Nucleotide and deduced amino acid sequence of the structural protein genes of Japanese encephalitis viruses from different geographical locations

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Strain variation among Japanese encephalitis (JE) virus isolates has been previously demonstrated by many workers using different methods. We report the nucleotide sequence of the 5' non-coding region and the nucleotide and deduced amino acid sequences of structural protein genes for eight wild-type JE virus strain isolated from different Asian countries (Vietnam, Nepal, Indonesia, Thailand, India, Japan and China). We compare these with five other published wild-type JE virus strains isolated from Japan and the mainland of China. No strain variation could be related to geographical location on the basis of the wild-type JE virus strains studied. The viruses differed from each other by between 21 (0.9%) and 111 (4.6%) nucleotides and by between 3 (0.4%) and 31 (4.2%) amino acids over the 2434 nucleotides examined. The amino acid divergence of the envelope (E) protein gene of the viruses was 4-2% or less among the 13 strains compared. Thus there is little genetic divergence in the strains studied. However, four variable E protein amino acids (E-51, E-209, E-244 and E-366) were identified. Residue positions E-51, E-244 and E-366 are found in peptides with functional T helper cell epitopes in two other flaviviruses. Therefore, these amino acids may be important in defining the various immunotypes of JE virus identified by antibody reactivity patterns.

Introduction

Japanese encephalitis (JE) virus belongs to genus Flavivirus and the family Flaviviridae. The genome of JE virus, like other flaviviruses, is a positive-polarity ssRNA approximately 11 kb in length, capped at the 5' terminus but lacking a poly(A) tract at its 3' end (Brinton, 1986). The genomic RNA contains one long open reading frame encoding three structural proteins, the capsid (C), membrane (M) and envelope (E) proteins, at its 5' end and the non-structural proteins in the 3' three-quarters of the genome. The structural proteins are involved in the majority of the biological properties of the virus, such as binding to cell receptors, invoking immunological responses (neutralization, passive protection and antibody-dependent enhancement), virion assembly and fusion activity at low pH (Rice et al., 1986; Monath, 1990).

JE virus is found throughout Asia from India in the west to Japan in the east, the Commonwealth of Independent States in the north and Indonesia in the south. Antigenic, biochemical and genetic differences between JE virus strains isolated from different areas have been observed by cross-neutralization (Banerjee, 1975), oligonucleotide fingerprinting (Banerjee & Ranadive, 1984; Hori, 1986), monoclonal and polyclonal antibody reactivities (Kobayashi et al., 1984, 1985; Wills et al., 1992) and by limited (240 bp) genomic sequence comparison (Chen et al., 1990, 1992; Huong et al., 1993).

Chen et al. (1990, 1992) suggested that JE viruses could be divided into four genotypes using 12% nucleotide divergence as a cut-off point for virus relationships when comparing prM gene segments.

To date, the nucleotide and deduced amino acid sequence of the structural protein genes of wild-type JE virus have only been published for virus strains isolated in Japan (Sumiyoshi et al., 1987) and the mainland of China (Hashimoto et al., 1988; Ni et al., 1994). In this paper we report the nucleotide sequence of structural protein genes and deduced amino acid sequences of eight wild-type JE virus strains isolated from several Asian countries and compare them to each other and five other published wild-type JE virus strains.

Methods

Cells and viruses. Vero cells, LLC-MK2 cells and C6-36 (Aedes albopictus) cells were grown at 37°C or 28°C (mosquito) in Eagle's MEM (EMEM; Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma), 2 mM-L-glutamine (Sigma) and antibiotics.
Table 1. Viruses used for analysing genetic relationships

<table>
<thead>
<tr>
<th>Virus name</th>
<th>Place of isolation</th>
<th>Country</th>
<th>Host</th>
<th>Year</th>
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<th>Accession number</th>
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<td>Nepal</td>
<td>Human</td>
<td>1985</td>
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<td>KPP034-35CT</td>
<td>Kampanghet</td>
<td>Thailand</td>
<td>Mosquito</td>
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<td>U03693</td>
</tr>
<tr>
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<td>Mosquito</td>
<td>1949</td>
<td>This paper</td>
<td>U03695</td>
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<tr>
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<td>U04522</td>
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<tr>
<td>HK526</td>
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<td>U03691</td>
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<tr>
<td>826309</td>
<td>Goa</td>
<td>India</td>
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<td>U03689</td>
</tr>
<tr>
<td>Saigon</td>
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<td>1962</td>
<td>This paper</td>
<td>U03696</td>
</tr>
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<td>Japan</td>
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<td>1966</td>
<td>Hesegawa et al. (1992)</td>
<td></td>
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<td>JaOAaS892</td>
<td>Osaka</td>
<td>Japan</td>
<td>Mosquito</td>
<td>1982</td>
<td>Sumiyoshi et al. (1987)</td>
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</table>

* NK, Not Known.

