Genome Analysis of Influenza C Viruses Isolated in 1981/82 from Pigs in China

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SUMMARY

The genomes of influenza C viruses isolated from pigs in Beijing, China during 1981/82 and of human influenza C virus strains isolated between 1947 and 1981 were analysed by comparison of RNA migration patterns on gels and by two-dimensional oligonucleotide (ON) mapping. The genomes of the pig isolates were closely related to one another, though in part distinguishable by point mutations. They were similar to but more distantly related to the genomes of human influenza C viruses. The genome of the C/pig/Beijing/10/81 isolate differed from that of the C/pig/Beijing/32/81 isolate obtained on the same day at the same place by a number of mutations which were all located in RNA segments 1 and 2 as shown by ON mapping. This result suggests that the two isolates are genetically related by a reassortment event which is likely to have occurred in nature. The question whether or not pigs are a natural reservoir for human influenza C viruses cannot be answered at present.

INTRODUCTION

Influenza C viruses usually cause only a mild upper respiratory tract infection in humans, or the infection is inapparent (Francis et al., 1950; Quilligan et al., 1954; Katagiri et al., 1983). However, as most of the young adult population possesses antibody against influenza C virus, the infection seems to be widespread in the human population (Chakraverty, 1978; O'Callaghan et al., 1980; Homma et al., 1982), and the virus can be readily isolated when searched for prospectively (Katagiri et al., 1983). Influenza C viruses have many properties in common with the more pathogenic influenza A and B viruses, but also differ in a number of important features. They contain a segmented genome of single-stranded RNA of negative polarity, but there are only seven RNA segments as opposed to eight in influenza A and B viruses (Palese et al., 1980). The 3' and 5' terminal sequences of the RNA segments of influenza C viruses are conserved in a very similar way to those of influenza A and B viruses (Desselberger et al., 1980). More detailed genetic analyses and genome structure data of different influenza C viruses are scarce. Reassortment of different influenza C viruses has been observed in vitro under appropriate conditions (Racaniello & Palese, 1979). When the genomes of different influenza C viruses isolated from man over a period of 32 years were investigated by comparative oligonucleotide mapping (Meier-Ewert et al., 1981), much less genome variation was observed than had been found for influenza A viruses isolated over a shorter period of time (Desselberger et al., 1978; Nakajima et al., 1978; Young et al., 1979; Young & Palese, 1979). Influenza C viruses lack neuraminidase (Kendal, 1975; Nerome et al., 1976) but the single surface glycoprotein (gp88) has a receptor-destruction function (Meier-Ewert et al., 1978). Preliminary gene coding assignments have been made (Air & Compan, 1983), but with the exception of RNA segment 4, which is likely to code for the glycoprotein gp88 and sequences of which have been published recently (Nakada et al., 1984; Pfeifer & Compan, 1984), no firm data are available yet. Influenza C viruses show a remarkable antigenic stability (Chakraverty, 1978) since they were first isolated (Taylor, 1949, 1951). Until 1981, influenza C viruses, like B but unlike A viruses, had only been isolated from man.
In 1981, a number of influenza C virus isolates were obtained from pigs in an abattoir in Beijing, China (Guo et al., 1983a, b). The pig influenza C viruses cross-reacted broadly with recent and earlier human influenza C virus strains; when experimentally reinoculated into pigs they replicated and were also transmitted to uninfected contact pigs (Guo et al., 1983a, b). The animals developed specific antibody within 2 to 3 weeks after infection, and approximately 4% of the sera of the abattoir pigs investigated during 1981 contained antibody directed against influenza C virus, indicating that Chinese domestic pigs are infected with type C influenza viruses in nature (Guo et al., 1983a). This was the first time that influenza viruses other than influenza A viruses had been found to cause natural infection in animals. It is still unclear from which source the pigs became infected, and the potential role of pigs as an animal reservoir for the spread of influenza C viruses in man remains to be elucidated.

