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Sequential Mutations in the NS Genes of Influenza Virus Field Strains

M. KRYSTAL, D. BUONAGURIO, J. F. YOUNG, AND P. PALESE*
Mount Sinai School of Medicine of CUNY, New York, New York 10029

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The complete nucleotide sequences of the NS genes from three human influenza viruses, A/FM/1/47 (H1N1), A/FW/1/50 (H1N1), and A/USSR/90/77 (H1N1), were determined. Only five single-base differences were found within the sequences of the A/FW/1/50 and A/USSR/90/77 NS genes, thus confirming earlier data suggesting that the 1977 H1N1 viruses are closely related to virus strains that were circulating around 1950. Comparison of all three sequences with those from A/PR/8/34 and A/Udorn/72 viruses illustrates that these genes (with the exception of that of the A/USSR/90/77 strain) evolve through cumulative base changes along a single common lineage. A nucleotide sequence variation of approximately 2.2 to 3.4% per 10 years was determined for the NS gene segments. Extensive size variation was also observed among the NS1 proteins of the various human viruses. The A/FM/1/47 NS1 protein, which consists of 202 amino acids, is 15% shorter than the A/Udorn/72 NS1 protein, which consists of 237 amino acids.

The influenza A virus genome is comprised of eight negative-stranded RNA segments which code for at least 10 distinct polypeptides (for review, see references 16, 28, 43). The smallest of these gene segments codes for the NS1 (14, 21, 22, 31, 33) and the NS2 polypeptides (13, 17, 18), which are not found in mature virions. The mRNA molecule coding for NS2 is derived from the NS1 mRNA through a splicing event, so that only the first 10 amino acids of NS2 are identical to those of NS1 (13, 17, 18, 30). During the last 60 years, reassortment of the surface protein genes of influenza viruses has been observed, resulting in antigenic shifts from H1N1 to H2N2 and from H2N2 to H3N2 subtypes. Earlier studies based on RNA:RNA hybridization (32, 33, 35) and partial RNA sequencing of various NS genes (11) suggested that conservation of the NS segment occurred during that period. Complete sequences of the NS RNA segments from the two human virus isolates, A/PR/8/34 (H1N1) and A/Udorn/72 (H3N2), which were isolated 38 years apart, revealed a greater than 91% nucleic acid homology (2, 19). The 91% sequence conservation between the NS genes of these viruses compares with sequence differences of approximately 50% between their hemagglutinin subtypes.

In this paper, we report the sequences of the NS genes of three additional field strains which were isolated in 1947, 1950, and 1977. The purpose in further examining NS gene sequences was twofold. First, we wished to determine whether sequential mutations are occurring in genes other than the hemagglutinin gene. The extent and nature of such changes can only be determined through the analysis of complete gene sequences from several field strains.

Second, we wished to analyze in more detail the H1N1 viruses which reappeared in 1977. Oligonucleotide mapping, hybridization analysis of the RNAs, and serological characterization of the 1977 strains had revealed a close relationship with viruses isolated around 1950 (15, 25, 34). Our data on the NS gene sequences confirm these earlier findings.

MATERIALS AND METHODS

Viruses. Influenza viruses A/USSR/90/77, A/FM/1/47, and A/FW/1/50 were grown in embryonated chicken eggs. Virus purifications through sucrose gradient centrifugation and RNA extractions were done as described previously (31).

Cloning and sequencing. Double-stranded cDNA was synthesized from purified influenza viral RNA by using reverse transcriptase and the synthetic oligonucleotide primers d(AGCAAAAGCAG)rG and d(AGTAGAAACAAG) (Collaborative Research, Inc., Waltham, Mass.) as described previously (2). Virus-specific double-stranded cDNA was inserted into pBR322 DNA by using synthetic *Eco*RI linkers. *Escherichia coli* cells were transformed with the DNA, and clones containing NS-specific cDNA were selected by hybridization to nitrocellulose filters (10) by using the previously cloned A/PR/8/34 NS gene as a hybridization probe (2). NS gene-containing plasmids were isolated from bacterial cells through a modification of the alkaline extraction procedure (4).