**Viruses.** The geographical location, year and host of isolation of the JE virus strains used in this study are shown in Table 1. All the strains have received less than 10 passages since isolation except for strains P3 and Saigon which had been extensively passed in mouse brain prior to this study. The viruses were propagated by infection of confluent monolayers of monkey kidney Vero or LLC-MK2 cells, or mosquito C6-36 cells at a m.o.i. of 1 p.f.u./cell. After 30 min adsorption, EMEM containing 2% FCS and 2 mM-L-glutamine was added and cultures were incubated at 37 °C for monkey kidney cells or 28 °C for mosquito cells for up to five days.

**Viral RNA extraction.** Virus stock (1 ml) was incubated at 37 °C for 30 min with 100 µg proteinase K; subsequently 100 µl 10% SDS was added and the samples were incubated for a further 15 min. Twenty µg of tRNA (Sigma) was then added to the samples which were mixed by vortexing. Viral RNA (vRNA) was extracted with an equal volume of phenol-chloroform (1:1) and then precipitated with two volumes of ice-cold ethanol and one-tenth volume of 3 M-sodium acetate at −20 °C overnight. The vRNA was subsequently resuspended in TNE (50 mM-Tris-HCl, 10 mM-sodium chloride and 5 mM-EDTA) and stored at −20 °C until used.

**Cloning and sequencing.** Seven oligonucleotide primers were synthesized based on the published SA14 genomic sequence (Nitayaphan et al., 1990). Four sense primers used were: 5' TATCTGTGTGAACTCTTGTGGC 3' (primer 1, nucleotides 8-28), 5' GGTGGGGCAACGGATGTGGA CTITTTCGGGG 3' (primer 2, nucleotides 1276-1303); 5' TCTGAAAGGGCAACACTATGGC 3' (primer 3, 1863-1882); 5' CAATGCCTTCCCTCCCGAAA 3' (primer 4, nucleotides 895-1014) and three antisense primers: 5' TCTTGTCCTCCTCTCGTTT 3' (primer 5, nucleotides 1298-1317); 5' TGGTCGATGCACATGGTG-GTC 3' (primer 6, nucleotides 2463-2483), 5' GAGTTCAATGACA-TCTTGGC 3' (primer 7, 1936-1956). The genomic RNA was first transcribed into cDNA by extending one of three antisense primers using Roas-associated virus type 2 reverse transcriptase (Amersham) and incubating at 55 °C for at least 1 h after denaturation by boiling. First-strand cDNA was amplified using Taq polymerase (Boehringer-Mannheim) and a hot-start program with one of three pairs of primers (primers 1 and 5, primers 2 and 6, or primers 3 and 4 and 7). The amplification proceeded through a total of 30 cycles consisting of denaturation at 94 °C (40 s), annealing at 53 °C (1 min) and primer extension at 72 °C (10 min). The cDNAs were directly ligated into the TA cloning vector pCR II (Invitrogen).

The nucleotide sequences of the cDNA were determined by the dideoxynucleotide chain termination method. Universal and specific primers were annealed to template dsDNA denatured with sodium hydroxide. The clones were sequenced in both directions using the Sequenase version 2.0 sequencing kit (USB). The [35S]dATP-labelled reaction products were separated on 5% polyacrylamide and 7 M-urea 0.4 mm thick gels. For each virus strain two or more clones were sequenced from RT-PCR product(s).

**Sequence analysis.** Computer analyses of nucleic acid data and deduced amino acid sequences were accomplished using the MICROGENIE (Queen & Korn, 1984) and PCGENE programs. A dendrogram was generated with the neighbour-joining algorithm in MEGA (Kumar et al., 1993) after nucleotide and deduced amino acid sequences were aligned using the CLUSTAL V (Higgins et al., 1992) program.

**Results**

**Nucleotide sequence of the prototype strain of JE virus**

The prototype strain of JE virus, Nakayama, was the first nucleotide sequence published for JE virus (McAda et al., 1987). The complete nucleotide sequence of the prM, M and E protein regions were reported but no sequence data were generated for the 5' non-coding region and the first 75 amino acids of the C protein.

