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Evolutionary Analysis of Influenza C Virus M Genes

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Abstract. The previous study of the 25 hemagglutinin-esterase (HE) glycoprotein genes of influenza C viruses identified four discrete lineages represented by C/Yamagata/26/81, C/Aichi/1/81, C/Aomori/74 and C/Mississippi/80, respectively. Here we compared the M gene sequence among the 24 viruses isolated between 1964 and 1991. A phylogenetic analysis showed that these genes have evolved into three distinct lineages. Lineage I included most of viruses with the HE genes of C/Yamagata/26/81-related lineage. The predominant members of lineage II were viruses having the HE genes of either C/Aichi/1/81- or C/Mississippi/80-related lineage. Lineage III contained only C/Aomori/74. Phylogenetic positions of several strains (C/Yamagata/64, C/Kanagawa/1/76, C/Miyagi/77 and C/Nara/1/85) were different between the M and HE gene trees, suggesting that they are reassortants. Furthermore, phylogenetic relationships between C/Mississippi/80-like and C/Aichi/1/81-like viruses were much closer for the M gene than the HE gene, raising the possibility that these two virus groups are genetically related by a reassortment event. Nucleotide changes in the M genes occurred at about 7% positions with a uniform distribution throughout the molecules. However, the predicted amino acid sequence of the matrix protein (M1) was conserved almost completely among the isolates analyzed. The amino acid sequence of the second protein (CM2) encoded by M gene was also highly conserved, but was more divergent than the M1 protein sequence, suggesting that the two M gene products are evolving differently in response to selective pressures or structural and functional constraints.

Key words: influenza C virus, matrix protein gene, evolution, M1 protein, CM2 protein, reassortment

Introduction

The genome of influenza C virus consists of seven single-stranded RNA segments. The RNA segment 6 (M gene) is 1180 or 1181 nucleotides in length and contains a single open reading frame that could code for a polypeptide of 374 amino acids (1,2). The predominant mRNA synthesized from this RNA segment, however, lacks a region from nucleotides 755 to 982 (numbering is based on the M gene sequence composed of 1181 nucleotides) and encodes a 242 amino acid M1 protein (1). In this spliced mRNA the spliced junction (nucleotides 753, 754 and 983) creates a termination codon. The M1 protein underlies the lipid bilayer, adding rigidity to the virion envelope, and is thought to play a key role in the assembly and budding of the virus by mediating interactions of the viral glycoproteins and the ribonucleoprotein complex. Unspliced mRNA from RNA segment 6, which was recently detected in infected cells (2), potentially encodes a polypeptide of Mr 42000 that contains additional 132 amino acids on the C-terminus of M1. Immunoprecipitation experiments with antiserum to the glutathione S-transferase (GST) fusion protein containing the extra C-terminal domain, however, identified a protein of Mr 18,000 (designated CM2) in infected cells (2). Although the mechanism by which CM2 is synthesized from the unspliced mRNA remains to be clarified, it is possible

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers AB000604 to AB000614 and AB000702 to AB000728.

that the mRNA may be translated from the initiation codon at nucleotides 732 to 734 to the termination codon at nucleotides 1149 to 1151, generating a protein of 139 amino acids (2). CM2 is an integral membrane protein that shares many structural features of influenza A virus M2 (3), a protein which has a proton channel activity (4,5). One of the aims of this study was to compare the mode of evolution between the two M gene products (M1 and CM2) with different functions.

Earlier studies on the RNA genome of various influenza C 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 cocirculate at any one time (6–8). We compared recently the hemagglutinin-esterase (HE) glycoprotein gene sequence among the 25 isolates obtained during 1964/1988 and revealed the existence of four discrete lineages represented by C/Yamagata/26/81, C/Aichi/1/81, C/Aomori/74 and C/Mississippi/80, respectively, three of which (C/Yamagata/26/81-, C/Aichi/1/81- and C/Mississippi/80-related lineages) cocirculated in Japan in the 1980s (9). It is likely, therefore, that mixed infection with influenza C viruses belonging to different lineages occurs in nature, resulting in the emergence of reassortment viruses. In fact, we recently obtained evidence that several virus strains (C/England/83, C/Nara/1/85, C/Yamagata/9/88 and C/Yamagata/5/92) have arisen by reassortment from parents belonging to different lineages (10,11). The second aim of the present study was to compare the phylogenetic positions of the individual isolates between the evolutionary trees for the HE and M genes, in order to identify additional reassortment viruses. For these purposes, we analyzed the genetic relationships among the M genes of 24 influenza C virus strains isolated between 1964 and 1991.