The sequences of the NS gene inserts were determined by using the Maxam and Gilbert chemical modification procedure (23). DNA fragments suitable

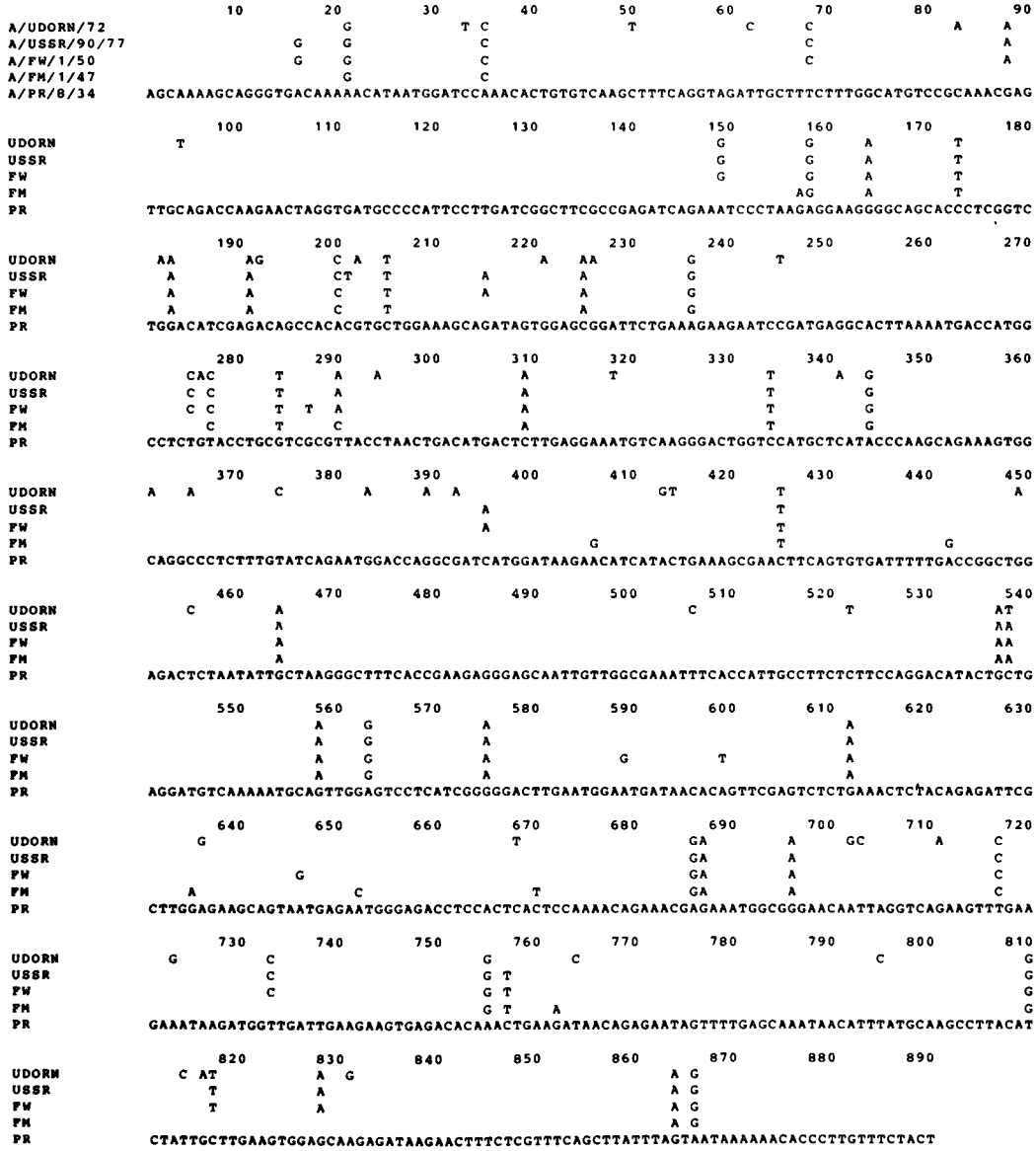

FIG. 1. Nucleotide sequences of human influenza viral NS genes. The complete sequence of the A/PR/8/34 NS gene (2) is shown. For the other sequences, only bases which are changed relative to the A/PR/8/34 sequence are indicated. The A/Udorn/72 NS sequence was reported previously (16).

for sequence analysis were obtained either by strand separation or by secondary cleavage of 5' end-labeled restriction enzyme fragments. The complete sequence of both DNA strands was determined for the A/USSR/90/77 virus NS gene. Large portions of the A/FM/1/47 and A/FM/1/50 virus NS genes were sequenced by using only one strand of the DNA. However, in all instances, the sequences across the boundaries of the restriction enzyme sites used to generate fragments were confirmed by using overlapping DNA fragments.

