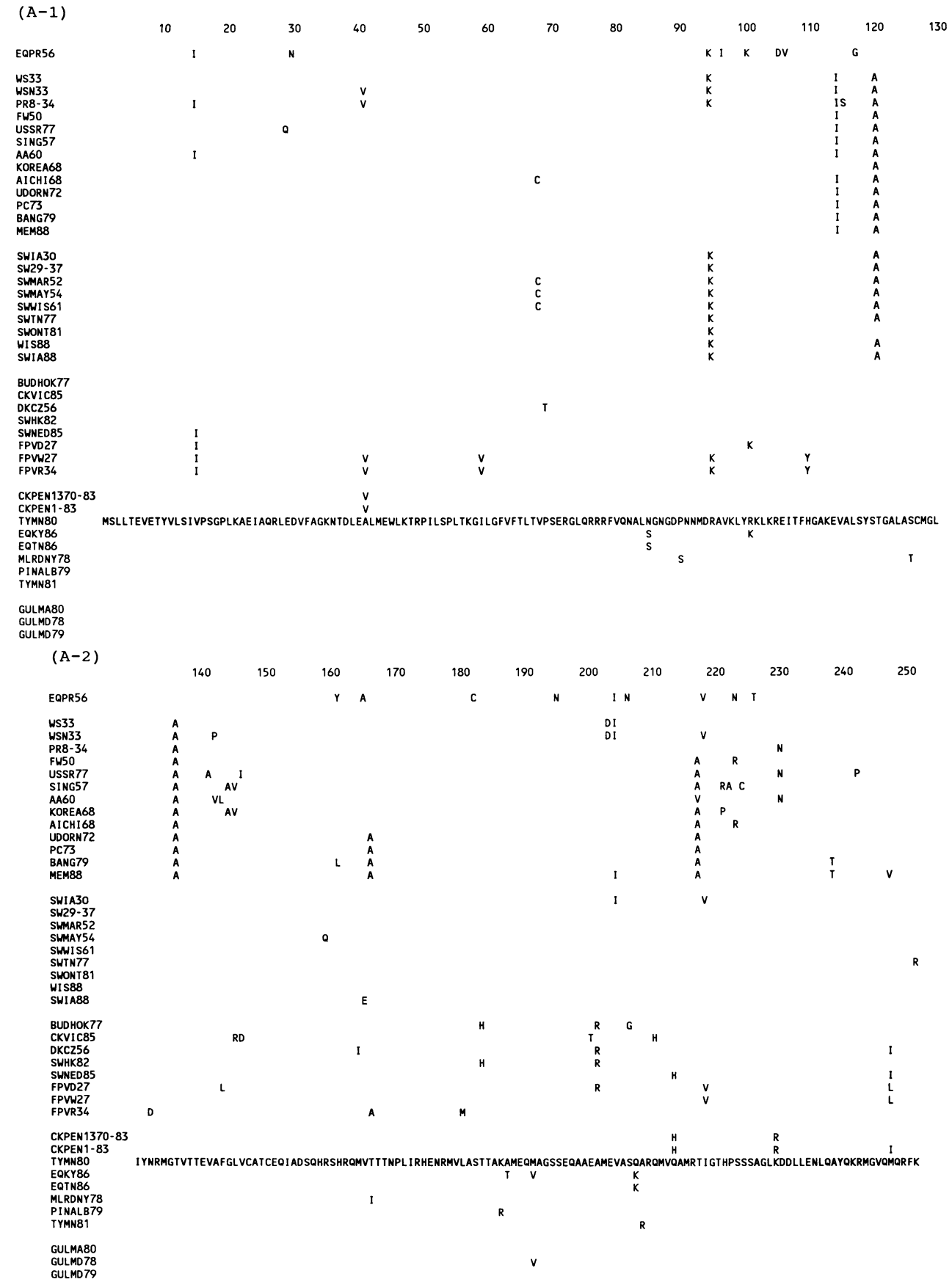

FIG. 2. Predicted amino acid sequences of the M1 (A-1 and A-2) and M2 (B) proteins. To facilitate detection of patterns of derived amino acid substitutions, we have written in full the sequence of the M gene of TYMN80, which is the most primitive gene (the closest to the root of the tree). Only differences from the TYMN80 baseline are shown for other sequences. All of the strains represented are listed in Table 1.

