Pathways of Evolution of Influenza A (H1N1) Viruses from 1977 to 1986 as Determined by Oligonucleotide Mapping and Sequencing Studies

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SUMMARY

The evolutionary relationships of epidemic influenza A (H1N1) viruses isolated between 1982 and 1986 have been examined by oligonucleotide mapping and partial DNA sequencing. The T1 mapping studies confirmed our previous report that the evolution of the influenza virus genome generally results in an average of four to six oligonucleotide changes per year. Between 1982 and 1986, however, two apparent exceptions to this finding occurred. H1N1 antigenic variants (including the A/Chile/83 and A/Victoria/83 reference strains) that caused influenza outbreaks and epidemics from 1983 to 1984 differed by 20 to 30 oligonucleotides from viruses isolated during the previous influenza season. T1 mapping of individual RNA segments and sequencing revealed that all six internal genes of a representative 1983 A/Chile-like virus were more closely related to genes of non-reassortant H1N1 viruses that circulated from 1977 to 1982 than to genes of H3N2 viruses. Therefore, the 1983 variant viruses were not H1N1-H3N2 reassortants. The A/Taiwan/86-like H1N1 antigenic variants that emerged in south-east Asia in the spring of 1986 and caused epidemic activity the following winter also exhibited changes of 20 to 30 oligonucleotides from the A/Chile/83-like or A/Victoria/83-like H1N1 viruses that circulated during the previous influenza season. Fewer oligonucleotide changes were observed between the 1986 A/Taiwan/86-like and H1N1 viruses isolated before 1983, however, suggesting that the former evolved from viruses that circulated before the 1983 antigenic variants became the predominant H1N1 epidemic virus strains. This was confirmed by sequencing the HA1 domain of the haemagglutinin genes of three A/Taiwan/86-like viruses. These studies provide evidence that other genes of influenza A viruses, in addition to the haemagglutinin gene, may evolve concurrently along two or more separate pathways.

INTRODUCTION

Influenza A (H1N1) viruses similar to strains that circulated in 1950 reappeared in epidemic form in 1977 in the midst of an influenza A (H3N2) epidemic (Kendal et al., 1978, 1979a; Scholtissek et al., 1978; Nakajima et al., 1978). This co-circulation of virus subtypes led to mixed infections from which H1N1–H3N2 reassortants could be isolated (Kendal et al., 1979b). Both RNA oligonucleotide mapping and RNA–RNA hybridization techniques readily detected the widespread circulation from 1978 to 1980 of reassortant viruses containing four or five RNA segments from the H3N2 virus and other genes, including those coding for the haemagglutinin (HA) and neuraminidase antigens, from the H1N1 virus (Young & Palese, 1979; Bean et al., 1980; Nakajima et al., 1981). Such reassortant viruses, however, appeared not to circulate after 1981 (Cox et al., 1983). Because influenza A H1N1 and H3N2 viruses have continued to co-circulate we have applied the technique of oligonucleotide mapping to over 130 H1N1 viruses from more than 20 countries to detect isolates with genomes that, like those of the 1978 reassortants, have diverged markedly from their predecessors.
By quantifying numbers of oligonucleotide changes between pairs of T1 oligonucleotide maps of total viral RNA of viruses isolated during a 3-year interval, we had found that for each genotype of virus circulating at the time (i.e. reassortant H1N1, non-reassortant H1N1, or H3N2), the number of oligonucleotide differences between viruses gradually increased as the length of time between their isolations increased (Cox et al., 1983). Here we report the molecular analysis and evolutionary relationships of H1N1 variants that were responsible for major influenza outbreaks and epidemics in 1983 and 1984 (Kendal & Cox, 1985) as well as the more recent 1986 antigenic variants that caused outbreaks in several Asian countries during the first half of 1986 and in the U.S.A. during the 1986 to 1987 influenza season (Centers for Disease Control, 1986, 1987).