The nucleotide and amino acid sequences were stored, edited, and compared in an IBM 370 computer at the University Computing Center of the City University of New York by using published programs (39–41).

RESULTS

Cloning and sequencing. Two alternative approaches are available for sequencing related RNAs from influenza viruses. Direct sequencing

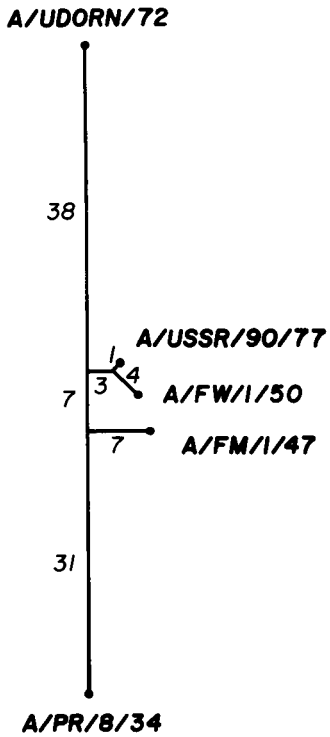

FIG. 2. Evolutionary tree using nucleotide differences among NS genes. Numbers indicate single-base differences. For the construction of this tree, 3 nucleotide positions out of 890 were omitted. In positions 290, 538, and 757, a second nucleotide change would have had to occur if the mutations were to be sequential. Inclusion of these hypothetical nucleotide changes would not permit construction of a linear evolutionary tree.

of virion RNAs by using reverse transcriptase and primers isolated from previously cloned DNA has been successfully used to sequence influenza A (7) and influenza B (M. Krystal and P. Palese, unpublished data) virus hemagglutinins. A second method entails cloning of the specific virion RNA followed by sequencing. We chose the latter approach because in previous experiments we always obtained a large number of full-length NS clones. This is probably due to the small size of the NS RNA segment. Starting with 10 μg of purified A/FM/1/47 virion RNA, we obtained almost 1,000 recombinant clones. Of the first 300 that were screened, 6 clones were identified as NS positive, and 3 of the 6 migrated as full-length clones in agarose gels. The other viral RNAs yielded similar numbers of full-length NS clones.

Nucleotide sequences. The cDNA inserts of the NS genes of A/FM/1/47, A/FW/1/50, and A/USSR/90/77 viruses were sequenced as described above. A comparison of these three sequences with those of the previously published sequences from A/PR/8/34 (2) and A/Udorn/72 (19) viruses permits analysis of their relatedness (Fig. 1). Nucleotide variations are seen throughout the segments, which are all 890 bases long. Excluding the USSR/90/77 gene, mutations occurring at a particular base in early isolates are usually conserved in the later strains, suggesting an evolutionary relationship. By using the sequence of the oldest isolate in this study (A/PR/8/34) as a baseline, it is seen that 31 nucleotide changes are shared among the remaining NS genes (Fig. 2). Only seven nucleotide substitutions are unique for the NS gene of the A/FM/1/47 virus. Seven shared changes then define the next branch point of the evolutionary tree. Finally, there are 38 unique changes in the A/Udorn/72 NS gene (Fig. 2).

The NS gene nucleotide sequences of A/USSR/90/77 and A/FW/1/50 viruses are nearly identical, differing at only five nucleotide positions. Clearly, the A/USSR/90/77 NS gene is closely related to the A/FW/1/50 gene, as shown in Fig. 2 and 3. It should be noted, however, that the A/USSR/90/77 virus was isolated 27 years later than the A/FW/1/50 virus. Thus, this virus appears to be anomalous with respect to the temporal relationship established by the other strains.

An alternative method of analysis of the sequence data involves comparison of the silent mutations in the different genes. In the case of

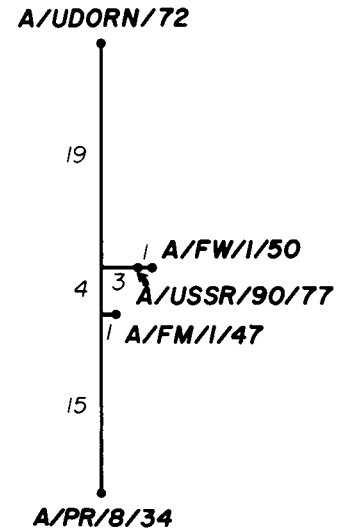

FIG. 3. Evolutionary tree using silent nucleotide changes in the NS genes. The entire gene (including untranslated regions) was used for this analysis. A single silent nucleotide change at position 757 was omitted to allow for construction of a tree with additive genetic distances (see the legend to Fig. 2).