(B)

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                    10        20        30        40        50        60        70        80        90
EQPR56              IKS    R N    L  AI   T         F       A   FRH       G I             SD N  N

WS33                I  E G R N           N                      F                    K
WSN33               I  E G R N     F     N                      F                    K
PR8-34              I  E G R NG      A   N                      F     G       K      K   S   A       S
FW50                I  E G R N         V                        LF H                 K   S   A  S
USSR77              I  Q G R N         V                        LF H                 K       A  S
SING57              I  E G R N         V                        FF H                 K   S   A  S    S
AA60                I  E G R N         V                 H      FF H                 K   S   A  S    S
KOREA68             I  E G R N         V          F             FF H                 K   S   A  S    S
AICHI               I  E G R N         V                        FFEH                 K   S   A  S    S
UDORN72             I  E G R N         V                        FFEH                 K   S   A  S    S
PC73                I  E G R N         V                        FFEH                 K   S   A  S    S
BANG79              I  E G R N         V                        FF H                 K       A  S    S
MEM88               I  E G R N         V                        LF H                 K       A  S    S

SWIA30                 E G R N         A                                             K  S
SW29-37             I  E G R N         A                          E                  K  S
SWMAR52             I SE G R N         A                          E          L       K  S
SWMAY54             I SE G R N         A                          E          L       K  S
SWWIS61               SE G R N         A                          F                  K  S
SWTN77              I SE G R N  G      A                      H   F                  K  S             A
SWONT81             I  E G R N         A                          F                  K  S             V
WIS88               I  E G   N         A                    L     F                  K  S             V
SWIA88              I  E G   N         AV                         F                  K  S

BUDHOK77             K                 I                                                    SV
CKVIC85                                                                                     S          A
DKCZ56                     RY G                                    C  H          K
SWHK82                                                             L                       S
SWNED85                 G RF                                       L
FPVD27                     S
FPVW27                     S                       F
FPVR34                     R N       I                 N                                   S

CKPEN1370-83        L                 I                 FY                    A           S      V
CKPEN1-83           L          Y      I                                       A           S      V
TYMN80      MSLLTEVETPTRNGWECKCSDSSDPLVIAASIIGILHLILWILDRLFFKCIYRRLKYGLKRGPSTEGVPESMREEYRQEQQNAVDVDDGHFVNIELE
EQKY86                        G                                 F   L
EQTN86                        G                                 F         G
MLRDNY78                                                                                    S
PINALB79                                                                                    S      S
TYMN81                                                             L                        S

GULMA80             H  S    R N                                                  Q  K S
GULMD78             H  S    R N                       F                          Q  K S
GULMD79             H  S    R N                                                  Q  K S
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FIG. 2—*Continued.*

protein; differences were distributed uniformly throughout the protein (Fig. 3). Amino acid sequence divergence was greater in the M2 protein, with 47 (48.5%) of the 97 amino acids showing differences, mainly within residues 10 to 28, 54 to 57, and 77 to 93. A minimum of 18 N-terminal amino acids of the M2 protein are exposed at the cell surface (extracellular domain, Fig. 3, A [24]), a single hydrophobic domain of 19 amino acids is anchored in the cell membrane (transmembrane domain, Fig. 3, B), and 54 C-terminal residues are located on the cytoplasmic side of the cell membrane (cytoplasmic domain, Fig. 3, C). An extensive region of high divergence is evident between the extracellular and transmembrane domains of the M2 protein. Two less-extensive regions of amino acid substitutions are evident in the cytoplasmic domain.