METHODS

Viruses. All influenza viruses examined in this study were grown in the allantoic cavity of 10- or 11-day-old embryonated eggs at a low m.o.i. Virus preparations used in this study varied in egg passage level from E3 to E6. The A/USSR/90/77 and A/England/332/80 viruses are well characterized prototype H1N1 viruses, while the A/Texas/12/82 virus was typical of H1N1 viruses isolated during the 1981 to 1982 influenza season. The H1N1 viruses A/Hong Kong/2/82 and A/Hong Kong/32/83 were selected for study here because of their HA sequences (Raymond et al., 1986; Daniels et al., 1985). Variant H1N1 viruses isolated in 1983 selected for study included A/Dunedin/27/83, A/Chile/8/83, A/Georgia/79/83 and A/Victoria/7/83, the antigenic properties and HA1 sequences of which have been reported previously (Raymond et al., 1986). Variant H1N1 viruses isolated in 1986 examined included A/Taiwan/1/86, A/Singapore/17/86, A/Singapore/6/86 and A/Beijing/1/86. The antigenic properties of these viruses were also reported previously (Centers for Disease Control, 1986), as was the sequence of the HA1 of the A/Taiwan/1/86 virus (Robertson, 1987). Influenza A (H3N2) viruses used in T1 mapping studies were the A/Philippines/2/82 and A/Korea/1/83 (data not shown) prototype strains.

T1 oligonucleotide mapping. Procedures for virus purification, extraction of RNA and oligonucleotide mapping have been described previously (Cox et al., 1983; Cox & Kendal, 1984). Briefly, purified RNA was digested with T1 ribonuclease, and the resultant oligonucleotides were labelled at the 5' ends using [γ-32p]ATP and T4 polynucleotide kinase. In the subsequent two-dimensional mapping, electrophoresis in the first dimension was performed at pH 3.5 in a 10% polyacrylamide, 6 M-urea gel, and in the second dimension was performed at pH 8.3 in a 21.8% polyacrylamide gel buffered with Tris-borate.

DNA sequencing. DNA sequencing studies were conducted using the Sanger dideoxynucleotide sequencing method (Sanger et al., 1977) modified for sequencing directly from RNA using synthetic oligodeoxynucleotide primers and reverse transcriptase (Air, 1979; Cox et al., 1986). For RNA segments 1, 5, 6, 7 and 8 of the A/Georgia/79/83 and reference H1N1 viruses, the individual segments were separated by PAGE as described previously (Cox et al., 1985), and approximately 150 to 200 nucleotides were sequenced from the 3' end of the virion RNA using the universal primer 5' (AGCAAAAGCAGG). Primers 5' d(GCTAGCAAGCAT), 5' d(CCACATCCACCT) and 5' d(CACAACATCCA) corresponding to viral RNA sequences beginning at RNA 2 nucleotide 1140 and RNA 3 nucleotides 226 and 452, respectively, were used to obtain sequences from RNA segments 2 and 3 that were inadequately separated by gel electrophoresis. Primers 5' d(TAAAACACCAAAATG), 5' d(AATCATGGTCTACATTG), 5' d(TTTTACAGAATTGCTA) and 5' d(CATAATATTGGGCA) corresponding to viral RNA sequences beginning at RNA 4 nucleotides 19, 304, 513 and 799, respectively, were used to obtain sequences of the HA1 domain of the HA gene. The reaction products were separated by electrophoresis in 0.2 mm thick, 6% or 8% acrylamide gels containing 100 mm-Tris–borate pH 8.3, 5 mm-EDTA and 8 M-urea. After electrophoresis, gels were autoradiographed at -70 °C for 48 h. Nucleotide sequences were stored and analysed in a Digital Corporation VAX computer by using the sequence analysis software package of the University of Wisconsin Genetics Computer Group (Version 5).

RESULTS

Genetic analysis of 1983 antigenic variants

The viruses examined by T1 oligonucleotide mapping and used in pairwise comparisons to calculate the rate of change were chosen for analysis because of their antigenic characteristics (i.e. they were representative of the predominant epidemic strains or had unusual antigenic properties), or because of their geographical or temporal distribution. T1 maps are shown here for only a few of the viruses examined.