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                      10        20        30        40        50        60
A/UDORN/72      S                 Q V                              N  A HV
A/USSR/90/77                      Q                                N    CV
A/FW/1/50                         Q                                N     V
A/FM/1/47                                             K            N     V
A/PR/8/34     MDPNTVSSFQVDCFLWHVRKRVADQELGDAPFLDRLRRDQKSLRGRGSTLGLDIETATRA

                      70        80        90       100       110       120
UDORN               K                T     I     I  L    F  M     E      I
USSR                                 A           I       F  M
FW                                   A           I       F  M
FM                                   A           I       F  M
PR            GKQIVERILKEESDEALKMTMASVPASRYLTDMTLEEMSRDWSMLIPKQKVAGPLCIRMD

                     130       140       150       160       170       180
UDORN                 M                                    F    I        I
USSR                                                            N        I
FW                                                              N        I
FM                  S           G                               N        I
PR            QAIMDKNIILKANFSVIFDRLETLILLRAFTEEGAIVGEISPLPSLPGHTAEDVKNAVGV

                     190       200       210       220       230
UDORN                        K       G                K  R A  K RRDKMAD
USSR                         K                        K  R      RRNKMAD
FW                   S       K          S             K  R      RRNKMAD
FM                           K      ***********************************
PR            LIGGLEWNDNTVRVSETLQRFAWRSSNENGRPPLTPKQKREMAGTIRSEV*******
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FIG. 4. Comparison of amino acid sequences of NS1 proteins. The single-letter amino acid code is used. The complete sequence of the NS1 protein from A/PR/8/34 virus is shown. For the other viruses, only the amino acid differences are illustrated. Termination codons are present in the A/FM/1/47 and A/PR/8/34 NS1 proteins at positions 203 and 231, respectively.

the NS gene, a silent mutation is a nucleotide change which does not alter the protein sequence of either the NS1 or NS2 proteins. Since these changes are presumably not subjected to selective pressure, comparison of the silent mutations should offer a more reliable marker for evolutionary analysis. Fifteen silent base changes accrued during the evolution of the NS gene from A/PR/8/34 to the A/FM/1/47 virus. These changes are also observed in the other NS genes. In essence, comparison of the silent mutations in the different genes reveals a comparable evolutionary pattern to that obtained when total nucleotide changes are used.

Amino acid sequences. Figure 4 shows the deduced amino acid sequences of the NS1 proteins. The NS1 polypeptides of A/FW/1/50, A/USSR/90/77, and A/Udorn/72 viruses are 237 amino acids long, whereas the A/PR/8/34 NS1 protein is 7 amino acids shorter. A nonsense mutation in position 635 of the A/FM/1/47 NS gene results in an NS1 polypeptide of only 202 amino acids. Comparison of the N-terminal 202 amino acids of the five NS1 polypeptides reveals an evolutionary pattern identical to that established by analysis of the nucleotide changes in their RNAs (Fig. 5).

Figure 6 compares the deduced NS2 polypeptides of the different NS genes. Since the splicing signals identified for the NS2 messenger RNA of the Udorn strain are conserved in the sequences of all other NS genes, it is likely that all NS2 polypeptides share the 10 N-terminal amino acids with their corresponding NS1 polypeptides. If this assumption is correct, the NS2 polypeptides of the five NS genes all have an identical length of 121 amino acids. Because of the small number of changes involved, no attempt was made to establish an evolutionary tree of the NS2 polypeptides.

DISCUSSION

We have obtained the complete sequences of the NS genes from three influenza virus field isolates and compared them with those of two previously sequenced NS genes (2, 19). Evolutionary trees were drawn for the five human NS genes based on total nucleotide, silent nucleotide, or amino acid changes. The results indicate that the NS genes in these human strains evolve along a common lineage. Similar observations were made previously for the hemagglutinin genes when amino acid and nucleotide sequences from H3 subtype viruses were compared (7, 36–38). Antigenic drift in the hemagglutinin was shown to be the result of these cumulative changes. Less conclusive evidence based on partial RNA sequencing (11) and oligonucleotide mapping (26, 48) has suggested that sequential mutations are also occurring in the