The amino acid terminal region shared by the M1 and M2 genes (nucleotides 26 to 51; nine amino acid residues) was completely conserved in all strains used in our study (Fig. 2 and 3). In the region of overlap between M1 and M2, constraints imposed by the M1 protein have clearly affected the changes in the M2 protein. Although this region of M2 shows high variability (10 of 13 amino acids vary), all of these mutations are second-codon position changes that result in third-position, silent changes in M1. In this region, there are only three third-codon changes in M2, and only two amino acid substitutions in M1.

To compare the evolution of M1 and M2, we constructed phylogenetic trees for each of the proteins, showing the amino acid changes occurring on the evolutionary pathways

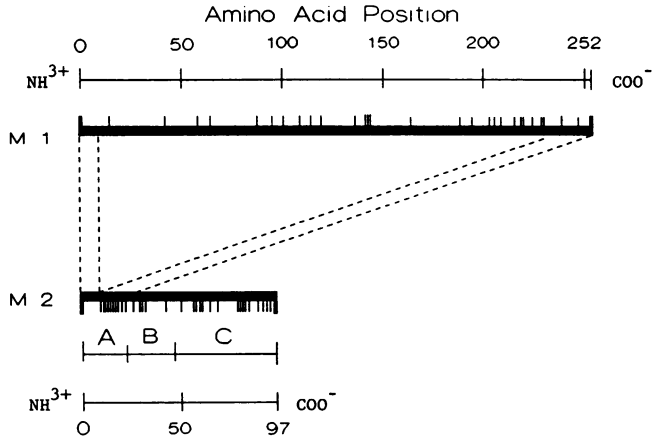

FIG. 3. Summary of the amino acid substitutions in 42 M proteins of influenza A viruses. Vertical lines show the positions of amino acid substitutions in more than two strains. The dashed lines indicate the region where nucleotides of the M1 and M2 genes overlap. A, B, and C indicate the extracellular, transmembrane, and cytoplasmic domains of M2 protein, respectively, as suggested by Lamb et al. (24).

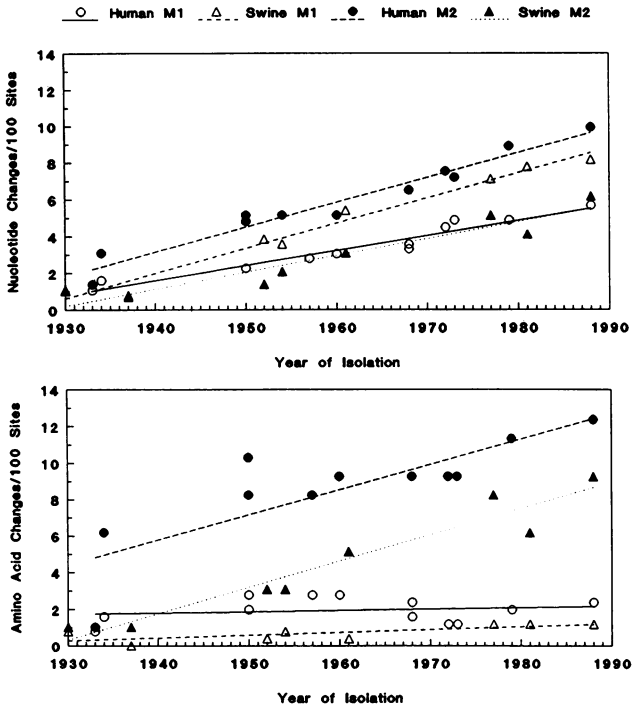

FIG. 4. Comparison of evolutionary rates for M genes (top) and their products (bottom). The slopes were estimated by regression of year of isolation against nucleotide or amino acid changes from the common ancestral node of the phylogenetic trees per 100 sites (Fig. 2).

calculated from the complete nucleotide sequence. This allows direct comparison of homologous branches in nucleotide and amino acid trees for differences in the effect of coding and noncoding genetic changes among lineages (14). Lineages in the M1 amino acid tree show much less evolution than does the whole M gene segment, indicating that most nucleotide changes in the M gene are silent. Thus, the M1 protein is uniformly conserved across the various host-specific viruses, resulting in relatively few amino acid differences. The one exception was found in fowl plague virus (FPV) strains, which showed appreciable evolution relative to other avian virus M1 proteins. By contrast, the M2 proteins showed considerable evolution among the human and swine lineages but virtually none in the avian lineages.