We report here analyses of the genomes of seven influenza C virus isolates obtained from Chinese pigs in 1981 and 1982, and for comparison genome data of six human influenza C virus strains isolated between 1947 and 1981. These studies were undertaken in order to determine the relationship between the strains and the degree of genomic variation that has occurred over time. This was done by analysis of RNA migration patterns in polyacrylamide gels and by oligonucleotide (ON) mapping. Changes observed were further investigated by comparing ON maps of individual RNA segments of selected strains.

Influenza C virus-specific proteins in infected Madin–Darby canine kidney (MDCK) cells were analysed comparatively for the pig isolates and for human strains, and the results are described in the accompanying paper (Elliott et al., 1984).

METHODS

Viruses. The influenza C viruses investigated were: C/pig/Beijing/10/81 (C/P/B/10/81), C/pig/Beijing/32/81 (C/P/B/32/81), C/pig/Beijing/107/81 (C/P/B/107/81), C/pig/Beijing/115/81 (C/P/B/115/81), C/pig/Beijing/123/81 (C/P/B/123/81), C/pig/Beijing/818/81 (C/P/B/818/81) (Guo et al., 1983a) and C/pig/Beijing/439/82 (C/P/B/439/82) which has recently been isolated by one of us (G.Y.J.); the human strains used were: C/Taylor/1233/47 (C/Taylor/47), C/Great Lakes/1167/54 (C/GL/54), C/Georgia/1/69 (C/Georgia/69), C/New Jersey/1/76 (C/NJ/76), C/Yamagata/10/81 (C/Ya/10/81) and C/Yamagata/11/81 (C/Ya/11/81). The two Yamagata isolates were kindly provided by Drs K. Nakamura and K. Sugawara (both Department of Bacteriology, Yamagata University School of Medicine, Yamagata, Japan). The other human reference strains except the C/Taylor/47 strain, were kindly sent by Dr A. P. Kendal (Center for Disease Control, Atlanta, Ga., U.S.A.); the C/Taylor/47 strain was from the virus Type Culture Collection of the Institute of Virology, China National Centre for Preventive Medicine.

For comparison, the A/PR/8/34 (H1N1) and B/Hong Kong/8/73 viruses were used.

Virus propagation. Viruses were propagated as described by Guo et al. (1983a).

Virus purification and RNA extraction. Viruses were purified by differential and sucrose gradient ultracentrifugation and the RNA extracted as described previously (Ritchey et al., 1976). The final RNA pellets were dissolved in small volumes of TE buffer (20 mM-Tris-HCl pH 7.5, 2 mM-EDTA). Purity and concentration were checked spectrophotometrically by recording the u.v. spectrum from 220 to 300 nm wavelength (1 µg RNA/ml corresponding to an absorbance of 0.025 at 260 nm wavelength in a cell of 10 mm width; Maniatis et al., 1982).

RNA gel electrophoresis, gel staining and electroelution of RNA. Electrophoresis of RNA was carried out on 3.5 or 4% polyacrylamide slab gels containing 6 M-urea as described (Ritchey et al., 1976) except that a Tris–borate–EDTA buffer (50 mM-Tris–borate pH 8.3, 1 mM-EDTA) was used. Gels were silver-stained as described by Whitton et al. (1983) or stained by soaking for 30 min in running buffer containing 4 µg/ml ethidium bromide. Gel strips containing segments were cut out from ethidium bromide-stained gels and the RNAs electroeluted according to the procedure described by Maniatis et al. (1982).

Oligonucleotide mapping. Labelling and separation of ONs was carried out as described previously (Desselberger et al., 1978; Nakajima et al., 1978; Pedersen & Haseltine, 1980). In brief, 1 µg of total viral RNA or approximately 1 µg of isolated individual RNA segment were cleaved after guanosine residues by ribonuclease T1, and the T1-resistant ONs were 32P-labelled at their 5' ends using [γ-32P]ATP (Amersham, sp. act. 3000 Ci/mmol) and polynucleotide kinase (New England Biolabs). The mixture of 5'-32P-labelled ONs was then fractionated by two-dimensional PAGE. Differences in the large ONs in maps of different RNAs were verified by co-electrophoresis of mixtures of the labelled ONs of the RNAs being compared.
Genome analysis of pig influenza C viruses