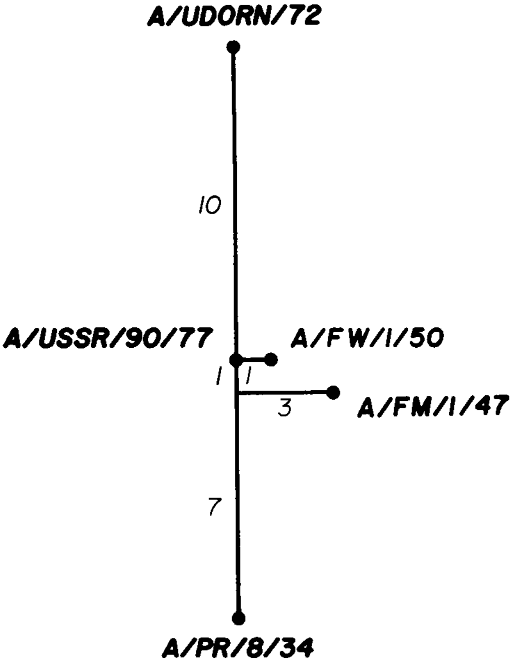

FIG. 5. Evolutionary tree using amino acid changes in the NS1 proteins among the human influenza viruses. Because of the early termination at amino acid position 203 in the A/FM/1/47 NS1 protein, only the first 202 amino acids were used in the construction of this tree. Amino acid positions 59 and 84 were not included because more than one sequential amino acid change occurred in these positions. Inclusion of these changes would not permit construction of an evolutionary tree with additive genetic distances. Amino acid 171 was also omitted because it was affected by nucleotide 538, which was not included in the nucleotide analysis (see the legend to Fig. 2).

genes coding for nonsurface proteins. Data presented in this paper further support the notion that cumulative mutations contribute to the genetic evolution of influenza virus genes. Since the observed mutations in the NS genes appear to be sequential, this suggests that reassortment has probably not contributed significantly to the evolution of the NS gene of these human viruses. This is in agreement with previous hybridization studies (33, 35). Although reassortment of NS genes may occur between cocirculating strains, the close similarity in these genes would normally preclude the detection of such an event. However, the NS genes of the H1N1 and H3N2 viruses which have been cocirculating since 1977 have significant differences which could allow detection of an NS gene reassortment. It should be noted that H1N1 viruses isolated in 1978 which derived genes from cocirculating H3N2 viruses contained the NS gene from the H1N1 parent (49).

An approximate rate of variation in the NS gene can be calculated by using the A/PR/8/34, A/FM/1/47, A/FW/1/50, and A/Udorn/72 sequences. This averages to 2.2 to 3.4% sequence divergence per 10 years. Obviously, this value represents only a crude estimate and does not take into account sampling problems inherent in this analysis. It is not known whether the strains used are representative of the strains circulating at a particular time. Furthermore, the A/PR/8/34 virus has undergone repeated egg and animal passages which may have contributed to additional changes. This may account for the small number of differences observed between the NS sequences of the A/PR/8/34 virus used in our laboratory and that used in another laboratory (47). However, it should be mentioned in this context that repeated (12 times) egg passage of the same virus isolate under low dilutions did not change the oligonucleotide map pattern of the RNA of the strain (8).

The variation rate observed among the NS genes can be compared with rates calculated for the subtype 3 hemagglutinin genes. Comparison of the complete HA sequences from A/Aichi/2/68 (42), A/MEM/102/72 (37), A/Vic/3/75 (24), and A/BK/1/79 (7) reveals a sequence variation rate between 4.5 and 6.5% per 10 years (Table 1). This rate appears to be higher than that observed for the NS genes. Since the pressure imposed on the hemaggluti-

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                        10        20        30        40        50        60
A/UDORN/72        S                  G   E                              L
A/USSR/90/77                   M       G   E
A/FW/1/50                      M       G   E    V  L               V
A/FM/1/47                      M       G   E                   E     L     F
A/PR/8/34       MDPNTVSSFQDILLRMSKMQLESSSGDLNGMITQFESLKLYRDSLGEAVMRMGDLHSLQN

                        70        80        90       100       110        121
UDORN             G                      R  T              Q  F
USSR              G                      R                 Q  F
FW                G                      R                 Q  F
FM                G                      R                 Q
PR              RNEKWREQLGQKFEEIRWLIEEVRHKLKITENSFEQITFMQALHLLLEVEQEIRTFSFQLI
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FIG. 6. Comparison of NS2 amino acid sequences. The single-letter amino acid code is used.