Comparison of the nucleotide and deduced amino acid sequence of 'Nakayama original' (i.e. the original isolate

**prM**

(i) **G****N**L**S**H**P**Q**X**X**Q**X**L**T**U**N**T**G**V**I**V**P**H**S**9**G**E**N**R**C**W**A**I**D**V**G**T**M**C**E**T**T**T**E**C**P**E**U**L**N

(ii) **G****N**L**S**H**P**Q**X**X**Q**X**L**T**U**N**T**G**V**I**V**P**H**S**9**G**E**N**R**C**W**A**I**D**V**G**T**M**C**E**T**T**T**E**C**P**E**U**L**N

**M**

(i) **G****N**D**P**E**V**O**C**K**D**O**Q**X**V**X**Y**V**G**R**C**T**T**S**R**S**K**R**S**E**V**S**W**W**Q**G**E**S**L**L**W**E**A**W**L**G**T**E**A

(ii) **G****N**D**P**E**V**O**C**K**D**O**Q**X**V**X**Y**V**G**R**C**T**T**S**R**S**K**R**S**E**V**S**W**W**Q**G**E**S**L**L**W**E**A**W**L**G**T**E**A

Fig. 1. Alignment of the deduced amino acid sequence in the prM and M protein regions of JE virus Nakayama, reported by McAda et al. (1987) and determined in this paper. (i), Amino acid sequence deduced from the nucleotide sequences of JE virus 'Nakayama original' published by McAda et al. (1987). (ii), The deduced amino acid sequence from JE virus 'Nakayama original' determined in this paper.
### Comparison of wild-type JE viruses

**Fig. 2.** Alignment of deduced amino acid sequences of the structural proteins of 13 JE virus strains.
Table 2. Percentage divergence of nucleotide and amino acid sequences* of the structural proteins of 10 wild-type JE virus strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>SA14/USAa</th>
<th>S892b</th>
<th>Beijing-1c</th>
<th>HK8526</th>
<th>826309</th>
<th>DH20</th>
<th>Indonesia</th>
<th>Saigon</th>
<th>P3</th>
<th>Nakayama</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA14/USA</td>
<td>0.4†</td>
<td>0.8</td>
<td>0.9</td>
<td>1.5</td>
<td>0.4</td>
<td>2.3</td>
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<tr>
<td>Saigon</td>
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<td>2.4</td>
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</tbody>
</table>

* Sequences are taken from a, Ni et al. (1994); b, Sumiyoshi et al. (1987); c, Hashimoto et al. (1988).
† Highest and lowest percentage divergences are shown in bold.

Comparison of the nucleotide and amino acid sequences of wild-type strains of JE virus

Prior to this study, only the entire sequence of the 5' non-coding region and structural protein genes of strains Beijing-1 (Hashimoto et al., 1988) and SA14/USA (Ni et al., 1994) from China, and JaOArS892 (abbreviated to S892) (Sumiyoshi et al., 1987) from Japan had been published. The nucleotide sequence of the E protein gene had also been published for JE virus strains Kamiyama (Hesegawa et al., 1992) and Nakayama (McAda et al., 1987) from Japan, while only the amino acid sequence of the E protein of a strain from Sarawak had been published (Cecilia & Gould, 1991). In this paper we have determined the nucleotide sequences of the 5' non-coding region and structural protein genes of seven wild-type JE virus strains isolated from China (strain P3), Japan (Nakayama original), Nepal (DH20), Vietnam (Saigon), India (826309), Indonesia and Taiwan (HK8256). The nucleotide sequence of only the E protein gene of strain KPPO34-35CT (abbreviated to KPPO34) from Thailand was also determined. Thus, there are a total of 10 structural protein gene regions, and 12 nucleotide and 13 amino acid sequences of E protein available for comparison. Fig. 2 shows the deduced amino acid alignment of the sequences of the viruses examined in this study and those previously published.

Over 2434 nucleotides, including the 5' non-coding region and structural protein genes, the 10 strains available for analysis differed from each other by between 25 (0.9 %) and 111 (4.6 %) nucleotides and by between 3 (0.4 %) and 31 (4.2 %) amino acids (Table 2). All nucleotide differences between the strains were base changes, no insertions or deletions were found. Most of the base differences in the strains were silent and did not alter the encoded amino acids (Fig. 2). In the 5' non-coding region of the 10 strains studied only two nucleotide differences were found, at nucleotides 61 and 73 (data not shown).

The highest amino acid divergence (4.2 %) of the structural proteins as a whole was between P3 from China and Saigon from Vietnam (Table 2). The amino acid divergence between the other strains was 3.3 % or less. The greater divergence of strains P3 and Saigon may be due to their multiple passages in mouse brain.

