Frequent occurrence of genetic reassortment between influenza C virus strains in nature

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Previous studies of the haemagglutinin–esterase (HE) genes of various influenza C isolates suggested the existence of three distinct virus lineages (C/Yamagata/26/81-, C/Aichi/1/81- and C/Mississippi/80-related lineages) in Japan in the 1980s. Here we analysed the genetic properties of three strains (C/Yamagata/5/92, C/Miyagi/3/93 and C/Miyagi/4/93) isolated in Yamagata and Sendai Cities, Japan, in 1992/1993. Comparison of total or partial nucleotide sequences of the seven RNA segments of C/Yamagata/5/92 with those of 11 previous isolates suggested that the 1992 strain is a reassortant which inherited HE, P3, NP and M genes from a C/Mississippi/80-like virus and PB2, PB1 and NS genes from a C/pig/Beijing/115/81-like virus. Furthermore, it became evident that at least two (C/England/83 and C/Yamagata/9/88) of the 11 reference strains are also reassortants.

The genome of influenza C virus consists of seven segments of RNA (Lamb, 1989). Reassortment characterized by the exchange of genomic segments between two different strains occurs in vitro at a very high frequency (Nishimura et al., 1994). Earlier studies on the RNA genomes of various isolates demonstrated that the extent of genetic difference did not correlate with the time of virus isolation, suggesting that variants from multiple evolutionary lineages may co-circulate at any one time (Buonagurio et al., 1985, 1986). Further evidence was obtained by Matsuzaki et al. (1994), who demonstrated that two strains belonging to different lineages were isolated only 1 day apart from two children living within walking distance. Thus circumstances exist that might lead to the formation of influenza C virus reassortants in humans. We have shown recently that a virus strain isolated in 1985 in Nara prefecture, Japan [C/Nara/1/85 (NA185)] has arisen by reassortment from parents closely related to two strains isolated in the same prefecture in 1982 [C/Nara/82 (NA82)] and 1985 [C/Nara/2/85 (NA285)] (Gao et al., 1994). However, there has been no information on how often this event occurs in nature.

Sequence analysis of the haemagglutinin-esterase (HE) genes of various isolates demonstrated cocirculation of at least three distinct virus lineages (C/Yamagata/26/81 (YA2681)-, C/Aichi/1/81 (AI181)- and C/Mississippi/80 (MS80)-related lineages) in Japan in the 1980s (Muraki et al., 1996). The surveillance of influenza virus infections initiated in Yamagata City in 1988 and in the adjacent Sendai City in 1990 has succeeded in isolating a number of viruses belonging to either the YA2681- or AI181-related lineages (Matsuzaki et al., 1994). A virus belonging to the MS80-related lineage, however, was not isolated in this geographical area until the autumn of 1992. Influenza C virus antigenically indistinguishable from MS80 was first isolated in Yamagata City in November 1992, followed by isolation of four additional strains having the same HE antigenicity as MS80 in Sendai City between February to May 1993. This implied the introduction of an MS80-like virus which then began to spread in Yamagata and Sendai Cities. The results of preliminary experiments, however, indicated that at least three (C/Yamagata/5/92 (YA592), C/Miyagi/3/93 (MI393) and C/Miyagi/4/93 (MI493)) of the five 1992/1993 isolates may be reassortant viruses which received their HE genes from an MS80-like parent. In this report, we determined the parental origin of each of the genome segments of isolates YA592, MI393 and MI493, and also provide evidence which suggests that reassortment of the genome between different influenza C strains occurs rather frequently in nature.

YA592, MI393 and MI493 were isolated between November 1992 and April 1993 from paediatric patients with acute respiratory illness who lived in Yamagata (YA592) or Sendai City (MI393 and MI493), Japan, by inoculating undiluted throat swab specimens into the amniotic cavities of 9-day-old embryonated hen's eggs. After three passages in

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The nucleotide sequence data in this paper have been submitted to the DDBJ/EMBL/GenBank databases and assigned the accession numbers D78383 to D78453.
eggs, the isolates were each cloned by the limiting dilution method and then propagated in the same host. The following 11 strains, which had been isolated, passaged, cloned and propagated in eggs, were also used for comparison: MS80, YA2681, C/pig/Beijing/115/81 (PB11581), AI181, NA82, C/Kyoto/41/82 (KY4182), C/England/83 (EG83), NA185, NA285, C/Yamagata/7/88 (YA788) and C/Yamagata/9/88 (YA988).

First, strains YA592, MI393 and MI493 were examined in ELISAs for reactivity with four anti-HE monoclonal antibodies (U9, K16, MS22 and S16), directed to four different antigenic sites (A-1 to A-3, and B-1), that had been prepared and characterized previously (Sugawara et al., 1993). The three isolates were antigenically very similar to one another. Comparison of their reactivity patterns with those of the previously isolated strains showed that the Yamagata/Sendai isolates had an HE antigenicity closely similar to that of the MS80-related viruses but different from that of the YA2681-related and AI181-related viruses (data not shown).