TABLE 1. Variation of influenza A viral genes

Gene[a]	% Variation per 10 yr	% Silent mutations[b] per 10 yr
NS	2.2–3.4	3.1–4.2
M	1.7	3.3
N2 subtype (NA)	10.2	14.4
NP	2.2	5.2
H3 subtype (HA)	4.5–6.5	7.1–8.6
Pac	2.1	4.6
Pb1	4.7	12.2

[a] Sequences used for these comparisons are as follows. NS: A/PR/8/34 (2), A/FM/1/47 (this paper), A/FW/1/50 (this paper), A/Udorn/72 (19), M: A/PR/8/34 (44), A/Udorn/72 (20), NA: A/NT/60/68 (3), A/Udorn/72 (22), NP: A/PR/8/34 (45), A/NT/60/68 (12), HA: A/Aichi/2/68 (42), A/MEM/102/72 (37), A/Vic/3/75 (24), A/BK/1/79 (7), Pac: A/PR/8/34 (9), A/NT/60/68 (6), Pb1: A/PR/8/34 (46), A/NT/60/68 (5).

[b] A base change was counted as silent if it did not alter any known protein sequence. The silent mutation rate was calculated by dividing the number of silent mutations by the number of base positions at which silent mutations could conceivably occur. The latter number was calculated as in reference 6, except that each untranslated base was also counted.

nin by the host immune response is probably greater than that on the NS proteins, we wished to compare these two gene sets by using only silent mutations. This analysis again gave lower values for the genes coding for the NS proteins. The silent mutation rate per 10 years for the NS gene is 3.1 to 4.2%, whereas the silent mutation rate in the hemagglutinin genes appears to be 7.1 to 8.6% per 10 years. Obviously, more sequence data is needed to establish the significance of this finding.

Less reliable variation rates can be calculated for other influenza virus RNA segments. Only two gene sequences from viruses are available for comparison of the membrane (20, 44), neuraminidase (N2 subtype) (3, 22), Pac (6, 9), and Pb1 (5, 46) and nucleoprotein (12, 45) genes. Therefore, sampling problems may compromise the interpretation of these observed variation rates. The data are presented in Table 1. Although there appear to be differences in the variation rates of these genes, the changes observed among hemagglutinin and neuraminidase genes belonging to different subtypes are substantially higher. For example, the hemagglutinin genes of the H1 and H3 subtypes share only a 50% homology.

Comparison of amino acid sequences of the two NS polypeptides among the five viruses was also made. Size variation was not observed in the deduced amino acid sequences of the five NS2 proteins, with all strains exhibiting a length of 121 amino acids. Evaluation of the sequence data in these five viruses, however, shows that the length of the NS1 polypeptides varies from 202 to 237 amino acids. Large variations have previously been observed in the migration rates in polyacrylamide gels among NS1 proteins from various viral isolates (27). Presumably, these differences are due to either charge or polypeptide length. Petri et al. (29) have examined NS1 proteins from different viruses and found them to be heterogeneous with respect to charge and phosphorylation.

An evolutionary analysis was made of the A/USSR/90/77 NS gene sequence in relation to the four other NS sequences (Fig. 2, 3, and 5). By using total nucleotide, silent nucleotide, and amino acid changes, the A/USSR/90/77 NS gene falls on the lineage as if it were a strain circulating around 1950. In fact, only 5 out of the 890 nucleotides are different in the 1977 isolate as compared to the A/FW/1/50 strain. This confirms earlier data obtained by oligonucleotide mapping of the RNAs of these viruses (25), as well as by results based on nucleic acid hybridization (34) and partial RNA sequencing (1). The small number of mutations found strongly indicates that little if any viral evolution took place in the NS genes during a 27-year interval. Thus, the reemergence of the H1N1 subtype in 1977 and the remarkable similarity between the 1950 and the 1977 strains remain a puzzling feature of influenza virus genetics and evolution.

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LITERATURE CITED

1. **Air, G. M.** 1981. Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza virus. Proc. Natl. Acad. Sci. U.S.A. **78**:7639–7643.
2. **Baez, M., R. Taussig, J. J. Zazra, J. F. Young, P. Palese, A. Reisfeld, and A. Skalka.** 1980. Complete nucleotide sequence of the influenza A/PR/8/34 virus NS gene and comparison with the NS genes of the A/Udorn/72 and A/FPV/Rostock/34 strains. Nucleic Acids Res. **8**:5845–5858.
3. **Bentley, D. R., and G. G. Brownlee.** 1982. Sequence of the N2 neuraminidase from influenza virus A/NT/60/68. Nucleic Acids Res. **10**:5033–5042.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Bishop, D. H. L., J. A. Huddleston, and G. G. Brownlee.** 1982. The complete sequence of RNA segment 2 of influenza A/NT/60/68 and its encoded P1 protein. Nucleic Acids Res. **10**:1335–1343.
6. **Bishop, D. H. L., K. L. Jones, J. A. Huddleston, and G. G. Brownlee.** 1982. Influenza A virus evolution: complete sequences of influenza A/NT/60/68 RNA segment 3 and its