Fig. 1. RNA migration patterns of five influenza C virus strains obtained from man (C/GL/54, C/Georgia/69, C/NJ/76, C/Ya/10/81, C/Ya/11/81), of six influenza C virus isolates from pigs (C/P/B/10/81, C/P/B/32/81, C/P/B/107/81, C/P/B/123/81, C/P/B/818/81, C/P/B/439/82) and of influenza virus strains A/PR/8/34 (H1N1) and B/Hong Kong/8/73. Approximately 1 μg of each RNA was applied to the slots of a 2.8% polyacrylamide–6 M-urea slab gel. Electrophoresis was at 200 V for 15 h using Tris–borate–EDTA buffer; migration was from top to bottom. Silver staining of the gel was as described by Whitton et al. (1983). RNA segments are numbered from top to bottom (1 to 8 for influenza A and B virus RNAs, 1 to 7 for influenza C virus RNAs). The two extra minor bands observed below RNA segments 5 and 7 of the C/P/B/439/82 isolate are believed to be due to RNA breakdown products.

RESULTS

Comparative migration patterns of the RNAs of influenza C viruses isolated from pigs and man

The RNAs extracted from egg-grown, gradient-purified virus were separated on polyacrylamide gels and the gels silver-stained (Whitton et al., 1983). It was found (Fig. 1) that all influenza C virus isolates from pigs contained seven RNA segments the migration patterns of which (under the conditions chosen) were indistinguishable from those of human influenza C virus strains obtained between 1954 and 1981. When the RNAs were separated using Loening’s buffer (Loening, 1967; Palese & Schulman, 1976) the resolution of RNA segments was very poor in that RNAs 6 and 7 appeared as a smear and could not be differentiated from the background. However, RNA segment 5 of all the pig isolates migrated faster than the corresponding RNA segments of the human influenza C virus strains (results not shown).

Size of the influenza C virus genome

By plotting the logarithms of the lengths of the sequenced segments of the A/PR/8/34 (H1N1) strain (Lamb, 1983) against the distance migrated (Fig. 2) a calibration curve was obtained (Peacock & Dingman, 1968) which was used to estimate the lengths of the influenza C virus RNAs.
Fig. 2. Plot of molecular weights (as number of nucleotides) of the sequenced RNA segments of the A/PR/8/34 (H1N1) strain (Lamb, 1983) against distance migrated on polyacrylamide gel (●). A calibration curve is obtained from which the molecular weights of the influenza C virus RNA electrophoresed on the same gel (arrows) are estimated.

Table 1. *Lengths of influenza C virus RNA segments*

<table>
<thead>
<tr>
<th>RNA segment</th>
<th>Clerx et al. (1983) cited by Air &amp; Compans (1983)</th>
<th>This paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2350</td>
<td>2400</td>
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<td>2</td>
<td>2350</td>
<td>2400</td>
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<tr>
<td>3</td>
<td>2150</td>
<td>2200</td>
</tr>
<tr>
<td>4</td>
<td>2000</td>
<td>2100*</td>
</tr>
<tr>
<td>5</td>
<td>1750</td>
<td>1850</td>
</tr>
<tr>
<td>6</td>
<td>1150</td>
<td>1200</td>
</tr>
<tr>
<td>7</td>
<td>975</td>
<td>960</td>
</tr>
<tr>
<td>Total</td>
<td>12725</td>
<td>13110</td>
</tr>
</tbody>
</table>


(Table 1). The values obtained were in close agreement with those reported by Clerx et al. (cited by Air & Compans, 1983), and the total number of nucleotides in the influenza C virus genome was estimated to be approximately 13100.