Table 1. Influenza C virus strains used in phylogenetic analyses

HE gene lineage[a]	Virus strain	Abbreviation	M gene lineage[c]	Accession number	Reference
C/Yamagata/26/81	C/Sapporo/71	SA71	I	AB000605	This report
	C/California/78	CAL78	I	AB000608	This report
	C/Kyoto/1/79	KY179	I	AB000609	This report
	C/Yamagata/26/81	YA2681	I	AB000721	This report
	C/pig/Beijing/115/81	PB11581	I	AB000722	This report
	C/England/83	EG83	I	AB000725	This report
	C/Nara/2/85	NA285	I	AB000727	This report
	C/Yamagata/1/88	YA188	I	D16261	2
	C/Yamagata/8/88	YA888	I	AB000612	This report
	C/Yamagata/10/89	YA1089	I	AB000613	This report
	C/Miyagi/9/91	MI991	I	AB000614	This report
C/Aichi/1/81	C/Johannesburg/66	JHG66	II	AB000604	This report
	C/Aichi/1/81	AI181	II	D16260	2
	C/Yamagata/1/86[b]	YA186	II	AB000611	This report
	C/Yamagata/9/88	YA988	II	AB000728	This report
C/Aomori/74	C/Yamagata/64	YA64	II	D16258	2
	C/Aomori/74	AO74	III	D16259	2
	C/Kanagawa/1/76	KA176	I	AB000606	This report
	C/Miyagi/77	MI77	I	AB000607	This report
C/Mississippi/80	C/Mississippi/80	MS80	II	AB000720	This report
	C/Nara/82	NA82	II	AB000723	This report
	C/Kyoto/41/82	KY4182	II	AB000724	This report
	C/Hyogo/1/83	HY183	II	AB000610	This report
	C/Nara/1/85	NA185	I	AB000726	This report

[a]The HE gene lineages to which the individual isolates belong have been determined previously (9).
[b]The HE gene sequence of C/Yamagata/1/86 has not yet been determined, but the HE protein of this strain was antigenically indistinguishable from that of C/Aichi/1/81 (16).
[c]Determined in this study.

Materials and Methods

Viruses and RNA Extraction

A total of 24 strains of influenza C virus isolated in Japan and other countries (China, England, the Republic of South Africa and the United States) during the period from 1964 and 1991 were used (Table 1). They were each cloned two times by means of limiting dilution method and then propagated in the amniotic cavity of 9-day-old embryonated chicken eggs. Viral RNA (vRNA) was isolated by treatment of purified virions with proteinase K and sodium dodecyl sulfate (SDS), followed by extraction with phenol-chloroform (12).

Polymerase Chain Reaction (PCR) Amplification of the M Gene and Nucleotide Sequencing

The vRNA was first transcribed into cDNA with avian myeloblastosis virus reverse transcriptase XL (Life Sciences) and a primer [5′d(AGCAGAAGCAGG)] complementary to positions 1 to 12 at the 3′ end of all influenza C virus RNA segments. Two asymmetric amplifications of the M gene-derived cDNA were carried out by using two primers, 5′d(AGAAGCAGGGGATTTCAAAA) corresponding to positions 4 to 23 in the mRNA (plus-strand) sense and 5′d(AGCAGTAGCAAGGGGATTTTT) corresponding to positions 1181 to 1161 in the vRNA (minus-strand) sense. The minus-strand amplification (100 µl) was done in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 µM each dNTP, 0.5 pmol mRNA sense primer, 50 pmol vRNA sense primer and 2.5 U ExTaq polymerase (Takara Biochemicals). The reaction mixture was then subjected to 35 cycles of amplification, each consisting of 0.5 min at 94°C (denaturation), 0.5 min at 55°C (annealing) and 1 min at 72°C (primer extention). In the plus-strand amplification the concentrations of mRNA and vRNA sense primers were 50 pmol and 0.5 pmol, respectively. The PCR products were purified by a rapid gel filtration using a CHROMA SPIN™ column (CLONTECH Laboratories), and their sequences were determined by the dideoxynucleotide chain termination method (13) using the Sequanase™ Version 2.0 7-deaza-dGTP Kit (Amersham) and the following primers: 5′d(4AGAAGCAGGGGATTTCAAAA23), 5′d(175TAATGTCTGGAGAAGCCACC194), 5′d(314GCAAATGAAAGCAGCTGGAG333), 5′d(453ACAATCACAGCTTGGTTGAG472), 5′d (581TGCTGGTATTACTGGCCTTG600), 5′d (677AATGAGACCCCACTTGGAAA696) and 5′d(1015CAATTATGCCTGAAATTGAC1034) for minus-strand sequencing, and 5′(1170GGGGATTTTTTCAAGGTAATTA1149), 5′d(1128TGGAAAAAGGAATTGGTGAG1109), 5′d(1041CCATCGAGTCAATTTCAGGC1022) and 5′d(148TTAGCTGCTGATTTGCAGGC129) for plus-strand sequencing. Sequence data were analyzed with PHYLIP program version 3.54c (14), and evolutionary relationships were calculated by the maximum parsimony method (15) by using the same software program.