Evolutionary rates of M genes and their products. The relatively high degree of divergence of M2 compared with M1 proteins (Fig. 2 and 3) suggests that they are evolving very differently in response to selective pressures or structural constraints. To investigate this possibility more closely, we estimated the evolutionary rates of human and classical swine virus M genes and their products by plotting the year of isolation for a virus against the branch distance to the ancestor node of the lineage (Fig. 4). Because the M1 and M2 proteins have different lengths, the rates are expressed as changes per 100 residues. A regression was not possible for avian M lineages because of a lack of correspondence between the dates of virus isolation and lineage position. Evolutionary rates for gull and equine lineages could not be estimated owing to the small number of datum points. Except for the placement of the USSR77 isolates, data for the human and swine lineages appear to be in order (Fig. 1). For the purpose of estimating evolutionary rates, we treated the USSR77 isolates as having appeared in 1950 because this virus was found to be very similar to 1950 human strains (2, 31, 41).

The rates of change per base for the human M1 and M2 genes were 0.83×10^{-3} and 1.36×10^{-3} substitutions per year, respectively; for the swine M1 and M2 genes, they were 1.43×10^{-3} and 0.91×10^{-3} substitutions per year, respectively (Fig. 4, top). These values are smaller than estimates reported for human NP genes (1.62×10^{-3} to 2.2×10^{-3} substitutions per site per year [2, 14]), PB2 genes (1.82×10^{-3} substitutions per site per year [15]), and NS genes (2×10^{-3} substitutions per site per year [8]). At the amino acid level, the evolutionary rate for the M1 proteins was much lower than that for the M2 proteins: human = 0.08×10^{-3} versus 1.38×10^{-3} amino acid changes per residue per year and swine = 0.16×10^{-3} versus 1.43×10^{-3} amino acid changes per residue per year (Fig. 4, bottom). The M1 proteins have evolved much more slowly than the M2 proteins (more than 15 times slower in human strains and more than 9 times slower in swine strains).

M2, a surface protein, may be subject to greater host-immune selective pressures than M1, an internal protein, although the latter may be recognized by cytotoxic T cells (16, 49). Other internal virus proteins are evolving faster than M1: human virus NP and PB2 proteins are evolving at 1.14×10^{-3} and 0.46×10^{-3} amino acid changes per residue per year (14, 15), respectively, compared with 0.08×10^{-3} amino acid changes per residue per year for human virus M1. The high degree of conservation among M1 proteins may be related to functional constraints that could arise from possible multiple interactions with other internal virus proteins. Although the functional relationships between the M1 protein and other influenza virus proteins are not yet clear, M1 does bind to ribonucleoprotein complexes (35, 51) and at least two RNA binding domains, centered around residue 80 to 109 and 129 to 164, have been suggested (47). Our results show that there are some amino acid substitutions in these sites (Fig. 2), but they do not change the hydrophilicity of the molecule, an important requirement for RNA binding activity. It has also been suggested that the N-terminal third of M1 serves to anchor the protein in the lipid bilayer of the viral envelope (48). This region is extremely conserved among all strains used in this study (Fig. 2).

Divergence dates for the hypothetical common ancestor. Estimates of evolutionary rates based on the whole M gene phylogeny were used to calculate dates of divergence from the hypothetical ancestral nodes of the human and classical swine lineages. This was done by dividing the branch-internodal distance by the evolutionary rate, yielding a distance in years (14). The estimated divergence dates for the human and classical swine lineages are 1905 and 1912, respectively (Fig. 1, left). These estimates are within the ranges estimated for the NP (1914) and PB2 (1910) genes (14, 15), supporting the idea that the human and classical swine virus lineages diverged from a common ancestor before 1918.

