

## Characterization of the 1918 “Spanish” Influenza Virus Matrix Gene Segment

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**The coding region of influenza A virus RNA segment 7 from the 1918 pandemic virus, consisting of the open reading frames of the two matrix genes M1 and M2, has been sequenced. While this segment is highly conserved among influenza virus strains, the 1918 sequence does not match any previously sequenced influenza virus strains. The 1918 sequence matches the consensus over the M1 RNA-binding domains and nuclear localization signal and the highly conserved transmembrane domain of M2. Amino acid changes that correlate with high yield and pathogenicity in animal models were not found in the 1918 strain. Phylogenetic analyses suggest that both genes were mammalian adapted and that the 1918 sequence is very similar to the common ancestor of all subsequent human and classical swine matrix segments. The 1918 sequence matches other mammalian strains at 4 amino acids in the extracellular domain of M2 that differ consistently between avian and mammalian strains, suggesting that the matrix segment may have been circulating in human strains for at least several years before 1918.**

The influenza virus pandemic of 1918 resulted in the deaths of up to 50 million people worldwide (23, 35). Recently, samples of the 1918 virus RNA have been obtained from several widely separated geographical sites (39). Analyses of viral hemagglutinin (HA), neuraminidase, and nonstructural genes suggest that the 1918 virus was genetically uniform and adapted to its human host (3, 39, 40). The sequences of these genes, however, did not reveal features that could account for the virulence of the virus, although several hypotheses could be discounted. Explanations for the lethality of the virus might therefore lie in the other five viral gene segments.

Influenza A virus RNA segment 7 encodes two proteins, the matrix proteins M1 and M2. M1 mRNA is colinear with the viral RNA, while M2 mRNA is encoded by a spliced transcript (29, 52). The proteins encoded by these mRNAs share their initial 9 amino acids and also have a stretch of 14 amino acids in overlapping reading frames. The M1 protein is a highly conserved 252-amino acid protein. It is the most abundant protein in the viral particle, lining the inner layer of the viral membrane and contacting the ribonucleoprotein core. M1 has been shown to have several functions (28), including regulation of the nuclear export of viral ribonucleoproteins (vRNPs), both permitting the transport of vRNP particles into the nucleus upon infection and preventing newly exported vRNP particles from reentering the nucleus (8, 32). M1 may also be involved in the inhibition of viral transcription in the late stages of infection and regulation of the switch from replication to viral assembly (36, 59, 60). M1 binds RNA (41, 49, 57, 59), vRNPs (34, 38, 41, 57), and lipids (7, 15); dimerizes with other

M1 molecules (16, 41); and interacts with both the HA and neuraminidase proteins (1, 11). It is also involved in export to the cytoplasmic membrane, virus assembly, and budding (12, 19, 31).

The 97-amino acid M2 protein is a homotetrameric integral membrane protein that exhibits ion channel activity (4, 20, 21, 30, 37, 46, 50) and is the target of the drug amantadine (18). The ion channel activity of M2 is important both during virion uncoating and during viral budding (14, 37, 46, 47; reviewed in reference 28).

The disease course and pathological damage caused by the 1918 virus are consistent with a virus capable of replicating to titers higher than those of other strains (53, 54). The matrix segment has been shown in several experimental systems to have a significant effect on virulence and viral replication rate (6, 13, 43, 44, 51, 55). In some of these studies, matrix proteins differing by as little as 1 or 2 amino acids conferred striking differences in phenotype. Certain mutations in the M2 gene lead to viruses that are resistant to antiviral drugs (17, 18). It is possible that unique characteristics of either M1 or M2 contributed to the lethality of the 1918 virus.

### MATERIALS AND METHODS

**Isolation of A/Brevig Mission/1/18 RNA and amplification of the matrix segment.** RNA was isolated by methods described in other publications (27, 48). The 982-nucleotide M1 and M2 complete coding region of the 1918 influenza virus segment 7 was amplified in 15 overlapping fragments varying in length from 97 to 142 nucleotides. Each fragment was reverse transcribed, amplified, and sequenced at least twice. Reverse transcription-PCR, isolation of products, and sequencing have been described previously (39, 40, 48). A list of the primers used is available upon request.