Nucleotide and amino acid homologies of individual structural protein genes

The nucleotide and amino acid sequences of C, PRM and M proteins for 10 strains, and E protein for 13 strains, were compared separately. Of the structural protein genes, the C protein region had the least variation at both the nucleotide and amino acid level (data not shown). SA14/USA differed from the other nine strains by 54 of 378 nucleotides and 10 of 126 amino acids. The nucleotide and amino acid homologies of the 10 strains are from 96.3 % to 99.7 % and 96.9 % to 100 %, respectively (data not shown). One important amino acid difference was noted at position C-123 where cysteine replaced tyrosine in strains SA14/USA, Beijing-1,
Comparison of wild-type JE viruses

Table 3. Percentage divergence of nucleotide and amino acid sequences* of the prM protein of 10 wild-type JE virus strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>SA14/USA</th>
<th>S892</th>
<th>Beijing-1</th>
<th>Amino acid divergence</th>
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<th>826309</th>
<th>DH20</th>
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<th>Saigon</th>
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<tbody>
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</table>

* Sequences are taken from a, Ni et al. (1994); b, Sumiyoshi et al. (1987); c, Hashimoto et al. (1988).
† Highest and lowest percentage divergences are shown in bold.

HK8526, Indonesia, DH20, P3 and Saigon. This changed the consensus cleavage sequence reported by Chambers et al. (1990) of anchored C and prM for JE virus from IAYAGM $ MKLSNF to IACAGM $ MKLSNF.

The nucleotide sequence of prM region was the most variable by up to 11 of 276 nucleotides (Table 3) but only a maximum of four of the 92 amino acids. Nucleotide divergence in prM region is between 0·4% and 6·5%, whereas the amino acid divergence of this region is between 0 and 5·5%. One unique amino acid difference was found at position prM-2 of strain P3, which changed the consensus cleavage sequence of anchored C and prM from IACAGA $ MKLSNF to IACAGA $ MKLSNF (Chambers et al., 1990).

The amino acid sequence of the M protein region (75 amino acids) was the most divergent amongst the structural proteins (data not shown); between 0 and 6·7% (i.e. zero to four amino acids). Nucleotide divergence of M protein region is between 0 and 4·9%.

An amino acid difference was found at position E-6 of strain Saigon, where isoleucine replaced methionine and at M-75 of strain Nakayama where a glycine substituted for serine. These amino acid differences would change the cleavage sequence between M and E proteins for strains Saigon and Nakayama from VAPAYS $ FNCLGM to VAPAYS $ FNCLGI and VAPAYG $ FNCLGM, respectively (Chambers et al., 1990).

Comparison of E protein nucleotide and amino acid sequences of the viruses revealed divergence of 5·7% or less and 4·2% or less, respectively (data not shown).

Variable amino acids in the structural proteins

Examination of the amino acid alignment in Fig. 2 identified positions that were variable depending on the JE virus strain. One variable amino acid was noted in the prM protein at position prM-14 (amino acid 141 in the polyprotein; Ile or Val) and another in the M protein at position M-37 (amino acid 256 in the polyprotein; Ile or Val); four variable amino acids were noted in the E protein at positions E-51 (amino acid 345 in the polyprotein; Ser or Val), E-209 (503 in the polyprotein; Lys or Arg), E-244 (537 in the polyprotein; Glu or Gly) and E-366 (660 in the polyprotein; Ala, Ser or Val).

Clustal analysis

In order to assess the genetic relationships between the strains examined in this study, a dendrogram (Fig. 3) was constructed using the neighbour-joining method in the
MEGA program (Kumar et al., 1993) based on calculating the similarity score obtained by pair comparison of the E protein amino acid sequences of 13 strains (12 strains described above plus a strain from Sarawak; Cecilia & Gould, 1991). The JE virus strains SA14/USA, S892, Kamiyama and Nakayama were defined in one group, HK8526 and Indonesia were in another group, strains Beijing-1 and KPP034 defined a third group, while the other strains were distinct and clustered independently from each other (Fig. 3). One strain isolated in Vietnam (Saigon) and another isolated from China (P3) were divergent from all other wild-type JE virus strains studied.

Discussion

Strain variation among JE virus isolates has been demonstrated by many workers using different methods (Okuno et al., 1968; Kobayashi et al., 1984; Hammam & Price, 1966; Hori, 1986; Banerjee & Ranadive, 1984; Chen et al., 1990; Wills et al., 1992). However, neither the molecular basis of strain variation nor the significance of the variation have been resolved.