The A/Georgia/79/83 virus was chosen as the representative of the 1983 H1N1 antigenic variants for detailed analysis because it was one of the first A/Chile/83-like variants isolated in
**Table 1. Oligonucleotide differences between pairs of H1N1 influenza viruses**

<table>
<thead>
<tr>
<th>H1N1 virus genomes compared (epidemic period)</th>
<th>Time interval of isolation (months)</th>
<th>Mean no. of spot differences (range)</th>
<th>No. of pairs examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1N1 non-recombinant and non-recombinant (1978–1982)</td>
<td>&lt;12</td>
<td>5 (2–8)</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>12–23</td>
<td>8 (4–10)</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>24–35</td>
<td>11 (10–13)</td>
<td>12</td>
</tr>
<tr>
<td>H1N1 recombinant and recombinant (1978–1981)</td>
<td>&lt;12</td>
<td>4 (2–7)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>12–23</td>
<td>9 (7–10)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>24–35</td>
<td>11 (10–12)</td>
<td>4</td>
</tr>
<tr>
<td>H1N1 non-recombinant and recombinant (1978–1980)</td>
<td>&lt;12</td>
<td>27 (26–28)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>12–23</td>
<td>31 (26–37)</td>
<td>5</td>
</tr>
<tr>
<td>H1N1 non-recombinant and 1983 variants (1980–1983)</td>
<td>&lt;12</td>
<td>22 (20–23)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>12–23</td>
<td>22 (18–28)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>12–23</td>
<td>27 (27)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>24–35</td>
<td>28 (27–30)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>48–59</td>
<td>15 (12–19)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>60–71</td>
<td>21 (17–25)</td>
<td>3</td>
</tr>
<tr>
<td>H1N1 1986 variants and H1N1 1986 variants</td>
<td>&lt;12</td>
<td>4 (3–6)</td>
<td>3</td>
</tr>
</tbody>
</table>

The earliest virus isolate examined that had an oligonucleotide map closely related to that of the A/Georgia/83 and the other 1983 antigenic variants was from a patient in Ohio during January 1983. Comparison of the T1 map of this virus (A/Ohio/4/83) with that of a virus isolated only 8 months earlier during the previous influenza season (A/Massachusetts/5/82) revealed 23 oligonucleotide differences, a number similar to the number of differences between the A/Georgia/83 and A/Texas/82 viruses.
Fig. 1. T1 oligonucleotide maps of H1N1 virus strains (a) A/England/333/80, (b) A/Texas/12/82 and (d) A/Georgia/79/83. (c) A diagram of oligonucleotides common and unique to A/Texas/12/82 and A/Georgia/79/83. The positions of dye markers bromophenol blue and xylene cyanol are shown by the letters B and X respectively. In (c) O and O denote oligonucleotides unique to A/Texas/12/82 and A/Georgia/79/83 respectively, and • denotes oligonucleotide common to A/Texas/12/82 and A/Georgia/79/83.
Table 2. Comparison of A/Georgia/79/83 partial gene sequences with H1N1 and H3N2 reference virus sequences

<table>
<thead>
<tr>
<th>RNA segment (encoded protein)</th>
<th>Position of nucleotides sequenced</th>
<th>No. of nucleotide differences between H1N1 and H3N2 viruses</th>
<th>Nucleotides in A/Georgia/83 similar to</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (PB2)</td>
<td>30-204</td>
<td>8*</td>
<td>H1N1 0</td>
</tr>
<tr>
<td>2 (PB1)</td>
<td>1162-1353</td>
<td>24†</td>
<td>H1N1 0</td>
</tr>
<tr>
<td>3 (PA)</td>
<td>252-675</td>
<td>13‡</td>
<td>H1N1 0</td>
</tr>
<tr>
<td>6 (NP)</td>
<td>46-207</td>
<td>10§</td>
<td>H3N2 0</td>
</tr>
<tr>
<td>7 (M)</td>
<td>22-224</td>
<td>7†</td>
<td>H3N1 0</td>
</tr>
<tr>
<td>8 (NS)</td>
<td>20-200</td>
<td>7¶</td>
<td>H3N1 0</td>
</tr>
</tbody>
</table>