predicted acidic P polypeptide compared with those of influenza A/PR/8/34. Virology 120:481–489.
7. Both, G. W., and M. J. Sleigh. 1981. Conservation and variation in hemagglutinins of Hong Kong subtype influenza viruses during antigenic drift. J. Virol. 39:663–672.
8. Brand, C., and P. Palese. 1980. Sequential passage of influenza virus in embryonated eggs or tissue culture: emergence of mutants. Virology 107:424–433.
9. Fields, S., and G. Winter. 1982. Nucleotide sequence of influenza virus segments 1 and 3 reveal mosaic structure of a small viral RNA segment. Cell 28:303–313.
10. Grunstein, M., and D. S. Hogness. 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. U.S.A. 72:3961–3965.
11. Hall, R. M., and G. M. Air. 1981. Variation in nucleotide sequences coding for the N-terminal regions of the matrix and nonstructural proteins of influenza A viruses. Virology 38:1–7.
12. 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. 10:1029–1038.
13. Inglis, S. C., T. Barrett, C. M. Brown, and J. W. Almond. 1979. The smallest genome RNA segment of influenza virus contains two genes that may overlap. Proc. Natl. Acad. Sci. U.S.A. 76:3790–3794.
14. Inglis, S. C., D. J. McGeogh, and B. W. J. Mahy. 1977. Polypeptides specified by the influenza virus genome. 2. Assignment of protein coding functions to individual genome segments by "in vitro" translation. Virology 78:522–536.
15. Kendal, A. P., G. R. Noble, J. J. Skehel, and W. R. Dowdle. 1978. Antigenic similarity of influenza A (H1N1) viruses from epidemics in 1977–1978 to "Scandinavian" strains isolated in epidemics of 1950–1951. Virology 89:632–636.
16. Kilbourne, E. D. (ed.). 1975. The influenza viruses and influenza. Academic Press, Inc., New York.
17. Lamb, R. A., and P. W. Choppin. 1979. Segment 8 of the influenza virus genome is unique in coding for two polypeptides. Proc. Natl. Acad. Sci. U.S.A. 76:4908–4912.
18. Lamb, R. A., P. W. Choppin, R. M. Chanock, and C. J. Lai. 1980. Mapping of the two overlapping genes for polypeptides NS1 and NS2 on RNA segment 8 of the influenza virus genome. Proc. Natl. Acad. Sci. U.S.A. 77:1857–1861.
19. Lamb, R. A., and C.-J. Lai. 1980. Sequence of interrupted and uninterrupted mRNAs and cloned DNAs coding for the two overlapping nonstructural proteins of influenza virus. Cell 21:475–485.
20. Lamb, R. A., and C.-J. Lai. 1981. Conservation of the influenza virus membrane protein (M1) amino acid sequence and an open reading frame of RNA segment 7 encoding a second protein (M2) in H1N1 and H3N2 strains. Virology 112:746–751.
21. Lazarowitz, S. G., R. W. Compans, and P. W. Choppin. 1971. Influenza virus structural and nonstructural proteins in infected cells and their plasma membranes. Virology 46:830–843.
22. Markoff, L., and C.-J. Lai. 1982. Sequences of the influenza A/Udorn/72 (H3N2) neuraminidase gene as determined from cloned full-length DNA. Virology 119:288–297.
23. Maxam, A., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavage. Methods Enzymol. 65:499–560.
24. Min Jou, W., M. Verhoeyen, R. Devos, E. Saman, R. Fang, D. Huylebroeck, and W. Fiers. 1980. Complete structure of the hemagglutinin gene from the human influenza A/Victoria/3/75 (H3N2) as determined from cloned DNA. Cell 19:683–696.
25. Nakajima, K., U. Desselberger, and P. Palese. 1978. Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950. Nature (London) 274:334–339.
26. Ortin, J., R. Najera, C. Lopez, M. Davila, and E. Domingo. 1980. Genetic variability of Hong Kong (H3N2) influenza viruses: spontaneous mutations and their location in the viral genome. Gene 11:319–331.
27. Oxford, J. S., T. Corcoran, and G. C. Schild. 1981. Intratypic electrophoretic variation of structural and nonstructural polypeptides of human influenza A viruses. J. Gen. Virol. 56:431–436.