Results and Discussion

Evolutionary Tree of M Gene Nucleotide Sequences

A phylogenetic tree for the M gene, constructed by using the nucleotide sequences of 24 influenza C strains isolated during 1964/1991, is shown in Fig. 1. The pattern indicates that these genes have evolved into at least three distinct lineages. Lineage I contains 14 virus strains isolated during 1971/1991 which include three foreign isolates from China (PB11581), England (EG83) and the United States (CAL78). Most of these viruses (except KA176, MI77 and NA185) were shown previously to possess the HE genes belonging to the YA2681 virus lineage (9). Lineage II contains seven Japanese and two foreign isolates (JHG66 from the Republic of South Africa and MS80 from the United States) obtained during 1964/1988. Our previous study showed that the HE genes of four strains of this group (MS80, NA82, KY4182 and HY183) are located on the MS80 virus lineage and those of three strains (JHG66, AI181 and YA988) on the AI181 virus lineage (9). The HE gene of YA186, although not sequenced as yet, is likely to belong to the AI181 virus lineage since this virus has the HE antigenicity indistinguishable from that of AI181 (16). The HE gene of YA64, on the other hand, has been shown to be within the AO74 virus lineage (9). Lineage III contains only one Japanese isolate of 1974 (AO74). Pairwise comparison of the M gene sequence among the 24 isolates (data not shown) revealed that the degrees of nucleotide sequence identity between

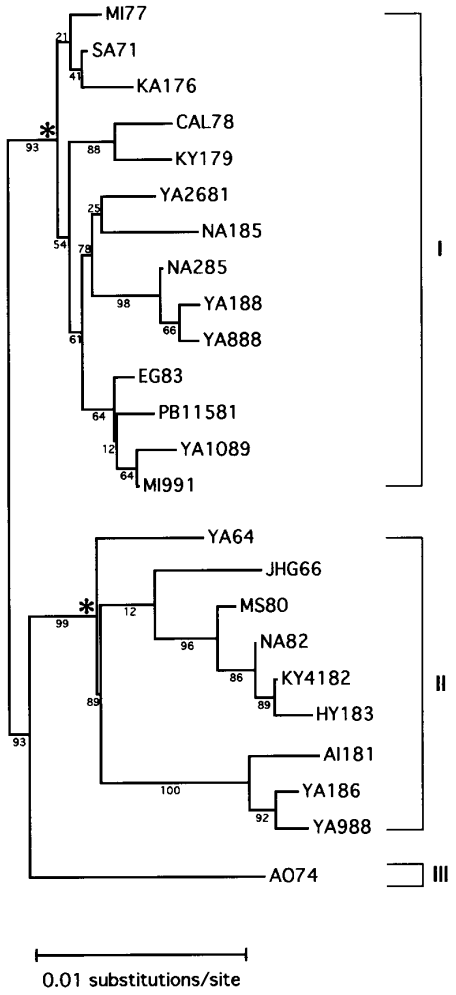

Fig. 1. A phylogenetic tree of influenza C virus M genes. The region from nucleotides 24 to 1160 was used for analysis. The horizontal distances are proportional to the minimum number of nucleotide differences needed to join nodes and M gene sequences. Numbers beneath or above the branches are the bootstrap probability of each branch (%). The asterisks indicate the hypothetical ancestors for lineages I and II.

the isolates on the same lineage were extremely high, differing only by $\sim 0.7\%$ (lineage I) or $\sim 1.1\%$ (lineage II). The M genes of the isolates that belong to different lineages were less homologous, differing by $\sim 1.9\%$ (lineages I and II, and lineages I and III) or $\sim 2.3\%$ (lineages II and III).