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REFERENCES

1. **Allen, H., J. McCauley, M. Waterfield, and M. J. Gething.** 1980. Influenza virus RNA segment 7 has the coding capacity for two polypeptides. Virology **107**:438–442.
2. **Altmuller, A., W. M. Fitch, and C. Scholtissek.** 1989. Biological and genetic evolution of the nucleoprotein gene of human influenza A viruses. J. Gen. Virol. **70**:2111–2119.
3. **Bean, W. J., G. Sriram, and R. G. Webster.** 1980. Electrophoretic analysis of iodine-labeled influenza RNA segments. Anal. Biochem. **102**:228–232.
4. **Bean, W. J., S. C. Threlkeld, and R. G. Webster.** 1989. Biological potential of amantadine-resistant influenza A virus in an avian model. J. Infect. Dis. **159**:1050–1056.
5. **Both, G. W., M. J. Sleigh, N. Cox, and A. P. Kendal.** 1983. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J. Virol. **48**:52–60.
6. **Briedis, D. J., R. A. Lamb, and P. W. Choppin.** 1982. Sequence of RNA segment 7 of the influenza B virus genome: partial amino acid homology between the membrane protein (M1) of influenza A and B viruses and conservation of second open reading frame. Virology **116**:581–588.
7. **Buckler-White, A. J., C. W. Naeve, and B. R. Murphy.** 1986. Characterization of a gene coding for M proteins which is involved in host range restriction of an avian influenza A virus in monkeys. J. Virol. **57**:697–700.
8. **Buonagurio, D. A., S. Nakada, J. D. Parvin, M. Krystal, P. Palese, and W. M. Fitch.** 1986. Evolution of human influenza A viruses over 50 years: rapid and uniform rate of change in the NS gene. Science **232**:980–982.
9. **Chen, E. Y., and P. H. Seeburg.** 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA **4**:165–170.
10. **Choppin, P. W., R. W. Compans, A. Scheild, J. Mcsharry, and S. Lazarowitz.** 1972. Structure and assembly of viral membranes. p. 163–179. *In* C. F. Fox (ed.), Membrane research. Academic Press, Inc., New York.
11. **Cox, N. J., F. Kitame, A. P. Kendal, H. F. Maassab, and C. Naeve.** 1988. Identification of sequence changes in the cold-adapted, live attenuated influenza vaccine strain, A/Ann Arbor/6/60 (H2N2). Virology **167**:554–567.
12. **Fang, R., W. Min Jou, D. Huylebroeck, R. Devos, and W. Fiers.** 1981. Complete structure of A/Duck/Ukraine/63 influenza hemagglutinin gene: animal virus as progenitor of human H3 Hong Kong 1968 influenza hemagglutinin. Cell **25**:315–323.
13. **Gammelin, M. A., A. Altmuller, U. Reinhardt, J. Mandler, V. R. Harley, P. J. Hudson, W. M. Fitch, and C. Scholtissek.** 1990. Phylogenetic analysis of nucleoproteins suggests that human influenza A viruses emerged from a 19-century avian ancestor. Mol. Biol. Evol. **7**:194–200.
14. **Gorman, O. T., W. J. Bean, Y. Kawaoka, and R. G. Webster.** 1990. Evolution of the nucleoprotein gene of influenza A virus. J. Virol. **64**:1487–1497.
15. **Gorman, O. T., R. O. Donis, Y. Kawaoka, and R. G. Webster.** 1990. Evolution of influenza A virus PB2 genes: implications for the evolution of the ribonucleoprotein complex and origin of human influenza A virus. J. Virol. **64**:4893–4902.
16. **Gotch, F., A. McMichael, G. Smith, and B. Moss.** 1987. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. J. Exp. Med. **165**:408–416.
17. **Hall, R. M., and G. M. Air.** 1981. Variation in nucleotide sequence coding for the N-terminal regions of matrix and nonstructural proteins of influenza A virus. J. Virol. **38**:1–7.
18. **Huddleston, J. A., and G. G. Brownlee.** 1982. The sequence of the nucleoprotein gene of human influenza A virus strain, A/NT/60/68. Nucleic Acids Res. **10**:1029–1037.
19. **Kawaoka, Y., S. Krauss, and R. G. Webster.** 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J. Virol. **63**:4603–4608.
20. **Kida, H., Y. Kawaoka, C. W. Naeve, and R. G. Webster.** 1987. Antigenic and genetic conservation of H3 influenza in wild ducks. Virology **159**:109–119.