*Oligonucleotide analysis of total RNAs of pig and human influenza C viruses*

The RNAs were subjected to two-dimensional ON mapping and the maps compared. The ON map of the C/P/B/32/81 isolate was taken as a reference (Fig. 3). It was found that the ON maps of most of the pig influenza C virus RNAs were remarkably similar to that of the C/P/B/32/81 RNA: ON maps of isolates C/P/B/107/81, C/P/B/115/81 and C/P/B/123/81 obtained 1 to 2 months after the C/P/B/32/81 isolate (Guo et al., 1983a) were identical (Fig. 3) and differed from the ON map of the C/P/B/32/81 RNA only by one missing and one additional ON (Fig. 3). The RNA of isolate C/P/B/818/81, obtained 10 months after the C/P/B/32/81 isolate (Guo et al., 1983a), differed by the same number of ON changes from the reference RNA, but the changes were located in a different area of the map (Fig. 3). The RNA of isolate C/P/B/439/82, obtained 18 months after the reference isolate, differed by more changes from the reference RNA (five missing, four additional large ONs, Fig. 3), but still showed an overall pattern of the large ONs similar to that of the reference RNA.
Fig. 3. Two-dimensional ON maps of the RNAs of influenza virus strains C/P/B/32/81, C/P/B/107/81, C/P/B/115/81, C/P/B/123/81, C/P/B/818/81 and C/P/B/439/82. Labelling and separation of RNase T1-resistant ONs were carried out as described under Methods. Migration in the first dimension was from left to right and in the second dimensions from bottom to top. The crosses (×) indicate the position of dye markers (xylene cyanol FF and the faster moving bromophenol blue). Using the ON map of the RNA of the C/P/B/32/81 isolate as a reference, ONs missing in the other ON maps are indicated by open circles (○) whereas additional ONs are marked by arrows (↑). Differences recorded were verified by co-electrophoresis of mixtures of labelled ONs of the RNAs being compared (results not shown). Only ONs below the dashed lines (---) were compared.
Fig. 4. Two-dimensional ON maps of the RNAs of influenza virus isolates C/NJ/76, C/Ya/10/81 and C/P/B/10/81. Labelling and separation of ONs and use of symbols are as described in the legend to Fig. 3. Missing (○) and additional (►) ONs are indicated using the ON map of C/P/B/32/81 RNA (Fig. 3, upper left) as a reference.

Table 2. Comparison of the large oligonucleotides of the RNAs of human influenza C viruses isolated between 1947 and 1981

<table>
<thead>
<tr>
<th>RNAs of strains compared*</th>
<th>Additional†</th>
<th>Missing‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/Taylor/47 and C/GL/54</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>C/GL/54 and C/Georgia/69</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>C/Georgia/69 and C/NJ/76</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>C/NJ/76 and C/Ya/10/81</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

* In the comparison of two patterns that of the more recent isolate was always defined as pattern II.
† Present in pattern II but not in pattern I.
‡ Present in pattern I but not in pattern II (Nakajima et al., 1978).
Furthermore, it was observed that pig isolate C/P/B/32/81, and with it the other pig isolates, differed by a higher number of large ONs from the RNAs of human influenza C virus strains. Fig. 4 shows the ON maps of the C/NJ/76 and C/Ya/10/81 RNAs; these differed from the C/P/B/32/81 RNA by numbers of missing/additional ONs in the range of 9/11 and 6/8, respectively. The RNAs of the C/Ya/10/81 and C/Ya/11/81 influenza virus strains yielded identical ON maps (K. Nakamura, personal communication; and our own result not shown). The comparison of the RNAs of the older human influenza C virus strains with the C/NJ/76 RNA (autoradiograms not shown) gave a result very similar to that reported by Meier-Ewert et al. (1981) and is summarized in Table 2: these strains, while exhibiting similar overall patterns of the large ONs, differed by a number of point mutations from one another.
Fig. 6. For legend, see p. 1867.
Fig. 6. (continued).
Fig. 6. (continued).
Fig. 6. Two-dimensional ON maps of isolated segments of influenza virus C/P/B/32/81, C/P/B/10J81 and C/NJ/76 RNAs. Labelling and separation of ONs and use of symbols are as described in the legend to Fig. 3. ON maps of RNA segments of the C/P/B/32/81 isolate were used as a reference with which the ON maps of the corresponding RNA segments of the other influenza C virus strains were compared.