**Phylogenetic analyses.** Nucleotide sequences of the M1 and M2 genes were aligned with the genes from other influenza virus isolates by using Lasergene software (DNASar, Madison, Wis.). Phylogenetic analyses were carried out by use of the neighbor-joining algorithm with distance parameter (p-distance) (Molecular Evolutionary Genetics Analysis, version 1.01, 1993). All analyses were bootstrapped 100 times.

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TABLE 1. Matrix gene sequences used in this analysis.

Abbreviation	Strain name	GenBank accession no.
BREVIG18	A/Brevig Mission/1/18 (H1N1)	AY130766
CH/BRESCIA02	A/chicken/Brescia/1902 (H7N7)	L37795
CH/HK/220/97	A/chicken/Hong Kong/220/97 (H5N1)	AAF74333
CH/HONGKONG76	A/chicken/Hong Kong/14/76 (H1N1)	U49119
DK/BAVARJA77	A/duck/Bavaria/2/77 (H1N1)	M55476
DK/CZECH56	A/duck/Czechoslovakia/56 (H4N6)	M63537
DK/HONGKONG79	A/cuck/Hong Kong/698/79 (H5N3)	AF098565
DK/PRIMORJE79	A/anas acuta/Primorje/695/79 (H3N2)	Z26858
ENGLAND69	A/England/939/69 (H3N2)	AJ298948
EQ/KENTUCKY81	A/equine/Kentucky/81 (H3N8)	AF001676
EQ/KENTUCKY86	A/equine/Kentucky/2/86 (H3N8)	M63540
EQ/MIAMI63	A/equine/Miami/63 (H3N8)	AF001674
EQ/PRAGUE56	A/equine/Praque/1/56 (H7N7)	M63533
FIJI83	A/Fiji/15899/83 (H1N1)	AJ298947
FM47	A/Fort Monmouth/1/47 (H1N1)	U02084
FPV/WEYBRIDGE34	A/FPV/Weybridge/34 (H7N7)	L37797
FTWAR50	A/Fort Warren/1/50 (H1N1)	J02125
FUKUSHIMA96	A/Fukushima/114/96 (H3N2)	AF038273
GS/GUANGDONG96	A/goose/Guangdong/1/96 (H5N1)	AAD51929
HK/1073/99	A/Hong Kong/1073/99 (H9N2)	AAC32081
HK/156/97	A/Hong Kong/156/97 (H5N1)	AAC32091
HK/482/97	A/Hong Kong/482/97 (H5N1)	AAC32091
HONGKONG97	A/Hong Kong/485/97 (H5N1)	AJ278648
MALL/NY78	A/mallard/New York/6750/78 (H2N2)	M12699
MALL/WISC75	A/mallard/Wisconsin/169/75 (H5N3)	AF073202
PR34	A/Puerto Rico/8/34 (H1N1)	NC 002016
QUAIL/ARK93	A/quail/Arkansas/29209-1/93 (H9N2)	AF156470
SINGAPORE57	A/Singapore/1/57 (H2N2)	X08093
SW/1937	A/swine/29/37 (H1N1)	M63517
SW/GERMANY91	A/swine/Germany/8533/91 (H1N1)	Z26861
SW/HONGKONG94	A/swine/Hong Kong/273/94 (H1N1)	U49115
SW/IOWA30	A/swine/Iowa/15/30 (H1N1)	M63534
SW/MARCH52	A/swine/March/52 (H1N1)	M63518
SW/NETH80	A/swine/Netherlands/25/80 (H1N1)	Z26862
SW/ONTARIO81	A/swine/Ontario/2/81 (H1N1)	M63520
SW/WISC61	A/swine/Wisconsin/1/61 (H1N1)	M63519
TEAL/HONGKONG97	A/teal/Hong Kong/W312/97 (H6N1)	AF250482
TY/NC88	A/turkey/North Carolina/1780/88 (H1N1)	M55479
USSR77	A/USSR/90/77 (H1N1)	M54941
WISC88	A/Wisconsin/3523/88 (H3N2)	M63521
WISC94	A/Wisconsin/4755/94 (H3N2)	U53169
WS33	A/WS/33 (H1N1)	X08089
WSN33	A/WSN/33 (H1N1)	X08088

**Viral strains.** Matrix segment sequences used in this analysis were obtained from GenBank. For a list of the strains and abbreviations used, see Table 1.

**Nucleotide sequence accession number.** The matrix gene segment coding sequence of A/Brevig Mission/1/18 has been deposited in GenBank under accession no. AY130766.