* Sequences were compared with those of A/NT/60/68 (Jones et al., 1983) and A/England/333/80.
† Sequences were compared with those of A/NT/60/68 (Bishop et al., 1982a) and A/USSR/90/77.
‡ Sequences were compared with those of A/NT/60/68 (Bishop et al., 1982b) and A/USSR/90/77.
§ Sequences were compared with those of A/NT/60/68 (Huddleston & Brownlee, 1982) and A/USSR/90/77.
¶ Sequences were compared with those of A/Udorn/72 (Lamb & Lai, 1981) and A/England/333/80.

strains A/Philippines/2/82 and A/Korea/1/83 with those of the H1N1 variants from 1983. Oligonucleotides present in 1983 H1N1 antigenic variants but absent in previous H1N1 strains did not correspond to oligonucleotides in H3N2 reference viruses (data not shown). This observation was confirmed by comparing T1 oligonucleotide maps of the isolated RNA segments of the A/Georgia/83, A/Texas/82 and A/Philippines/82 viruses (Fig. 2). Comparison of the individual segments of the 1983 H1N1 virus A/Georgia/83 with those of the A/Texas/82 virus revealed clear similarities. Oligonucleotide differences, however, were present in each segment of the A/Georgia/83 virus except that coding for neuraminidase (not shown). Thirteen oligonucleotide differences were found in RNA segments 1 and 2 combined (PB2 and PB1 genes), five changes in segment 3 (PA gene), seven in segment 4 (HA gene), three in segment 5 (nucleoprotein (NP) gene), and one change each in segments 7 (M1 and M2 protein genes) and 8 (NS1 and NS2 protein genes). No changes were observed in segment 6 (neuraminidase gene). Much greater numbers of oligonucleotide differences were observed between the T1 maps of the H3N2 gene segments and those of the A/Georgia/83 gene segments (Fig. 2).

To confirm the results of T1 mapping, we also determined sequences for between 150 and 420 bases for the PB2, PB1, PA, NP, M and NS genes of the variant A/Georgia/83 and of non-reassortant H1N1 reference strains A/England/333/80 or A/USSR/90/77. These sequences were compared with sequences already published for the H3N2 strains A/NT/60/68 or A/Udorn/72 in order to determine whether the 1983 variant sequences were similar to H1 or H3 sequences. An example of sequence data obtained for each gene is shown using data for the NP gene (Fig. 3). When NP gene sequences of the A/NT/60/68 (H3N2) and A/England/333/80 (N1H1) viruses were compared from nucleotides 46 to 207, differences were found in 10 positions. The A/Georgia/83 virus had the same nucleotide as the H1N1 virus in each of these 10 positions, and had two additional nucleotides differing from the A/England/333/80 sequence (Fig. 3). Likewise, comparing sequences obtained for the other five genes revealed that all genes of the H1N1 variant A/Georgia/83 virus contained H1N1-like sequences. In fact, for the areas of the PB2, PB1, PA, M and NS genes that were sequenced in the A/Georgia/83 virus, seven of eight, 23 of 24, 12 of 13, seven of seven, and seven of seven, respectively, of the nucleotide differences between reference H1N1 and H3N2 strains were found to be H1-like (Table 2).

Genetic analysis of 1986 antigenic variants

T1 oligonucleotide maps were also prepared for viruses representing the new antigenic variants of influenza A (H1N1) that appeared in south-east Asia in early 1986 (Centers for Disease Control, 1986). Both of the prototype strains, A/Taiwan/1/86 (Fig. 4a) and
Fig. 2. T1 oligonucleotide maps of RNA segments of H1N1 viruses A/Texas/12/82 (a to f) and A/Georgia/79/83 (g to k) and of the H3N2 virus A/Philippines/2/82 (l to q). (T1 maps are not shown for the gene segments encoding the HA and neuraminidase proteins.) Gene segments 1 and 2 of A/Georgia/79/83 did not separate well by PAGE and were analysed together. Arrows on the A/Texas/12/82 and A/Georgia/79/83 panels indicate oligonucleotide differences between the two viruses.
Fig. 3. Partial nucleotide sequences of the nucleoprotein genes of the 1983 variant A/Georgia/79/83 (H1N1), A/England/333/80 (H1N1) and A/NT/60/68 (H3N2) viruses written in the mRNA sense starting from the initiation codon.