21. **Kida, H., K. F. Shortridge, and R. G. Webster.** 1988. Origin of the hemagglutinin gene of H3N2 influenza viruses from pigs in China. Virology **162**:160–166.
22. **Lamb, R. A.** 1989. The genes and proteins of the influenza viruses, p. 1–87. *In* R. M. Krug (ed.), The influenza viruses. Plenum Press, New York.
23. **Lamb, R. A., and C.-J. Lai.** 1981. Conservation of influenza virus membrane protein (M1) amino acid sequence and open reading frame of RNA segment 7 encoding a second protein (M2) in H1N1 and H3N2 strains. Virology **112**:746–751.
24. **Lamb, R. A., L. S. Zebedee, and C. D. Richardson.** 1985. Influenza virus M2 protein is an integral membrane protein expressed on the infected cell surface. Cell **40**:627–633.
25. **Laver, W. G., and R. G. Webster.** 1972. Studies on the origin of pandemic influenza. II. Peptide maps of the light and heavy polypeptide chains from the hemagglutinin subunits of A2 influenza virus isolated before and after the appearance of Hong Kong influenza. Virology **48**:445–455.
26. **Laver, W. G., and R. G. Webster.** 1979. Ecology of influenza viruses in lower mammals and birds. Br. Med. Bull. **35**:29–33.
27. **Markusin, S., H. Ghiasi, N. Sokolov, A. Shilov, B. Sinitsin, D. Brown, A. Klimov, and D. Nayak.** 1988. Nucleotide sequence of RNA segment 7 and the predicted amino acid sequence of M1 and M2 proteins of FPV/Weybridge (H7N7) and WSN (H1N1) influenza viruses. Virus Res. **10**:263–272.
28. **Martinez, C., L. Del Rio, A. Portela, E. Domingo, and J. Ortin.** 1983. Evolution of the influenza virus neuraminidase gene during drift of the N2 subtype. Virology **130**:539–545.
29. **McCauley, J. W., B. W. J. Mahy, and S. C. Inglis.** 1982. Nucleotide sequence of fowl plague virus RNA segment 7. J. Gen. Virol. **58**:211–215.
30. **Murphy, B. R., A. J. Buckler-White, W. T. London, and M. H. Snyder.** 1989. Characterization of the M protein and nucleoprotein genes of an avian influenza A virus which are involved in host range restriction in monkeys. Vaccine **7**:557–561.
31. **Nakajima, K., U. Desselberger, and P. Palese.** 1978. Recent human influenza viruses are closely related genetically to strains isolated in 1950. Nature (London) **274**:334–339.
32. **Nakajima, K., E. Nobusawa, and S. Nakajima.** 1990. Evolution of the NS genes of the influenza A viruses. II. Characterization of the amino acid changes in the NS1 proteins of the influenza A viruses. Virus Genes **4**:15–26.
33. **Okazaki, K., Y. Kawaoka, and R. G. Webster.** 1989. Evolutionary pathways of the PA genes of influenza A viruses. Virology **172**:601–608.
34. **Ortin, J., C. Martinez, L. del Rio, M. Davila, C. Lopez-Galindez, N. Villanueva, and E. Domingo.** 1983. Evolution of the nucleotide sequence of the influenza virus RNA segment 7 during drift of the H3N2 subtype. Gene **23**:233–239.
35. **Rees, R. J., and N. J. Dimmock.** 1981. Electrophoretic separation of influenza virus ribonucleoproteins. J. Gen. Virol. **53**:125–132.
36. **Rota, R. A., E. P. Rocha, M. W. Harmon, V. S. Hinshaw, M. G. Sheerar, Y. Kawaoka, N. J. Cox, and T. F. Smith.** 1989. Laboratory characterization of a swine influenza virus isolated from a fatal case of human influenza. J. Clin. Microbiol. **27**:1413–1416.
37. **Samokhvalov, E. I., V. A. Karginov, V. E. Chizhikov, V. M. Blinov, V. P. Yuferov, S. K. Vasilenko, L. V. Uryvaev, and V. M. Zhdanov.** 1985. Primary structure of RNA segment 7 of A/USSR/90/77(H1N1) influenza virus. Bioorg. Khim. **11**:1080–1085.
38. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
39. **Schild, G. C.** 1972. Evidence for a new type specific structural antigen of the influenza virus particle. J. Gen. Virol. **15**:99–103.
40. **Scholtissek, C., W. Rohde, V. Von Hoyningen, and R. Rott.** 1978. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology **87**:13–20.
41. **Scholtissek, C., V. Von Hoyningen, and R. Rott.** 1978. Genetic