Fig. 7. Diagram of the ON map of whole RNA of isolate C/P/B/32/81 (Fig. 3, upper left). ONs were numbered, taking their segment derivation (Fig. 6) into account, as follows: ONs 101, 102 etc. are derived from segments 1 or 2; ONs 301, 302 etc. from segment 3; ONs 401, 402 etc. from segment 4; ONs 501, 502 etc. from segment 5; ONs 601, 602 etc. from segment 6; ONs 701, 702 etc. from segment 7; ONs 01, 02 could not be assigned to segments. The use of crosses (×) and of the dashed line (---) is explained in the legend to Fig. 3.
The ON map of the C/P/B/10/81 RNA yielded a very interesting result (Fig. 4). The RNA of this isolate, which was obtained in January 1981 on the same day in the same place as reference isolate C/P/B/32/81, differed from the C/P/B/32/81 RNA (Fig. 3) in a number of large ONs (six missing, four additional); this difference is equivalent to that observed in the comparison of the reference RNA with the RNA of the C/P/B/439/82 isolate obtained 18 months later and is in contrast to the much greater similarity of the ON maps of the other 1981 influenza C virus isolates from pigs.

Oligonucleotide analysis of individual RNA segments of selected influenza C virus strains

In order to elucidate the question of how the changes observed in the C/P/B/10/81 RNA were distributed over the genome, ON maps of isolated individual segments of the reference C/P/B/32/81 RNA, of the C/P/B/10/81 and (for comparison) of the C/N J/76 RNAs were produced. Approximately 25 μg of total viral RNAs were separated on a polyacrylamide gel which was stained with ethidium bromide (Fig. 5). Gel pieces containing the RNA segments were cut out (RNA segments 1 and 2 co-migrated and were cut out together; the other RNA segments were obtained individually), electroeluted and the RNAs subjected to the procedure of two-dimensional ON mapping. The results are shown in Fig. 6. When comparing the ON maps of corresponding RNA segments of the two pig isolates it was found that the changes recorded in the ON maps of the whole RNAs (Fig. 3 and 4) were exclusively located in segments 1 and 2 whereas in the comparison of ON maps of corresponding RNA segments of the C/P/B/32/81 and the C/N J/76 isolates changes were found to be distributed more widely over the genome: although they were predominantly in segments 1 and 2, several changes were also found in segments 3, 4 and 7, while segments 5 and 6 revealed no changes (Fig. 6). The results are summarized in Tables 3 and 4 in which the numbers of large ONs found to be unique or in common (co-migrating) are listed for corresponding segments of the strains compared. The comparison of the two pig isolates (Fig. 6, Table 3) suggests that they are genetically related by a reassortment event which is likely to have occurred in nature. The comparison of the segmental ON maps of the C/P/B/32/81 and the C/N J/76 strains (Fig. 6, Table 4) shows changes in five of the seven genome segments. These changes are more likely to be accumulated point mutations, although they are not evenly distributed and hence reassortment events in the ancestry of these strains cannot be excluded. The comparison is also hampered by the fact that the isolates were obtained from different hosts and that the relationship of the two hosts with regard to interspecies spread of influenza C viruses remains to be elucidated (see below).

Assignment of oligonucleotide changes in maps of total RNAs to individual RNA segments

The assignment of most large ONs of the C/P/B/32/81 reference RNA to given segments (Fig. 7) allows the location of changes in the RNAs of other isolates (as indicated by missing ONs) to certain segments even if there are no segmental ON maps available. Thus, the missing ON 313 in isolates C/P/B/107/81, C/P/B/115/81 and C/P/B/123/81 relates the change to segment 3, the missing ON 407 in the RNA of isolate C/P/B/818/81 indicates a mutation in segment 4, and the missing ONs 103, 109, 118, 501 and 01 of the C/P/B/439/82 RNA show changes in segments 1/2 and 5. The C/Ya/10/81 RNA has changes in segments 1/2, 4, 5 and 7 compared to the C/P/B/32/81 RNA as indicated by the missing ONs 113, 123, 404, 505, 506, 702 in the map of the whole RNA and also by ON maps of isolated segments (results not shown).