A/Singapore/6/86 (not shown), exhibited 25 to 30 oligonucleotide changes when compared with representative H1N1 viruses isolated in 1984 and 1985 (Table 1). The 1986 variant viruses, however, exhibited only 13 to 15 changes when compared with viruses that had circulated just before the emergence of the 1983 antigenic variants (Table 1), and when the T1 map of the A/Taiwan/86 virus (Fig. 4a) was compared with that of the A/Texas/82 virus (Fig. 1b) only 12 oligonucleotide differences were detected (Fig. 4d). When the A/Taiwan/86 and A/Alaska/84 viruses were compared, however, 30 oligonucleotide differences were observed (Fig. 4c). The A/Texas/82 and A/Alaska/84 viruses were isolated 51 and 26 months, respectively, before the isolation of the A/Taiwan/86 virus. This suggested that the A/Taiwan/1/86 virus was more closely related to the A/England/80-like viruses than to the A/Chile/83 or A/Victoria/83-like antigenic variants. The T1 maps of the A/Taiwan/86-like viruses were also compared with those of a variety of other viruses including the A/Hong Kong/2/82 and A/Hong Kong/32/83 viruses. It was observed that the A/Singapore/6/86 and A/Hong Kong/2/82 T1 maps differed from each other by 14 oligonucleotides, indicating that the total genomes of these two viruses are also closely related even though they were isolated 41 months apart.

To examine the relationship of the A/Taiwan/86-like viruses to previously isolated viruses in more detail, we obtained nucleotide sequences of the HA1 domains of the HA genes of the A/Taiwan/1/86, A/Singapore/6/86 and A/Beijing/1/86 viruses. These sequences were compared with those already published for H1N1 viruses isolated between 1977 and 1983 (Raymond et al., 1986; Daniels et al., 1985). A direct comparison of the nucleotide and deduced amino acid (Fig. 5) sequences revealed that the HA genes of the three viruses isolated in 1986 were very closely related to each other and more closely related to the HA gene of the A/Hong Kong/2/82 virus than to any of the other HA genes sequenced.

A computer program (written and run by Dr Mark Pallanch, Centers for Disease Control; Rico-Hesse et al., 1987) was used to prepare a dendrogram showing sequence relationships among H1N1 HA sequences based on the numbers of nucleotide differences between HA genes (dendrogram not shown). Using this analysis, the HA genes of the A/Taiwan/86-like-viruses were most closely related to the HA gene of the A/Hong Kong/2/82 virus. The difference table generated above along with the amino acid substitutions were then used to draw an expanded evolutionary tree based on one published previously for viruses isolated up to 1983 (Raymond et al., 1986). This analysis clearly placed the HA of the A/Chile/83- and A/Taiwan/86-like viruses on separate evolutionary branches but placed the A/Hong Kong/2/82 and A/Taiwan/86-like viruses on the same side-branch (Fig. 6a). This analysis was repeated after excluding sequence changes at amino acids 138, 163, 189, 190 and 225 which have been suggested to be dependent on virus growth in eggs (Robertson et al., 1987); however, there was little change in the evolutionary tree (Fig. 6b).