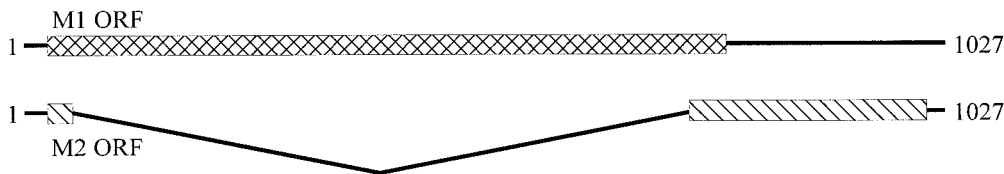
## RESULTS

**Sequence of the matrix gene segment.** The entire matrix coding sequence (982 nucleotides) from the shared start codon at nucleotide 26 of the matrix segment to the stop codon of the M2 open reading frame (ORF) at nucleotide 1007 was determined from the frozen sample obtained from Brevig Mission, Alaska (A/Brevig Mission/1/18 [H1N1]), Brevig18. The 1918 sequence and a map of the two overlapping reading frames of an influenza A virus matrix segment are shown in Fig. 1. The theoretical translations of the two ORFs, M1 and M2, are indicated.

**M1 gene.** The 1918 matrix segment has an M1 ORF of 756 nucleotides, with a predicted 252-amino acid M1 protein. Several functional domains have been mapped to the M1 protein. The theoretical translation of the Brevig18 M1 sequence predicts that the strain contains the consensus RNA-binding and nuclear localization signal (RKLKR) at residues 101 to 105 (10, 41, 58). The Brevig18 M1 sequence also matches the consensus at residues 148 to 162 (CATCEQIADSQHRSH), where a putative zinc-binding motif has been identified (9, 49). Two residues, those at positions 115 and 137, may be of potential interest. They are V (position 115) and T (position 137) in the 1918 strain (and in all avian and swine strains) and I and A, respectively, in other human viruses. Neither of these residues is located in a known functional domain of the protein.

**M2 gene.** The 1918 matrix segment has a spliced M2 ORF of 291 nucleotides, with a predicted 97-amino acid M2 protein. Like other influenza A viruses, the Brevig18 M2 protein has

A



B

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M S L L T E V E T Y V L S I V P S G P L K A E I A Q R L E D
26-ATGAGTCTTTTAAACCGAGGTCGAAACCTACGTTCTCTATCGTCCCGTCAGGCCCCCTCAAAGCCGAGATCGCGCAGAGACTTGAAGAT-115
M S L L T E V E T

V F A G K N T D L E A L M E W L K T R P I L S P L T K G I L
GTCTTTGCAGGGAAGAACACCGATCTTGAGGCTCTCATGGAATGGCTAAAGACAAGACCAATCTGTACCTCTGACTAAGGGGATTTTA-205

G F V F T L T V P S E R G L Q R R R F V Q N A L N G N G D P
GGATTTGTGTTCCAGCTCACCGTCCCGAGTGAGCGAGGACTGCAGCGTAGACGCTTTGTCCAAAATGCCCTTAATGGGAACGGGGATCCA-295

N N M D R A V K L Y R K L K R E I T F H G A K E V A L S Y S
AATAACATGGACAGAGCAGTTAAACTGTACAGGAAGCTTAAGAGGGAGATAACATTCATGGGGCCAAAGAAGTAGCAGTCAAGTATTCC-385

A G A L A S C M G L I Y N R M G T V T T E V A F G L V C A T
GCTGGTGCACCTGCCAGTTGTATGGCCCTCATATACAACAGGATGGGACTGTGACCCTGAAGTGGCATTGGCCCTGGTATGCCCAACC-475

C E Q I A D S Q H R S H R Q M V T T T N P L I R H E N R M V
TGTGAACAGATTGCTGATCCAGCATCGGTCTCACAGGCAATGGTGACAACAACCAATCCACTAATCAGAGATGAGAACAGAAATGGTA-565

L A S T T A K A M E Q M A G S S E Q A A E A M E V A S Q A R
CTGGCCAGCAGTACGGCTAAGGCTATGGAGCAATGGTGGATCGAGTGAGCAAGCAGCAGAGGCCATGGAGTTGCTAGTCAGGCTAGG-655