There were several noticeable differences in the evolutionary trees of the M and HE genes. The HE gene of NA185, together with those of MS80, NA82, KY4182 and HY183, formed an MS80 virus lineage (9). However, the M gene of NA185 was found to fall within lineage I, in contrast to the M genes of the other four strains which were all within lineage II. This confirms the previously described idea that NA185 is a virus arisen by reassortment from two viruses closely related to NA82 and NA285 (10). Virus strains KA176 and MI77 have been demonstrated to possess the HE genes highly homologous to that of AO74 (9). It became evident here, however, that the M genes of the 1976 and 1977 isolates were dissimilar to that of AO74, the former belonging to lineage I and the latter to lineage III. Rather, the M genes of KA176 and MI77 had a close relatedness to that of SA71 having the HE gene of the YA2681 virus lineage (9). These data suggest strongly that KA176 and MI77 are both reassortants which received HE genes from an AO74-like virus and M genes from a SA71-like virus. The HE gene of YA64, like those of AO74, KA176 and MI77, was within the AO74 virus lineage (9). By contrast, the M gene of YA64 was located on lineage II, separately from those of the 1974–1977 isolates which were either on lineage I or on lineage III. These observations also raised the possibility that YA64 may be genetically related to the 1974–1977 strains by a reassortment event(s). Finally, the phylogenetic positions of viruses having the HE genes of the MS80 virus lineage (MS80, NA82, KY4182 and HY183) were markedly different between the trees for the M and HE genes. In the HE gene tree, the evolutionary relationship between the MS80 and AI181 virus lineages was much more distant than that between the AI181 and YA2681 virus lineages (9). In the M gene tree, in contrast, the four isolates described above, as well as the isolates with the HE genes of the AI181 virus lineage (JHG66, AI181, YA186 and YA988), were located on lineage II although they appeared to form two distinct subgroups within the lineage. The observation that the phylogenetic relationship between MS80-like and AI181-like viruses was much closer for the M gene than the HE gene led us to a conclusion that one of these two virus groups acquired their M (or HE) genes from the other through a reassortment event, as has previously been suggested from the sequence data obtained with a limited number of virus isolates (11). The observations described above (summarized in Table 1), altogether, support the notion that reassortment of the genome between influenza C viruses occurs frequently in nature and plays a significant role in generating genetic variation of the viruses.

Nucleotide Sequences of M Genes and Deduced Amino Acid Sequences of M1 and CM2 Proteins

No deletions and insertions were detected in the coding region of the 24 M genes analyzed. As has been described for the M gene of YA188 (2), an insertion of one extra A residue was observed in the 5′ noncoding region (position 24) of the M genes of several virus strains (YA2681, NA185, NA285 and YA888) isolated in Japan during 1981/1988. Interestingly, these five isolates (including YA188) made up a branch cluster within lineage I. Nucleotide substitutions were observed at 84 positions (7.4%), including an insertion at position 24. These substitutions were uniformly distributed throughout the molecule, with 54 (7.4%) of the 726 nucleotides (positions 27 to 752) and 30 (7.2%) of the 417 nucleotides (positions 732 to 1148) showing differences in the M1 and CM2 coding regions, respectively. However, the initiation signals and the termination signals for the synthesis of M1 and CM2 proteins as well as the nucleotide sequences around the 5′ and 3′ end junctions of the M1 mRNA were strictly conserved among the 24 M gene sequences, suggesting that all the viruses analyzed are capable of synthesizing the M1 and CM2 proteins composed of 242 and 139 amino acid residues, respectively. In fact, radioimmunoprecipitation experiments with anti-M1 monoclonal antibody and antiserum to the GST/CM2 fusion protein revealed that M1 and CM2 were synthesized in HMV-II cells (a human malignant melanoma cell line) infected with any of the 24 virus strains and that their electrophoretic mobilities were identical among the isolates, except that the CM2s of JHG66 and YA188 migrated slightly faster than those of the other strains (data not shown).