In the present study we compared the nucleotide sequences of 10 JE virus strains over their 5' non-coding region and structural protein genes and of 13 strains over their E protein gene. The genetic homologies of these strains are considerably higher than indicated by antigenic analyses of the same viruses with monoclonal and polyclonal antibodies (Wills et al., 1992). We have also examined the mouse virulence of the strains used in this study (Wills et al., 1992). All the viruses were neurovirulent following intracerebral inoculation while only some were virulent following intranasal inoculation. We were unable to identify any correlation between differences in the primary amino acid sequence and virulence of the viruses following intranasal inoculation.

We have found that the E protein of 13 strains of JE virus differed by up to 4.2% at the amino acid level, which would indicate that JE virus shows similar variation in the E protein to dengue 3 viruses (Lanciotti et al., 1994). However, if the mouse brain-passaged strains P3 and Saigon are not included, the maximum variation is 1.8%, which is consistent with the results for dengue 1 (Chu et al., 1989) and Murray Valley encephalitis (MVE) viruses (Lobigs et al., 1990).

The dendrogram in Fig. 3 indicates that strain variation amongst the JE viruses studied is not related to their geographic locations, which agrees with studies of Okuno et al. (1968), Wills et al. (1992), Hammam & Price (1966), Banerjee & Ranadive (1984) and Hori (1986). However, our results are not entirely consistent with the hypothesis made by Chen et al. (1990). They analysed a 240 nucleotide portion of the prM region of 46 JE virus isolates. Forty-five strains differed from S892 by 2% to 16%. Similar results were obtained by Huong et al. (1993) who compared 16 JE viruses from Vietnam and found that the homologies of these strains were over 95%. In agreement with Chen et al. (1990), our results show that the nucleotide sequence of the prM protein region was the most variable among the structural protein genes (Table 3). The 10 strains examined in this study differed from each other by between 0.4% and 6.5% in the prM protein genes. Also, the amino acid divergences in the report of Chen et al. (1990) were between 0 and 7.5%, which agree with our calculations (between 0 and 5.5%; Table 3). Although the prM region of JE virus strains does differ markedly at the nucleotide level compared to the structural protein genes as a whole, which as determined by Chen et al. (1990) is useful in the genetic comparison of wild-type JE virus strains, the functional significance of the prM gene nucleotide variation remains to be established.

The most probable explanation for strain variation of JE virus is variation in particular amino acids in the structural proteins. Combinations of these variable amino acids plus a limited number of unique amino acid changes may have resulted in the strain variation detected by serological tests. Of six variable JE virus amino acids found in the prM, M and E protein sequences (prM-14, M-37, E-51, E-209, E-244 and E-366), E-51 is potentially important since amino acid E-52 is part of the epitope recognized by JE-specific monoclonal antibody Narma3 (Hesegawa et al., 1992). The amino acid change at E51 would change the conformation of the E protein in this region. The 10-fold haemagglutination inhibition activity difference of Narma3 between strains Nakayama and Kamiyama may result from their amino acid difference at E-51 (Kobayashi et al., 1985).

Previous studies have shown that the antibody response induced by JE virus is dependent on the mouse strain used and that C57BL/6 (H-2b) mice respond better than BALB/c (H-2d) or C3H (H-2k) mice (Jan et al., 1993; Wills et al., 1993). Furthermore, cross neutralization of JE viruses is more uniform in sera prepared in C57BL/6 mice than BALB/c mice (Wills et al., 1993). The region of the E protein defined by MVE virus peptide 239–251 is highly conserved among JE serocomplex viruses and contains a dominant IA3-restricted T helper cell epitope (Matthews et al., 1992). This may account for the enhanced antibody response to JE virus in C57BL/6 mice. This may also be of biological importance, since T helper cells from C57BL/6 mice primed with a MVE virus peptide containing this important T helper cell epitope were able to recognize JE virus in lymphoblastogenesis assays (J. H. Mathews, personal communication). Therefore, the various immunotypes of JE virus as seen at the level of the B cell
response may represent differences in T helper cell activation due to residue changes at E-51, E-244 and E-366. This proposal is supported by studies with MVE virus and the more distantly related dengue 2 virus, which share T helper cell epitopes in the E protein regions E-35–55 and E-365–376 (Mathews et al., 1992; Roehrig et al., 1994). Clearly, studies on the characterization of T helper cell epitopes in the E protein of JE virus are required to investigate the immune response to JE virus and their potential role in differentiating immunotypes of JE virus.

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