Q M V Q A M R T I G T H P S S S A G L K D D L I E N L Q A Y
CAATGGTGCAGGCGATGAGAACCATTGGGACTCATCTAGCTCCAGTGTGGTCTGAAAGACGATCTTATTGAAATTTGCAGGCTAC-745
P T

Q K R M G V Q M Q R F K stop
CAGAACGAATGGGGTGCAGATGCAACGATCAAGTGATCCTCTCGTTATTGCCGCAAGTATCATTGGGATCTTGCACTTGATATTGTG-835
R N E W G C R C N D S S D P L V I A A S I I G I L H L I L W

GATCTTGATCGTCTTTTTTCAAATGCATTTATCGTCCGCTTAAATACGGTTTGAAGAGGGCCCTTCTACGGAAGGAGTCCCGGAGTC-925
I L D R L F F K C I Y R R L K Y G L K R G P S T E G V P E S

TATGAGGGAAGAAATATCGAAAGGAACAGCAGATGCTGTGGATGTTGACGATGGTCATTTGTCAACATAGAGCTGGAGTAA-1007
M R E E Y R K E Q Q S A V D V D D G H F V N I E L E stop
    
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FIG. 1. Sequence of the 1918 matrix segment. (A) Diagram of the matrix gene segment of an influenza A virus showing the positions of the two overlapping ORFs. Nucleotides 1 to 25 and 1008 to 1027 are noncoding. The M1 and M2 ORFs share the first nine codons. (B) Sequence of the A/Brevig Mission/1/18 (H1N1) matrix gene segment coding region (GenBank accession no. AY130766). The coding sequences start at nucleotide 26; M1 and M2 share the initial nine codons. The M1 theoretical translation is shown above the line; that of M2 is shown below the line. The M2 transmembrane is boxed. The M2 ectodomain consists of the first 24 M2 protein amino acids (see also Fig. 3). The numbering of the gene segment is aligned to A/USSR/90/77 (GenBank accession no. M54941) and refers to the sequence in the sense (mRNA) orientation.

the potentially palmitoylated C at position 50 (45) and the potentially phosphorylated S at position 64 (22) within the cytoplasmic domain. Within the transmembrane domain, residue 28 is I in Brevig18 but V in most other human viruses.

**Amantadine resistance region.** Five amino acid sites that have been identified in the transmembrane region of the M2 protein are involved in resistance to the antiviral drug amantadine: sites 26, 27, 30, 31, and 34 (5, 18, 21). The Brevig18 sequence is identical at these positions to that of the amantadine-sensitive strain Singapore57. Thus, it is predicted that the M2 protein of Brevig18 would be sensitive to amantadine.

**Phylogenetic analyses.** Thirty-seven M1 nucleotide sequences were analyzed by neighbor joining, with the proportion of differences as the p-distance. Four clades were differentiated: human, swine, Eurasian avian, and equine plus North

American avian. Brevig18 was located within and near the root of the human clade (Fig. 2A). Synonymous and nonsynonymous changes were analyzed separately. Synonymous changes gave a result essentially identical to that shown in Fig. 2A. Little information was obtained by analyzing nonsynonymous changes, which produced a tree with virtually no horizontal depth for most branches. This was expected given the high degree of conservation of the M1 protein.

Thirty-seven M2 nucleotide sequences were analyzed by the neighbor-joining method as described above. A tree very similar to that seen with the M1 sequences was obtained (Fig. 2B), except that only three clades were differentiated: human, swine, and avian plus equine. Brevig18 was located within and near the root of the human clade. Analyzing synonymous positions alone gave a similar result. The analysis of nonsynonymous substitutions