	M1			CM2										
	10	62	165	244	247	253	255	267	273	308	345	354	359	371
SA71	T	V	S	L	I	T	Q	N	D	I	S	D	T	I
KA176	·	·	·	·	·	·	·	·	·	·	·	·	·	·
MI77	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CAL78	·	·	·	·	·	·	K	·	·	·	·	·	·	·
KY179	·	·	·	·	·	·	K	·	·	T	·	·	·	·
YA2681	·	·	·	·	·	·	·	·	·	·	·	·	·	·
PB11581	·	·	·	·	·	·	·	·	·	·	·	·	·	·
EG83	·	·	·	·	·	·	·	·	·	·	·	·	·	·
NA185	·	·	·	·	·	·	·	·	E	·	·	·	·	·
NA285	·	·	·	·	·	·	·	·	·	·	·	·	·	·
YA188	·	·	·	·	·	·	·	·	·	·	F	·	·	·
YA888	·	·	·	·	·	·	·	·	·	·	·	·	·	·
YA1089	·	·	·	·	·	·	·	·	·	·	·	·	·	·
MI991	·	·	·	·	·	·	·	·	·	·	·	·	·	·
YA64	·	·	·	·	·	·	·	K	·	T	·	·	I	·
JHG66	A	·	·	·	·	·	·	K	·	·	F	G	·	·
MS80	·	I	·	·	·	·	·	K	·	·	·	·	·	·
AI181	·	·	G	·	·	A	R	K	·	·	·	·	·	V
NA82	·	·	·	·	·	·	·	K	·	·	·	·	·	·
KY4182	·	·	·	·	·	·	·	K	·	·	·	·	·	·
HY183	·	·	·	·	·	·	·	K	·	·	·	·	·	·
YA186	·	·	G	·	T	A	·	·	·	·	·	·	·	·
YA988	·	·	G	·	·	A	·	·	·	·	·	·	·	·
AO74	·	·	·	F	·	·	·	K	·	·	·	·	·	·

Fig. 2. Comparison of deduced amino acid sequences of the M1 and CM2 proteins among 24 influenza C virus strains. Amino acid differences from the sequence of SA71 are shown. Numbering of amino acid residues is based on the sequence of the putative 374 amino acid protein.

The deduced amino acid sequences of the M1 and CM2 proteins were compared among the isolates, and the results are summarized in Fig. 2. The amino acid sequence of M1 was strikingly conserved, with only three (1.2%) out of the 242 residues showing differences. It was particularly impressive that the M1s of the 14 lineage I viruses isolated over a period of 20 years had amino acid sequences completely identical to one another and that their sequences were also the same as those of the M1s of five strains (YA64, AO74, NA82, KY4182 and HY183) belonging to either lineage II or lineage III. Among the 139 amino acid residues of CM2, substitutions were detected at 11 positions (7.9%), indicating that the amino acid sequence divergence in this protein is greater than that in M1.

Amino acid sequence of CM2, although more divergent than the sequence of M1, was conserved fairly well, as evidenced by the finding that 10 out of the 14 isolates belonging to lineage I (including SA71 and MI991 isolated 20 years apart) showed no amino acid differences. Furthermore, cysteine residues (positions 260, 265, 279 and 324) as well as the potential site for N-glycosylation (positions 270 to 272) were completely conserved among the 24 virus strains. It was also interesting that only one amino acid substitution (Ile 308 → Thr) occurred in the transmembrane domain composed of residues 287 to 318, whereas six and four substitutions were seen in the extracellular (positions 253 to 286) and cytoplasmic (positions 319 to 374) domains, respectively. A high degree of conservation of the transmembrane domain relative to the other two domains, which has also been observed with the M2 protein of influenza A virus (17), provides support for its functional importance. As described above, the CM2s of strains JHG66 and YA188 migrated in SDS-polyacrylamide gel electrophoresis slightly faster than those of the remaining 22 isolates. Fig. 2 indicates that amino acid substitution at position 345 (Ser → Phe) occurred in JHG66 and YA188 but in none of the others, suggesting that this amino acid change may have resulted in an increase in the electrophoretic mobility of CM2.

The relatively low degree of amino acid sequence divergence in M1 compared to the divergence in CM2 suggests that these two proteins are evolving differently in response to selective pressures or structural and functional constraints. To examine this possibility in more detail, nucleotide differences per sites at synonymous sites (KS) and amino acid changing sites (KA) between each pair of the M gene sequences were calculated by the method of Nei and Gojobori (18) for the regions encoding the M1 and CM2 proteins. Part of the results (data concerning the lineage II viruses) are shown in Fig. 3. In almost all of the pairs the KA value determined for the M1 coding region was very low compared to the value determined for the CM2 coding region, which confirms that amino acid change has occurred much less frequently in M1 than CM2. The extremely high degree of the M1 protein sequence conservation may be related to functional constraints that could arise from possible multiple interactions with other viral components including the ribonucleoprotein complex, the cytoplasmic tails of the HE and CM2 glycoproteins and the lipid envelope.