Examination of the sequence changes in the three A/Taiwan/86-like viruses compared to the A/Chile/83 virus (present in the previous vaccine) revealed that the changes relevant to antigenic reactivity are likely to be those in antigenic site Sb at the tip of the HA molecule at amino acids 189, 192, 193, 196 and 197 and the additional glycosylation site caused by the amino acid substitution from lysine to asparagine at position 63. Thus, our data agree with the sequence
Fig. 4. T1 oligonucleotide maps of H1N1 virus strains (a) A/Taiwan/1/86 and (b) A/Alaska/4/84. (c) Diagram of oligonucleotides common and unique to A/Taiwan/1/86 and A/Alaska/4/84. (d) Diagram of oligonucleotides common and unique to A/Taiwan/1/86 and A/Texas/12/82. The position of the dye markers bromphenol blue and xylene cyanol are shown by the letters B and X, respectively. • denotes oligonucleotides unique to A/Taiwan/1/86 (c and d) and ○ denotes oligonucleotides unique to A/Alaska/1/84 (c) or A/Texas/12/82 (d), while ● denotes oligonucleotides common to A/Taiwan/1/86 and A/Alaska/1/84 (c) or A/Taiwan/1/86 and A/Texas/12/82 (d).
Fig. 5. The deduced amino acid sequence of the HA1 region of the HA of three A/Taiwan/86-like viruses compared with sequences of other H1N1 viruses isolated since 1977. Differences between A/USSR/77 and all other strains are shown. The amino acids are numbered to correspond to the numbering for the H3 subtype according to the alignment of Winter et al. (1981) with additional amino acid residues present in the H1 subtype sequence marked by an asterisk. These additional residues are numbered by reference to the previous residue followed by a letter designation, e.g. 53a, 77a, 125a, 125b, 125c etc.
Fig. 6. The evolutionary pathway of the 1977 to 1986 influenza strains deduced from nucleotide changes detected in the HA1 region of the HA. Distances between strains reflect total numbers of nucleotide changes between virus sequences. Numbers refer to the amino acid changes that are either conserved when these numbers are a main branch or strain-specific when the numbers are on side-branches. Numbering is as described in Fig. 5. (a) Evolutionary tree drawn to include all detected changes. (b) Evolutionary tree drawn without changes attributed by Robertson et al. (1987) to the egg-selection process. Amino acid sequences for the A/Beijing/1/86 and A/Singapore/6/86 viruses were identical, so only the position of the A/Singapore/86 virus is shown.
data published by Robertson (1987) for the A/Taiwan/1/86 virus with three exceptions: serine at amino acid position 138, glycine at position 240 and lysine at position 314, where alanine, glutamic acid and arginine respectively were reported previously. The third difference can be explained on the basis that a mixed population exists. We observed bands in both the A and G lanes at nucleotide 1021, with the band in the A lane being darker. Therefore, the codon could be AAA (our results) or AGA (Robertson, 1987) encoding lysine or arginine at amino acid 314, depending on relative band intensities.

DISCUSSION

The rate of evolution of an RNA genome should be approximately constant over time and dependent on several factors including the inherent frequency of error of the RNA-replicating enzymes, selection for variants by the immune system of a partially immune population (for the glycoprotein genes), and selection against variants imposed by structural and functional constraints of viral proteins. The evolution of individual influenza virus genes has been examined extensively using rapid sequencing methods and, where detailed statistical comparisons were made, the following conclusions were drawn. (i) As with other RNA viruses, the influenza virus genes evolve at a very rapid rate compared with DNA genomes (Hayashida et al., 1985; Holland et al., 1982; Buonagurio et al., 1986; Smith & Inglis, 1987). (ii) The rate of amino acid change differs from gene to gene (Hayashida et al., 1985; Ortín et al., 1983; Krystal et al., 1983) but the rate of silent substitution is approximately similar for all genes (Hayashida et al., 1985). (iii) Individual influenza virus genes generally accumulate mutations at approximately constant rates with respect to time during evolution (Hayashida et al., 1985; Buonagurio et al., 1986), with some exceptions where the apparent rate of evolution for a viral gene is higher than expected during a particular period (Raymond et al., 1986; Hayashida et al., 1985).

Evolution of total influenza virus genomes was examined by T1 oligonucleotide mapping supplemented with DNA sequencing to examine viruses of particular interest in more detail. Although T1 mapping is less precise than DNA sequencing, it has been shown to provide estimates of base sequence differences between RNAs that correlate well with values obtained from sequencing data (Young et al., 1979; Palese et al., 1981; Aaronson et al., 1982; Cox et al., 1983). Unlike DNA sequencing, oligonucleotide mapping allows one to examine relatively rapidly the total genome of numerous virus isolates and to determine which of the viruses have genomes that differ substantially from those of co-circulating or previously circulating viruses. Because viruses were not plaque-purified or cloned by terminal dilution in eggs, results are representative of the consensus sequences present in the virus population. Sequence differences in minor subpopulations would not be detected by either oligonucleotide mapping or DNA sequencing.