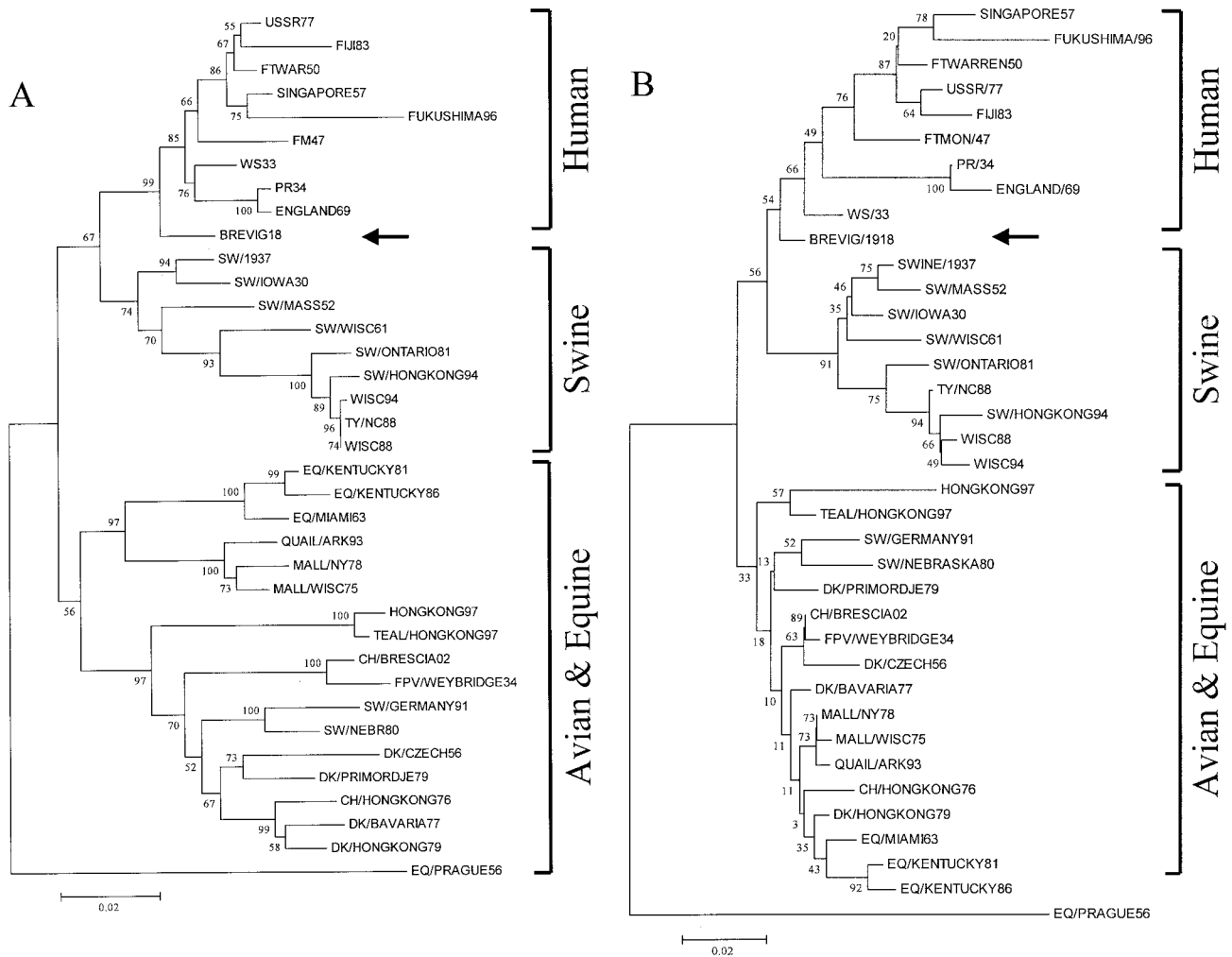


FIG. 2. Phylogenetic trees of the Brevig18 M1 (A) and M2 (B) nucleotide sequences. Both trees are neighbor-joining trees determined by p-distance. The Brevig18 strain is indicated by an arrow. Bootstrap values are given for selected nodes, and distance bars are shown below the trees.

alone resulted in a tree in which Brevig18 and Sw/Iowa30 were placed within and near the root of the avian-equine clade. However, bootstrap values for this region of the tree were extremely low, suggesting little real support for this topology.

The phylogenetic analyses suggest that the Brevig18 matrix genes, while more avian-like than those of other mammalian influenza viruses, were mammalian adapted. For example, the extracellular domain of the M2 protein contains 4 amino acids that differ consistently between the avian and mammalian clades (M2 residues 14, 16, 18, and 20). Brevig18 matches the mammalian sequence at all 4 of these residues (Fig. 3).