DISCUSSION

Serologically identified influenza C viruses, for a long time thought to be restricted to growth in man, were isolated from pigs in China in 1981/82 (Guo et al., 1983a, b) and data on the genome structure of these viruses have been reported in this paper. For comparison, corresponding genome data of human influenza C virus isolates obtained between 1947 and 1981 have been produced. It was found that the genomes of the pig influenza C virus isolates had seven RNA segments of the same size as the RNA segments of human influenza C virus isolates.

Oligonucleotide mapping revealed that the genomes of most of the pig influenza C virus isolates of 1981 were very closely related but nevertheless distinguishable by a few point mutations;
Table 3. Comparison of the large oligonucleotides of individual RNA segments of influenza virus strains C/P/B/32/81 and C/P/B/10/81

<table>
<thead>
<tr>
<th>RNA segment</th>
<th>C/P/B/32/81 Unique</th>
<th>In common</th>
<th>C/P/B/10/81 Unique</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 + 2</td>
<td>6</td>
<td>41</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>25</td>
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</tr>
<tr>
<td>4</td>
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<tr>
<td>7</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4. Comparison of the large oligonucleotides of individual RNA segments of influenza virus strains C/P/B/32/81 and C/NJ/76

<table>
<thead>
<tr>
<th>RNA segment</th>
<th>C/P/B/32/81 Unique</th>
<th>In common</th>
<th>C/NJ/76 Unique</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 + 2</td>
<td>14</td>
<td>33</td>
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<tr>
<td>7</td>
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</table>

on the other hand they were clearly different from the genomes of recent human influenza C virus isolates. In addition, it should be mentioned that human influenza C virus strains were not available in the virus laboratory in Beijing until late in 1981, by which time many pig isolates had already been obtained (Guo et al., 1983a) and that attempts to isolate influenza viruses from man were not made at that time in the laboratory in Beijing either. This made it unlikely that the pig isolates were laboratory contaminants originating from human strains and strongly suggested them to be of genuine pig origin. The 1982 influenza C virus isolate available (C/P/B/439/82) had drifted from the isolates obtained 18 months earlier by a few more point mutations, but the estimated minimum number (seven) of base changes (Nakajima et al., 1978) was one-third to a quarter of that found in the genomes of Chinese human influenza A viruses over a comparable period of time (Young et al., 1979). When the ON map of the reference C/P/B/32/81 RNA was compared with that of the C/NJ/76 RNA, a minimum number of 16 base changes (over a period of 5 years) was calculated, which is in the same range as the changes recorded within the pig isolates obtained in 1981/82. It shows that the pig isolates are closely related to isolates obtained from man and that the genome changes over time recorded in the interspecies comparison are comparable to those found for pig isolates or for human isolates (Meier-Ewert et al., 1981; and our own data). However, a comparison of the ON maps of the pig reference C/P/B/32/81 RNA and the RNA of the C/Ya/10/81 isolate (obtained 2 months later from man in Japan) showed more differences than within the group of pig isolates, suggesting that accumulation of point mutations in influenza C virus genomes might go in different directions in different hosts or that other mechanisms of genome variation might be operative.