To ascertain further the relationship between strains YA592, MI393 and MI493, their total virion RNAs were subjected to ribonuclease T1-oligonucleotide fingerprinting (Kawamura et al., 1986), and the resulting maps were compared. The maps were remarkably similar to one another (data not shown). Unexpectedly, however, the patterns of these three isolates differed by a number of oligonucleotide spots (> 20) from that of NA82, a representative strain of the MS80-related viruses but different from that of the YA2681-related and AI181-related viruses (data not shown).

To confirm the results of the antigenic analysis, which suggested that the HE genes of the Yamagata/Sendai isolates originate from an MS80-like parent, we determined the HE gene sequence (nucleotides 64 to 1989) of YA592 by using cloned cDNA derived from virion RNA as described by Umetsu et al. (1992). This sequence, together with the previously determined HE gene sequences of the 11 reference strains listed above, was analysed with the ODEN program version 1.1.1 (Ina, 1994), and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). Fig. 1 shows that the HE genes analysed were split into three distinct lineages represented by YA2681, AI181 and MS80, respectively, the YA592 strain being located on the MS80 virus lineage.

In order to deduce the parental origins of the six other genes of YA592, partial nucleotide sequences of the PB2 (positions 52 to 520), PB1 (positions 50 to 426), P3 (positions 49 to 420), nucleoprotein (NP) (positions 71 to 670), matrix (M) protein (positions 51 to 421) and non-structural (NS) protein (positions 50 to 390) genes of YA592, as well as those of the 11 reference strains, were determined by the dideoxynucleotide chain termination procedure (Sanger et al., 1977) using purified virion RNA as template and synthetic oligonucleotide primers (Gao et al., 1994), followed by construction of the phylogenetic trees. As shown in Fig. 2, the
PB1, P3 and NS genes, like the HE gene, were each split into three distinct lineages represented by YA2681, AI181 and MS80, respectively. The phylogenetic position of YA592, however, was different among these RNA segments. The PB1 gene of YA592 was located on the YA2681 virus lineage whereas its P3 gene was on the MS80 virus lineage. The NS gene of this strain seemed to belong to the YA2681 virus lineage. The phylogenetic tree of the M gene sequences was unique in that only two major branch clusters could be distinguished, one containing YA2681 and the other both AI181 and MS80. The YA592 virus M gene was within the latter branch cluster. In the trees for the PB2 and NP genes, a separate, fourth lineage including two reference strains (PB11581 and EG83) could be identified, in addition to the three lineages described above. The PB2 gene of YA592 was on this fourth lineage whereas its NP gene was on the MS80 virus lineage. Based on the results in Figs 1 and 2, we infer that YA592 is a reassortant virus which has received HE, P3, NP and M genes from an MS80-like virus and PB2, P1 and NS genes from a PB11581-like virus. Additionally, the observation that oligonucleotide maps of M193 and M1493 were nearly identical with that of YA592 (data not shown) suggests strongly that the former are also reassortants possessing the same genome composition as the latter.

To rule out the unlikely possibility that a reassortant virus (YA592) might have arisen in eggs inoculated with an undiluted clinical sample containing two different viruses similar to MS80 and PB11581, a pair of virus clones was obtained from limiting dilutions of the throat swab specimen. Partial sequencing analyses showed that the composition of both clones was indistinguishable from the YA592 virus material used in the experiments described above.

The genome composition of the 12 isolates analysed in this study is summarized in Table 1. By comparing the nucleotide sequences of the genome segments of NA185 with those of NA82 and NA285, we suggested previously that NA185 may be a reassortant which derives HE and NP genes from an NA82-like virus and the other genes from an NA285-like virus (Gao et al., 1994). This was confirmed by phylogenetic analyses in this study. It should be noted that NA185 and YA592 possess a different genome composition although both have the same HE antigenicity as MS80, suggesting that MS80-like viruses reassorted separately with YA2681-like and PB11581-like viruses to generate NA185 and YA592, respectively. Clearly, EG83 and YA988 are also reassortants. The PB2, P3, HE, NP, M and NS genes of EG83 are closely related to those of PB11581 whereas its PB1 gene is not located on the YA2681 virus lineage containing PB11581 but on the AI181 virus lineage, indicating that EG83 emerged from PB11581-like and AI181-like viruses through a reassortment event. YA988 also appears to have inherited P3 and NS genes from a YA2681-like virus and the remaining five genes from an AI181-like virus. It is also noteworthy that PB11581 is within the branch cluster composed of several YA2681-related viruses in the phylogenetic trees for the PB1, P3, HE, M and NS genes, but that together with EG83, PB11581 forms a separate evolutionary lineage in the PB2 and NP gene trees. This could be accounted for if a YA2681-like virus reassorted with another parent not identified as yet, yielding PB11581. Furthermore, the finding that the phylogenetic relationship between AI181-related and MS80-related viruses is much closer for the M gene than the other genes tempts us to speculate that these two virus groups may also be genetically related by a reassortment event, members of one inheriting their M genes from the other. It seems likely, therefore, that reassortment of the genome between influenza C viruses occurs in nature rather frequently and thus contributes to genetic variation of the viruses.

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### References


### Table 1. Genome compositions of influenza C isolates, as determined by phylogenetic analysis

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* M, MS80 virus lineage; Y, YA2681 virus lineage; A, AI181 virus lineage; P, fourth lineage containing PB11581; M/A, a lineage containing both MS80 and AI181.


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