## DISCUSSION

Several model systems have indicated that small changes in the matrix proteins can have large effects on phenotype. Replacing the matrix segment of human viruses with that of PR34 consistently results in a higher yield of the strain for vaccine development (2). The PR34 M1 gene has been shown to confer high-growth characteristics in single-gene reassortant strains (55). The PR34 M1 protein, however, differs by fewer than 10

amino acids from modern human strains. Other studies indicate that the matrix segment of WSN33 (like PR34, a repeatedly passaged derivative of an early human strain) can also confer a high-yield phenotype (55). However, between them, the M1 proteins of WSN33 and PR34 share only a few amino acids that differ consistently from modern human strains and each has additional, unique differences. Therefore, it is not clear which amino acid changes are responsible for the high-yield phenotype or even if the same amino acids are responsible in both PR34 and WSN33. At those M1 sites where PR34 and WSN33 share amino acids found in few other strains, the Brevig18 sequence instead matches the human consensus. Only at M1 site 218 does Brevig18 share a characteristic with PR34 and WSN33 that distinguishes them from modern human strains. PR34, WSN33, and Brevig18 all retain the threonine that is found in avian strains, whereas all subsequent human strains have replaced the avian threonine with an alanine or valine.

While the PR34 and WSN33 matrix segments contribute to a high-growth phenotype when reassortant human viruses are grown on chicken eggs, other experiments suggest that minor

		1	1111111112	2222
	1234567890	1234567890	1234	
BREVIG18	MSLLTEVETP	TRNEWGCRN	DSSD	
WS33	.....I	.....	.....	
PR34	.....I	.....G	.....	
FTWAR50	.....I	.....	.....	
SINGAPORE57	.....I	.....	.....	
USSR77	.....I	.....	.....	
FIJI83	.....I	.....	.....	
ENGLAND69	.....I	.....G	.....	
FUKUSHIMA96	.....I	.....	.....	
HK/156/97	.....L	...G...S	.....	
HK/482/97	.....L	...G...S	.....	
CH/HK/220/97	.....Y	...G...S	.....	
HK/1073/99	.....L	...G.E.K.R	.....	
GS/Guangdong96	.....P	...K...E.K.S	.....	
CH/BRESCIA02	.....	...G.E...S	.....	
FPV/WEYBRIDGE34	.....	...G.E...S	.....	
DK/CZECH56	.....	...G.E...YS	G...	
MALL/WISC75	.....	...G.E...K.S	.....	
CH/HONGKONG76	.....	...G.E...K.S	.....	
DK/BAVARIA77	.....	...G.E...K.S	.....	
MALL/NY78	.....	...G.E...K.S	.....	
DK/HONGKONG79	??????	...G.E...K.S	.....	
DK/PRIMORDJE79	.....	...G.E...S	.....	
QUAIL/ARK93	.....	...G.E...K.S	.....	
TEAL/HONGKONG97	.....L	...G.E...K.S	.....	
EQ/KENTUCKY81	.....	...G.E...K.S	.....	
EQ/KENTUCKY86	.....	...G.E...K.S	G...	
EQ/MIAMI63	.....	...G.E...K.S	.....	
SW/NETH80	.....	...K.G.E...S	.....	
SW/GERMANY91	.....	...G.E...YS	.....	
SW/IOWA30	.....	.....	.....	
SWINE/1937	.....	I.....	.....	
SW/MARCH52	.....	I.S.....	.....	
SW/WISC61	.....	...S.....	.....	
SW/ONTARIO81	.....	I.....	.....	
TY/NC88	.....	I.....K.	.....	
WISC88	.....	I.....K.	.....	
WISC94	.....	I.....K.	.....	
SW/HONGKONG94	.....	I.....K.	.....	

FIG. 3. Alignment of M2 extracellular domain protein sequences. The first 24 residues of M2 are shown. Amino acids matching Brevig18 are shown as dots. The first 6 residues of Dk/HongKong/79 are unknown and are represented by question marks.

changes in the matrix proteins also contribute to higher growth in mouse lung. Passage of the FM47 virus through the mouse lung resulted in a single amino acid change in M1, T139A, that was shown in single gene reassortment assays to be responsible for higher yield in mouse lung (42, 43). Adaptation of another human virus (HK68) to mouse resulted in high-yield variants with one amino acid change in M1 (T167A) and one in M2 (D44N) (6). Govorkova et al. adapted one avian (A/black duck/NJ/1580/78 [H2N3]) and one human (A/Japan × Bel-lamy/57 [H2N1]) virus to the mouse lung and again found that high-growth variants had only 1 or 2 amino acid changes in the matrix proteins (13). Changes making viruses more virulent in the mouse lung also appear to increase growth rates in embry-onated chicken eggs and MDCK cells, suggesting that the changes may have functional significance beyond mouse adap-tation (13, 56). None of these changes were found in Brevig18.