Other mechanisms were also suspected from a further set of data. Among the 1981 influenza C virus isolates from pigs there was one, C/P/B/10/81 (obtained at the same place on the same day as the reference isolate C/P/B/32/81), the ON pattern of which differed to a much greater extent from that of the reference RNA than did the ON maps of the other 1981 pig influenza C virus RNAs. Oligonucleotide mapping of isolated RNA segments revealed that all the changes ob-
served on the maps of the whole RNAs were located in segments 1 and/or 2 whereas the rest of the genome (comprising approximately two-thirds of the whole genome, Table 1) was completely conserved in its large ONs. When a similar constellation was found in the genomes of natural isolates of different influenza A virus subtypes it was suggested that the genomes compared were genetically interrelated by a reassortment event which was likely to have occurred in nature (Desselberger et al., 1978; Young & Palese, 1979), and this same suggestion is made here. The exact parents of the reassortment event cannot be identified, nor can a statement be made of the host in which the event took place (see below). The potential of different human influenza C viruses to reassort under in vitro conditions has been demonstrated previously (Racaniello & Palese, 1979). The mutational changes observed in all the other pig strains isolated in 1981/82 did not seem to be confined to one particular segment, but were scattered over the genome. Similar observations were made with a series of human influenza A virus isolates obtained in China in 1977/78 (Young et al., 1979).

The isolation of genuine influenza C viruses from pigs in which they seem to be able to circulate naturally, and the observation of close serological and genomic relationships of human and pig influenza C viruses raise the question of whether pigs serve as a natural reservoir for human influenza C viruses. The data available so far do not suffice for an answer. Only when pig and human influenza C virus isolates obtained in close geographical and temporal proximity are available can this problem be elucidated further.

Sequence homologies range from 15 to 100% for influenza A viruses (of different subtypes) isolated between 1933 and 1976 from different species, and from 79 to 100% for influenza B viruses isolated between 1940 and 1973 as determined by cDNA-RNA hybridization followed by S1 digestion (Palese et al., 1981); in contrast, influenza C viruses isolated between 1947 and 1976 showed sequence homologies between 90 and 100%. Likewise Meier-Ewert et al. (1981) using ON mapping reported a remarkable genomic stability of human influenza C viruses over time. This was confirmed and extended in the present study by analysis of seven influenza C virus isolates obtained from pigs in 1981/82.

From sequence data the rates of sequence divergence have been calculated for different genes of influenza A viruses (Krystal et al., 1983a) and found to be 2-1 to 4-7% per 10 years for non-surface protein genes and 4-5 to 10-2% per 10 years for the haemagglutinin (HA) and neuraminidase (NA) genes of the same subtype. From some influenza B virus HA genes an overall nucleotide change of 2-0% over 10 years has been reported (Krystal et al., 1983b), which is much lower than the rate observed among HA genes of influenza A viruses. Such comparisons cannot be made yet for influenza C virus genes due to the lack of sufficient sequence data. But the ON data allow an approximate estimate of overall genome variation based on the assumptions that the large ONs evaluated (and changes recorded within this subset of sequences) are representative of the whole genome and comprise approximately 7 to 10% of it (Nakajima et al., 1978). Thus, it was calculated that a minimum number of seven for base changes observed between the RNAs of the C/P/B/32/81 and the C/P/B/439/82 isolates in 10% of the genome over a period of 18 months amounted to $7 \times 10 \times (10/1.5) \times (1/13100) \times 100 = 3.6\%$ nucleotide variation over 10 years. In this formula, 7 is the minimum number of base changes, 10 the correction factor to cover 100% of the genome, 10/1.5 the correction factor to cover 10 years, 13100 the number of nucleotides in the whole genome, and 100 the factor to convert the ratio into a percentage. The respective number for genomic change between the RNAs of the C/NJ/76 and the C/P/B/32/81 isolates was $16 \times 10 \times (10/5) \times (1/13100) \times 100 = 2.4\%$. From the comparisons of the whole genomes of human influenza C virus strains isolated between 1947 and 1981 (Table 2) it was calculated that their nucleotide changes over 10 years were between 1-0 and 2-2%. These values measure continuous genome variation only under the assumption that no reassortment has taken place. They show a degree of variation in influenza C virus genomes comparable to those of the relatively more conserved (non-surface protein gene) segments of the influenza A virus genome (2-1 to 4-7%) and of the HA genes of influenza B viruses (2-0%).

The fact that the proteins synthesized in MDCK cells infected with human and pig influenza C virus isolates were also very closely related (Elliott et al., 1984) is in excellent accord with the high degree of genomic stability which we have demonstrated.
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