28. Palese, P., and J. F. Young. 1982. Variation of influenza A, B and C viruses. Science 215:1468–1474.
29. Petri, T., S. Patterson, and N. J. Dimmock. 1982. Polymorphism of the NS1 proteins of type A influenza virus. J. Gen. Virol. 61:217–231.
30. Porter, A. G., J. C. Smith, and J. S. Emtage. 1980. Nucleotide sequence of influenza virus RNA segment 8 indicates that coding regions for NS1 and NS2 proteins overlap. Proc. Natl. Acad. Sci. U.S.A. 77:1857–1861.
31. Ritchey, M. B., P. Palese, and J. L. Schulman. 1976. Mapping of the influenza virus genome. III. Identification of genes coding for nucleoprotein, membrane protein and nonstructural protein. J. Virol. 20:307–313.
32. Rohde, W., E. Harms, and C. Scholtissek. 1977. Biochemical studies on influenza viruses. I. Comparative analysis of equine 2 virus and virus N gene and gene products. Virology 79:393–404.
33. Scholtissek, C., and V. von Hoyningen-Huene. 1980. Genetic relatedness of the gene which codes for the nonstructural (NS) protein of different influenza A strains. Virology 102:13–20.
34. Scholtissek, C., V. von Hoyningen, and R. Rott. 1978. Genetic relatedness between the new 1977 epidemic strains (H1N1) of influenza and human influenza strains isolated between 1947 and 1957 (H1N1). Virology 89:613–617.
35. 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.
36. Sleigh, M. J., and G. W. Both. 1981. The extent of hemagglutinin variation during antigenic drift in the Hong Kong subtype of influenza from 1968 to 1979, p. 341–354. *In* D. P. Nayak (ed.), Genetic variation among influenza viruses. Academic Press, Inc., New York.
37. Sleigh, M. J., G. W. Both, G. G. Brownlee, V. J. Bender, and B. A. Moss. 1980. The haemagglutinin gene of influenza A virus: nucleotide sequence analysis of cloned DNA copies, p. 69–89. *In* G. Laver and G. Air (ed.), Structure and variation of influenza virus. Elsevier/North Holland, New York.
38. Sleigh, M. J., G. W. Both, P. A. Underwood, and V. J. Bender. 1981. Antigenic drift in the hemagglutinin of the Hong Kong influenza subtype: correlation of amino acid changes with alterations in viral antigenicity. J. Virol. 37:845–853.
39. Staden, R. 1977. Sequence data handling by computer. Nucleic Acids Res. 11:4037–4051.
40. Staden, R. 1978. Further procedures for sequence analysis by computer. Nucleic Acids Res. 5:1013–1015.
41. Staden, R. 1979. A strategy of DNA sequencing employing computer programs. Nucleic Acids Res. 6:2601–2610.
42. Verhoeyen, M., R. Fang, W. Min Jou, R. Devos, D. Huylebroeck, E. Saman, and W. Fiers. 1980. Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75. Nature (London) 286:771–776.
43. Webster, R. G., W. G. Laver, G. M. Air, and G. C. Schild. 1982. Molecular mechanisms of variation in influenza viruses. Nature (London) 296:115–121.
44. 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.
45. Winter, G., and S. Fields. 1981. The structure of the gene encoding the nucleoprotein of human influenza virus A/PR/8/34. Virology 114:423–428.
46. Winter, G., and S. Fields. 1982. Nucleotide sequence of

human influenza A/PR/8/34 segment 2. Nucleic Acids Res. **10**:2135–2143.

47. **Winter, G., S. Fields, M. J. Gait, and G. G. Brownlee.** 1981. The use of synthetic oligonucleotide primers in cloning and sequencing segment 8 of influenza virus (A/PR/8/34). Nucleic Acids Res. **9**:237–245.
48. **Young, J. F., U. Desselberger, and P. Palese.** 1979. Evolution of human influenza A viruses in nature: sequential mutations in the genomes of new H1N1 isolates. Cell **18**:73–83.
49. **Young, J. F., and P. Palese.** 1979. Evolution of human influenza A viruses in nature: recombination contributes to genetic variation of H1N1 strains. Proc. Natl. Acad. Sci. U.S.A. **76**:6547–6551.