relatedness between the new 1977 epidemic strain (H1N1) of influenza and human influenza strains isolated between 1947 and 1957 (H1N1). Virology **89**:613–617.

42. **Smith, M., N. L. Brown, G. M. Air, B. G. Barrell, A. R. Coulson, C. A. Hutchinson, and F. Sanger.** 1977. DNA sequence at the C termini of the overlapping genes A and B in bacteriophage ΦX174. Nature (London) **265**:702–705.
43. **Strauss, J. H., and E. G. Strauss.** 1988. Evolution of RNA viruses. Annu. Rev. Microbiol. **42**:657–683.
44. **Sugrue, R. J., G. Bahadur, M. C. Zambon, M. Hall-Smith, A. R. Douglas, and A. J. Hay.** 1990. Specific structural alteration of the influenza haemagglutinin by amantadine. EMBO J. **9**:3469–3476.
45. **Webster, R. G.** 1972. On the origin of pandemic influenza viruses. Curr. Top. Microbiol. Immunol. **59**:75–105.
46. **Winter, G., and S. Fields.** 1980. Cloning of influenza cDNA into M13: the sequence of the RNA segment encoding the A/PR/8/34 matrix protein. Nucleic Acids Res. **8**:1965–1974.
47. **Ye, Z., N. W. Baylor, and R. R. Wagner.** 1989. Transcription-inhibition and RNA-binding domains of influenza A virus matrix protein mapped with anti-idiotypic antibodies and synthetic peptides. J. Virol. **63**:3586–3594.
48. **Ye, Z., R. Pal, J. W. Fox, and R. R. Wagner.** 1987. Functional and antigenic domains of the matrix (M1) protein of influenza A virus. J. Virol. **61**:239–246.
49. **Yewdell, J. W., and C. J. Hackett.** 1989. Specificity and function of T lymphocytes induced by influenza A viruses. p. 361–434. *In* R. M. Krug (ed.), The influenza viruses. Plenum Press, New York.
50. **Zebedee, S. L., and R. A. Lamb.** 1989. Nucleotide sequences of influenza A virus RNA segment 7: a comparison of five isolates. Nucleic Acids Res. **17**:2870.
51. **Zvonarjev, A. Y., and Y. Z. Ghendon.** 1980. Influence of membrane (M) protein on influenza A virus virion transcriptase activity *in vitro* and its susceptibility to rimantadine. J. Virol. **33**:583–586.