Influenza A (H1N1) virus genomes that differ substantially from genomes of viruses isolated previously have been identified by oligonucleotide mapping three times during circulation of this influenza subtype in man since 1978. Variant genomes were first detected in 1978 and were also detected in 1983 and 1986. The occurrence in 1978 was accounted for by the emergence of the H1N1-H3N2 reassortants with four or five RNA segments from viruses of the H3N2 subtype (Young & Palese, 1979; Bean et al., 1980). The large numbers of oligonucleotide changes, however, detected between 1983 H1N1 variants and 1980 to 1982 H1N1 non-reassortants cannot be accounted for on the basis of sequence divergence of the HA gene alone, nor by reassortment between H1N1 and H3N2 viruses. Another mechanism(s) must be postulated. Possible mechanisms include the following: (i) an unusual replication event (e.g. a mutation in a polymerase gene increasing its rate of copy error); (ii) a change in the number of total virus replications in humans before January 1983 resulting in a greater accumulation of mutations; (iii) replication in an unusual host with a consequent greater selection for mutations; (iv) reassortment between the previously circulating H1N1 viruses with a different animal or human H1N1 strain not yet determined; (v) rearrangements of viral genes other than gene reassortment; (vi) descent of 1983 variants from a lineage of influenza viruses that diverged from the main pathway in approximately 1979, but that had not been recognized previously because it had little or no epidemiological impact. We feel that the last possibility is the most
likely, based on the previous observations that at least the HA gene of influenza A viruses can
coevolve along separate pathways (Both et al., 1983; Raymond et al., 1986). Because only a small
fraction of the total number of influenza A viruses isolated each year are subjected to molecular
analysis, it is quite possible that viruses circulating at a low level would not be analysed.
Influenza viruses causing less serious or subclinical cases of influenza might not come to the
attention of medical practitioners and therefore would not be isolated. Such strains might
continue to circulate and acquire mutations during replication that increase their transmissi-
bility or virulence so that the mutated virus can cause epidemics; only then would these viruses
be recognized as important strains and be analysed in a variety of ways.

In the case of the 1986 variants, the large number of oligonucleotide differences between the
A/Taiwan/86-like viruses and viruses circulating between 1983 and 1985 can be accounted for
by the observation that these viruses lie on two different branches of the evolutionary tree, the
A/Taiwan/86-like viruses descending from viruses such as those isolated in Hong Kong 3 or 4
years previously. This is supported by sequence data for the HA gene (Fig. 5). The A/Hong
Kong/82-like viruses must have circulated (at least in south-east Asia) during the intervening
years and have acquired mutations that allowed the epidemiologically successful A/Taiwan/86-
like viruses to spread around the world and displace the 1983 antigenic variants during 1986 and
1987. A recent report by Brown (1988) suggests that there might be yet another branch on the
H1N1 evolutionary tree; however, sequence data are required to understand the relationship of
the Canadian viruses to those examined here.

Major epidemic activity was associated with separate, possible ‘dead-end’ branches in the
evolutionary tree for the H3N2 A/Victoria/3/75-like viruses and the influenza A H1N1-H3N2
reassortant viruses (Both et al., 1983; Cox et al., 1983). The branch on which the A/Chile/83-
like antigenic variants lie may also be such a ‘dead-end’ branch; however, we cannot exclude the
possibility that A/Chile/83-like viruses may continue to circulate at a low level and will
ultimately yield the next epidemic influenza A H1N1 virus strain. It is not currently possible to
predict with certainty which branch of the evolutionary tree will yield the next epidemic
influenza A H1N1 virus strain.

We thank the numerous participants in the WHO influenza surveillance program for a continual supply of
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