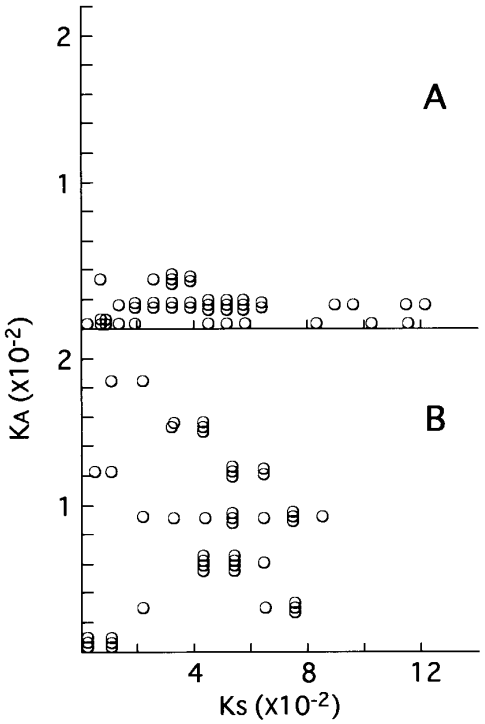

Fig. 3. KS and KA values in pairwise comparison of the regions encoding the M1 (A) and CM2 (B) proteins among influenza C virus M genes belonging to lineage II.

Evolutionary Rates of M Gene

We attempted to estimate the evolutionary rates of the M genes of lineages I and II by plotting the total branch distance from the ancestor nodes (marked by asterisks in Fig. 1) against the year of isolation for each virus. As seen in Fig. 4, the extent of nucleotide difference was roughly proportional to the time of interval of virus isolation, and the evolutionary rates were estimated by regression analysis to be 0.21 and 0.30×10^{-3} substitutions/site/year for lineages I and II, respectively. These values are both smaller than the estimates reported for influenza A virus M gene [1.08×10^{-3} substitutions/site/year (17)] and influenza C virus HE gene [0.49×10^{-3} substitutions/site/year (9)]. The difference in the evolutionary rate between the M genes of influenza A and C viruses seems to be at least in part due to a long replication cycle of the latter virus (19). The possible explana-

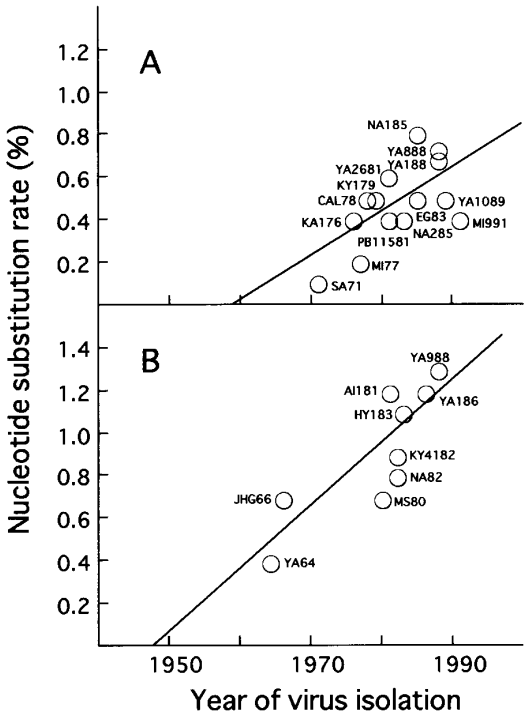

Fig. 4. Evolutionary rates for the M genes of lineage I (A) and lineage II (B). For estimating evolutionary rates, the total branch distance from the common ancestor nodes (asterisks in Fig. 1) was plotted against the year of isolation. The regression lines were drawn by the least squares method, with the determination coefficients (R^2) of 0.40 for A ($p < 0.05$) and 0.69 for B ($p < 0.01$).

tions for the difference between the M and HE genes of influenza C virus may be that the HE glycoprotein is subjected to greater immune selective pressures than the M1 and CM2 proteins and that a higher degree of structural and functional constraint is exerted on the change of M1 than HE.

Acknowledgments

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