Several closely related H5N1 viruses were isolated from humans in 1997. When the complete genomes of strains with a high-pathogenicity phenotype were compared to those of low pathogenicity, a total of 5 amino acid differences were found to correlate with the high-pathogenicity phenotype. One change was in M1 (V15I), a change that has also been found in PR34 but not in Brevig18 (24).

The amino acid changes found to increase virulence in these

studies are distributed over the M1 gene from amino acids 15 to 227 and often fall in areas of no known function. Almost all of the changes resulting in increased yield or virulence are not found in any other strain. Overall, the studies suggest that matrix sequence variation plays an important role in virulence but that virulence cannot be predicted directly from the matrix sequence. The 1918 matrix sequence does not code for any of the single amino acid changes that correlate with virulence in these studies.

While the PR34 matrix segment appears to confer a high-growth phenotype on a wide range of human viruses, other studies suggest that the phenotypic effect of the matrix segment depends on viral context. Murphy et al. showed that replacing the matrix segment of a human H3N2 virus (A/Udm/307/72 [H3N2]) with that of A/mallard/NY/6750/78 (H2N2) resulted in severe growth restriction in the squirrel monkey respiratory tree, while replacement with the matrix segment of A/pintail/Albarta/119/79 (H4N6) caused no growth restriction (33). The two avian segments differed at only 3 amino acids, 2 in M1 and 1 in M2. Similarly, Subbarao et al. showed that the matrix segment of A/Ann Arbor/6/60 (H2N2) attenuated A/Korea/82 (H3N2) but not A/Udm/72 (H3N2) (44).

The Brevig18 M1 and M2 sequences do not code for any of the unique amino acid changes that correlate with the high-yield phenotype in different model systems. The amino acids that are unique to the PR34 matrix segment, which may con-tribute to its ability to confer a high-yield phenotype on many human viruses, are not shared by Brevig18. There is not a single amino acid position in M1 or M2 that is unique to Brevig18, suggesting that if the matrix genes were involved specifically in viral lethality in 1918, a combination of substi-tutions may have played a role. There is, however, substantial evidence that small changes in matrix sequence, in a particular viral context, can result in a high-yield phenotype. It is possible that the unique Brevig18 matrix sequence would result in a high-yield phenotype in any human strain, but the possibility that it would contribute to lethality only in the context of the other 1918 viral proteins cannot be ruled out. It may well be that a particular constellation of amino acids in several pro-teins contributed to the virulence of the 1918 strain.

Influenza viruses must have at least a novel HA to cause a pandemic (26). However, they must also be readily transmis-sible between humans and capable of replicating in the human lung. In the pandemics of 1957 and 1968, all of the gene segments encoding internal proteins (with the exception of PB1) were retained from the strain previously circulating in humans (25). Determining whether the matrix segment was similarly retained in 1918 is complicated because the matrix proteins are very highly conserved. Only about 5% of M1 amino acids differ among the most divergent strains. While almost 20% of M2 amino acids vary, they tend to vary more between species than within them and do not appear to change progressively over time like the surface proteins. Phylogenetic analyses suggest that the matrix proteins are not under contin-uous positive selective pressure in humans; however, there are certain amino acid residues that distinguish the human clades from those of swine and birds. In M1, 3 residues (115, 121, and 137) distinguish the human clade from the 1930s to the present. Brevig18 matches the human consensus at one of the three, retaining the amino acid characteristic of avian strains at

the other two. By contrast, in M2, 7 amino acids are found in all human adapted strains; Brevig18 matches the human consensus at 5 of them (Fig. 3). Of interest is the fact that 4 of these 5 amino acids are coded for by the short stretch of nucleotides that are used in overlapping reading frames in both M1 and M2. Since the corresponding amino acids in M1 are extremely conserved, change in M2 in this area is highly constrained. If Brevig18 was acquired directly from an avian source, these four concerted changes had to have been made very quickly. While this observation might suggest that the 1918 matrix segment was retained from the previously circulating human strain, overall phylogenetic analyses suggest an intermediate position for Brevig18 between avian and subsequent mammalian strains, similar to the phylogenetic position of previously analyzed 1918 gene segments. It will probably not be possible to determine whether or not the 1918 matrix segment shifted immediately before the pandemic without samples of the influenza virus strain circulating in humans before 1918.

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